NCTIONAL DEFECTS IN HIV-1 INFECTION

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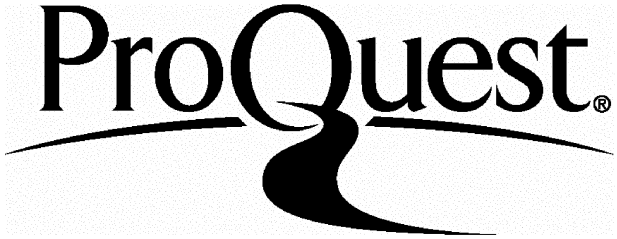

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ABSTRACT

The activation response of T lymphocytes was investigated and compared in HIV-1 infected and uninfected individuals and patients with acute viral infections (AVI). A spontaneous cell death was seen in unstimulated T cells from AVI patients and to a lesser extent in HIV-1 infection. This spontaneous death occurred by apoptosis, associated with a decrease in $CD3^+$ T cells expressing Bcl-2, a protein that blocks apoptosis. Bcl-2 negative lymphocytes expressed CD45RO, a marker of primed T cells that is greatly increased during viral infections. After activation, $CD45RA^+$ T cells from HIV-1^- individuals lost Bcl-2 expression as the T cells acquired CD45RO, indicting that the loss of Bcl-2 may occur as a normal consequence of acute stimulation, providing a mechanism for the removal of effector T cells. The presence of IL-2 greatly reduced spontaneous cell death, indicating the absolute requirement for IL-2 of this vulnerable population.

Cell death in HIV-1 infection was greatly increased after mitogenic stimulation. Activation associated death did not correlate with Bcl-2 expression and could not be prevented by IL-2. In addition, it did not appear to occur by apoptosis. This proliferative defect was due to the absence of the co-stimulatory molecule, CD28, on $CD3^+$,$CD8^+$ T cells. This increased $CD3^+$,$CD8^+$,$CD28^-$ population expressed the activation markers CD45RO, HLA-DR and CD38 and was responsible for the $CD8^+$ lymphocytosis observed throughout the course of HIV-1 disease. The $CD28^-$ T cells did not lack expression of Bcl-2 but did contain the cytotoxic granule proteins TIA-1 and perforin. Indeed, $CD8^+$ T cells from HIV-1^+ individuals were highly cytolytic in a redirected killing assay, indicating that this population may be terminally differentiated cytotoxic effector cells. Cell death after stimulation may be an abortive response that occurs because of the absence of a second signal normally provided through CD28.

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CHAPTER 1

INTRODUCTION AND AIMS

Infection with the human immunodeficiency virus type I (HIV-1) causes a progressive impairment of the immune system, most clearly demonstrated by the relentless decline in the number of $CD4^+$ lymphocytes. This ultimately results in opportunistic infections and cancers, the hallmark of AIDS. However, it is clear from many studies that factors other than $CD4^+$ T cell depletion also play an important role in AIDS pathogenesis. In particular the inability of T cells from infected individuals to respond normally to antigenic challenge is likely to contribute greatly to the disease. Paradoxically, this occurs not only in the presumptively HIV-1 infected $CD4^+$ cells but also within the expanded $CD8^+$ population. The role of $CD8^+$ T cells in HIV-1 infection is unclear since on one hand HIV-1 specific CTL might play a critical role in the removal of infected cells and the control of virus replication, while on the other they could contribute to the immunopathology through the lysis of uninfected cells or the release of immunosuppressive factors. The elucidation of the function of T cells in HIV-1 infection is therefore of crucial importance in understanding the pathogenesis of HIV-1 infection and AIDS.

The Human Immunodeficiency Virus (HIV)-1

The link between a viral pathogen and the acquired immune deficiency syndrome (AIDS) came with the isolation of a novel retrovirus from the lymph node of a patient with AIDS (Barre-Sinoussi et al 1983). The virus was originally called lymphadenopathy-associated virus (LAV), human T lymphotropic virus III (HTLV-III; Popovic et al 1984) and AIDS associated retrovirus (ARV; Levy et al 1984). HIV-1 is a retrovirus, so-called because it contains reverse transcriptase, an enzyme involved in the formation of DNA copies of the viral RNA genome. Unlike some other retroviruses, including Rous and murine sarcoma virus, HIV-1 is non-transforming but it does share structural and biological similarities with visna virus of sheep (Nathanson et al 1985) and equine infectious anaemia virus (EIAV) both of which, like HIV-1, cause a slowly progressive and ultimately fatal disease. It is also closely related to simian immunodeficiency virus (SIV), that causes an AIDS-like disease in macaque monkeys and to HIV-2, a human retrovirus prevalent

in West Africa that also causes AIDS (Brun-Vezinet et al 1987).

The Structure and Regulation of HIV-1

Under electron microscopy, HIV-1 has an electron dense core that contains viral RNA and the enzymes associated with the initial steps of virus replication. The core is surrounded by a lipid outer membrane lined on the inside with a matrix protein (p17) and on the outside with the envelope glycoprotein gp120 which is anchored into the membrane by a smaller, transmembrane glycoprotein, gp41. These viral proteins are encoded by three structural genes named *gag*, *pol* and *env* (Kieny 1990).

The life-cycle of HIV-1 begins with the binding of the virus to its specific receptor on the cell surface. This is mediated by the envelope glycoprotein gp120 which binds to the CD4 (Leu3/T4) antigen on the surface of target T-lymphocytes (Klatzman et al 1984a,b, Dalgleish et al 1994). Although CD4 is the primary receptor for HIV-1 a second receptor may also be involved. This has been proposed because mouse cells expressing human CD4 molecules cannot be infected with HIV-1. Recent studies have suggested that the co-receptor for HIV-1 might be dipeptidyl peptidase (CD26), a serine protease, thought to bind to the V3 loop of gp120 and aid virus penetration into the cell (Callebaut et al 1993). However, this suggestion could not be confirmed (Broder et al 1994, Patience et al 1994). After penetration, the viral ssRNA is converted to dsDNA by the action of polymerase and reverse transcriptase enzymes within the virus. The DNA migrates to the nucleus and is inserted into the host cell DNA by a viral integrase enzyme (Rabson 1990). A period of latency may then occur during which the virus is not expressed but remains hidden within the cellular DNA. This period of latency may persist for some time until an extracellular event triggers replication (Rabson 1988). Once triggered to replicate, the host cell RNA polymerase forms RNA copies of the viral DNA within the nucleus. Cellular enzymes process the initial RNA transcript to give a complex pattern of fragments that serve as the mRNA for viral proteins. The full length transcript is incorporated into newly formed virus particles which bud from the cell surface, acquiring their envelope as they do so (Kieny 1990).

HIV-1 replication is controlled by a set of regulator genes that determine when

latency is broken and how much virus is made. In resting cells very little if any viral RNA is made. However, when cells are activated, either specifically with antigen or non-specifically by mitogen, viral RNA is transcribed and replication begins (Rabson 1988). This association between T-cell activation and virus replication is due to the presence of binding sites in the long terminal repeat (LTR) of the virus for many cellular factors, including NF-κB. Mitogenic or antigenic stimulation of T-cells leads to the induction of κB binding proteins that interact with the HIV-1 enhancer element to initiate transcription of HIV-1 mRNA (Nabel & Baltimore 1987).

The transactivator (*tat*) gene is a positive feedback regulator that can accelerate viral production in HIV-infected cells (Sodroski et al 1986, Lee et al 1986, Rice & Mathews 1988). It encodes for a 14-kd protein which binds to the tat-responsive element (TAR) in the LTR and is thought to act by allowing transcription to bypass an early stop codon (Rice et al 1988, Kao et al 1987). The LTR also contains the negative regulatory element (NRE) which binds to the viral negative early factor (NEF) and suppresses the initiation of replication (Shaw et al 1988, Siekevitz et al 1987).

The regulator of virion protein expression (*rev*) gene encodes a 20-kd protein which inhibits the transcription of the regulatory genes and virion proteins (Sodroski et al 1986, Rosen et al 1988). This negative effect can be overcome to allow expression of viral proteins if the *rev* product binds to a cis-acting antirepressive sequence (CAR). This acts to relieve repression of the expression of the *env* and *gag* genes either by stabilizing the unspliced mRNA transcripts or by assisting in their transport from the nucleus to the cytoplasm (Felber et al. 1989; Malim et al. 1989).

GP120/CD4 Interactions

Once HIV-1 had been isolated, it was found to have tropism for $CD4^+$ cells and to use the CD4 molecule as its primary cellular receptor. It was shown by Klatzman et al (1984a,b) in PBL, that only $CD4^+$ lymphocytes could be infected with HIV-1 and that pre-incubation of cells with anti-CD4 antibodies could block infection as measured by reverse transcriptase activity. This was also true for cell lines, where only $CD4^+$ cell lines could be infected. The anti-CD4 antibodies not only prevented infection but also the formation of multi-nucleated giant cells or syncytia (Dalgleish

et al 1984) which occur when infected cells with viral proteins on their surface fuse with uninfected cells bearing the virus specific receptor (Hoshino et al. 1983).

The virus glycoprotein gp120 was implicated as the ligand for CD4 when it was discovered that this protein coprecipitated with CD4 from infected cells (McDougal et al 1986). Further evidence in support of gp120 came from studies using recombinant gp120 which was found to bind with high affinity to CD4 (Lasky et al 1987). It is now known that the gp120 binding site on the CD4 molecule is located in the first 179 amino acids of the N-terminal, V1 domain (Berger, Fuerst & Moss 1988).

The gp120 molecule has been fully sequenced. Although some regions have a high degree of variation between isolates, the cysteine residues that form disulphide links in the molecule and result in loop structures are highly conserved (Kieber-Emmons, Jameson & Morrow 1989). The CD4 binding activity is found between amino acids 422-437 with a critical residue at 417 (Richardson et al 1988). As HIV-1 seropositive individuals or animals immunised with gp120 fail to produce antibodies that prevent the virus from binding to CD4, it has been postulated that the CD4 binding site on the gp120 may be a valley or cleft similar to that proposed for rhinoviruses (Rossman et al 1985), that may be inaccessible to antibody.

Clinical Course of HIV-1 Infection

HIV-1 has been isolated from the blood, semen, vaginal secretions, urine, cerebro-spinal fluid, saliva, tears and breast milk of infected individuals (Osmond 1990). It is primarily sexually transmitted although direct injection of any of these fluids into the bloodstream could theoretically result in infection. The clinical course of HIV-1 disease has been classified by the U.S. Centers for Disease Control and Prevention (CDC; Anonymous 1986, Anonymous 1987, Anonymous 1993). Listed below is a summary of the CDC classification which was used to group the patients in this study.

(i) ***CDC stage I acute HIV-1 infection***

Within 3-6 weeks following infection with HIV-1, 50-70% of individuals have an acute clinical syndrome (Cooper et al 1985, Ho et al 1985) defined as "a mononucleosis-like syndrome, with or without

aseptic meningitis, associated with seroconversion for HIV antibody". This syndrome is analogous to what is observed in other acute viral infections (CMV, EBV; Sinicco et al 1990) and includes symptoms such as fever, lethargy, malaise, soar throat, headaches, lymphadenopathy, myalgias and a maculopapular rash. During this acute phase there is also a $CD8^+$ lymphocytosis with an increase in activated $CD8^+$ cells (Sinicco et al 1990).

(ii) ***CDC stage II asymptomatic HIV-1 infection***

Individuals then enter a prolonged period of clinical latency in which the parameters of virus replication are difficult to detect in peripheral blood (Fauci et al 1991). Although defined as an asymptomatic stage individuals still present with a variety of conditions which are not AIDS defining but which reflect the deterioration of the immune system during this period. These include oral thrush, diarrhoea and skin complaints which may either be a re-emergence of pre-existing problems eg psoriasis, acne or a new condition eg HIV folliculitis.

(iii) ***CDC stage III (PGL)***

Some individuals whilst still asymptomatic, present with persistent generalized lymphadenopathy (PGL; previously known as lymphadenopathy syndrome LAS). This is defined as lymph node enlargement of 1cm or more at two or more extrainguinal sites persisting for more than three months in the absence of a concurrent illness or condition other than HIV infection to explain the findings.

(iv) ***CDC stage IV***

The final stage of HIV-1 infection is symptomatic AIDS classified by the presence in the individual of an AIDS-defining illness. These are sub-grouped and include constitutional disease (subgroup A), neurologic disease (subgroup B), secondary infectious diseases (subgroup C), secondary cancers (subgroup D) and other conditions (subgroup E). This group contains individuals previously designated as AIDS related complex (ARC) which was defined as patients with constitutional disease (severe weight loss, night sweats) and recurrent

infection eg oral thrush.

More recently the CDC has revised the classification system for HIV-1 infection to emphasize the clinical importance of the $CD4^+$ lymphocyte count. The three $CD4^+$ T lymphocyte categories are defined as follows; category 1 >500 cells/μl; category 2 200-499 cells/μl; category 3 <200 cells/ul. The AIDS surveillance case definitions now include all HIV infected individuals who have $CD4^+$ lymphocyte counts of <200/μl or 14% of total lymphocytes (Anonymous 1993). This revised system was not used in the present study although many of the findings were compared to the $CD4^+$ lymphocyte count.

Virus Burden

The acute stage of HIV-1 disease is associated with high levels of viraemia in peripheral blood as measured by virus isolation and the detection of viral p24 in the plasma (Daar et al 1991, Clark Saag & Decker 1991, Graziozi et al 1992). This decreases with the emergence of HIV-1 immunity indicating that the host immune response can control viral replication. During the long clinically latent stage of the infection it had been assumed that the virus also entered a latent phase as indicated by the paucity of HIV-1 infected cells. However, as detection systems became more sensitive it has become clear that there is no true virus latency in HIV-1 infection. Although the number of $CD4^+$ T cells infected by HIV-1 remains low; estimates range from 1/100-1/10,000 for asymptomatics to 1/20-1/100 in full blown AIDS (Schnittman et al 1990a, Bagasra et al 1992), virus burden increases dramatically as patients progress to AIDS. For example, it was shown by Genesca et al (1990) using the polymerase chain reaction (PCR) to detect proviral DNA that although 96% of $HIV\text{-}1^+$ individuals contained HIV-1 DNA, the number of copies per 10^5 PBMC increased from 67 in CDCII patients to 802 in full blown AIDS. In addition, a longer term study showed that patients remaining asymptomatic had a lower virus burden than those who progressed to AIDS (Schnittman et al 1990a). Using PCR, viral RNA can be detected in the plasma in the majority of $HIV\text{-}1^+$ individuals at all stages of the disease (Holodniy et al 1991, Michael et al 1992). This may be explained by recent the findings that HIV-1 is active in lymphoid tissue during clinical latency (Pantaleo et al 1991, Pantaleo et al 1993) and may contribute to the

high levels of viral RNA detected in the serum. Thus, although there is a period of clinical latency in HIV-1 disease, this does not necessarily reflect a low virus burden or true viral latency.

The Cellular Immune Response to HIV-1 Infection

The host cell mediated immune response against a viral pathogen involves the expansion of specific $CD4^+$ T cells which are implicated in the subsequent generation of cytotoxic lymphocytes (Borysiewicz et al 1983). This initial cellular response can be measured by the induction of DNA synthesis after culture of T cells with viral antigens and has proved to be a good prognostic indicator of the ability of the host to overcome the infection (Levandowski Ou & Jackson 1986, Ljungman et al 1985). Using this assay, individuals infected with HIV-1 were found to have poor proliferative responses to HIV-1 antigens (Wahren et al 1986). This occurred even in asymptomatic patients but became more prevalent with disease progression (Wahren et al 1987) and could not be restored by exogenous IL-2. In addition, this lack of HIV-1 specific cell mediated immune responses occurred while patients retained responses to other viral infections such as cytomegalovirus (CMV) and Herpes simplex virus (HSV) (Wahren et al 1986, Wahren et al 1987).

Natural Killer Cells

Natural killer (NK) cells mediate two forms of cytolysis, MHC non-restricted killing and antibody dependant cytotoxicity (ADCC), both of which may be important in the control of viral infections. Morphologically NK cells are large granular lymphocytes which express CD16 and/or CD56 (Leu19) antigens but do not have a CD3/Ti complex or rearrange T-cell antigen receptor genes (Lanier et al 1986). A subset of NK cells expresses the CD57 (Leu7, HNK-1) antigen which is expressed on approximately half of the $CD16^+$ population and is associated with lower NK cytotoxic activity (Lanier et al 1983). Natural killer cells display MHC non-restricted killing *in vitro* against a variety of tumour cell targets eg K562 (Lanier et al 1983) and virally infected target cells (Diamond et al 1977). ADCC involves the interaction of $CD16^+$ lymphocytes with antibody coated target cells (Perussia et al 1983), antibody giving specificity to the activity.

The importance of NK cells in the anti-viral immune response *in vivo* has been demonstrated in mice with genetically low levels of NK cells. These animals are more sensitive to murine CMV than those with high levels (Bancroft Shellam & Chalmer 1981) while beige mice, deficient in NK cytolytic function, are highly susceptible to infection (Roder & Duwe 1979). It has been demonstrated *in vitro* using human effector cells that NK cells can lyse a number of virus infected targets including CMV infected fibroblasts (Borysiewicz et al 1985) and can also limit viral replication (Fitzgerald, Mendelson & Lopez 1985)). Resistance to infection by some viruses is also correlated with NK activity eg in mice resistant to severe infection by HSV-1 (Lopez 1980) and mouse CMV (Bancroft Shellam & Chalmer 1981).

NK Activity in HIV-1 Infection

The numbers of NK cells as measured by morphology or CD16 expression are normal throughout the course of HIV-1 disease (Lifson et al 1984, Poli et al 1985), however there is an increased expression of $CD57^+$ lymphocytes (Poon et al 1983, Lifson et al 1984, Poli et al 1985). These NK cells are active against HIV-1 infected targets *in vitro* and this activity can be augmented by IL-2 (Ruscetti et al 1986). Cytolytic activity against K562 or U937 is normal or only slightly decreased in the early stages of infection (Reuben et al 1983, Lazzarin et al 1984, Poli et al 1985, Lewis et al 1985, Burkes et al 1987) but is impaired in patients with AIDS (Gerstoft et al 1982, Poon et al 1983, Rook et al 1983, Lifson et al 1984, Poli et al 1985, Lewis et al 1985, Bonavida Katz & Gottlieb 1986). This is apparently due to a post-binding defect which may involve defective tubulin rearrangement (Sirianni et al 1988) as the binding frequency of the NK cells to the target is normal but no lysis takes place (Poli et al 1985, Bonavida Katz & Gottlieb 1986). The defect can be overcome if cells are stimulated with Con A or TPA or if IL-2, IL-12, INF-γ or IFN-β are added to the system (Bonavida Katz & Gottlieb 1986, Poli et al 1985, Chemimi et al 1992)

The ability of cells to acquire lymphokine activated killer (LAK) activity after culture with IL-2 is normal or even slightly increased in AIDS patients compared to HIV-1- individuals (Chin et al 1989). These LAK cells have been shown to have activity against HIV-1 infected monocytes (Melder et al 1990)

Several investigators have shown ADCC reactivity against HIV-1 envelope glycoproteins (Koup et al 1989b). In most cases normal donor lymphocytes were used with sera from HIV-1^+ individuals. Using this system the majority of HIV-1 infected individuals at all stages of the disease were shown to have high titres of anti-HIV-1 antibodies that could direct ADCC (Ljunggren et al 1987, Lyerly et al 1987, Emskoetter et al 1989) although some investigators have reported a rather low titre (Sinclair et al 1988). Using sera and effectors from the same individuals, Ljunggren et al (1989) reported ADCC activity in 70% of HIV-1^+ individuals tested. Nevertheless, killing was of lower efficiency than the control group, apparently due to an effector cell defect (Tyler et al 1990)

Cytotoxic T Lymphocytes (CTL)

CTL were first shown to recognize viral antigens in the context of self MHC by Zinkernagel & Doherty (1975) in the mouse. It was originally thought that CTL were directed against external viral proteins expressed on the surface of infected cells but it is now clear that CTL also recognize internal viral proteins (Townsend, Gotch & Davey 1985) which are processed by the infected cell and presented as peptides in association with the MHC class I molecule (Townsend et al 1986).

Initially virus specific CTL were thought to be exclusively $CD8^+$ and class I restricted, however, virus specific CTL which are $CD4^+$ and class II restricted have been described (Meuer et al 1982). These are directed towards MHC class II bearing target cells which include monocytes, macrophages, B-cells and activated T-cells and may arise because of the selective binding of different viral peptides to MHC class II molecules or because of the trophism of viruses to class II positive cells. Alternatively, it has been suggested that $CD4^+$ CTL are involved in the termination of a specific immune response through the lysis of antigen presenting cells (Braakman et al 1987).

Human class I restricted, virus specific CTL have been described against a number of different viruses including influenza (McMichael & Askonas 1978), EBV (Moss et al 1981), HSV (Yasukawa, Shiroguchi & Kolayashi 1983) and CMV (Borysiewicz et al 1983) and have been shown to play an important role in both the resolution of acute viral infections (Quinnan et al 1982) and the control of chronic or

persistent infection (Yao et al 1985). The importance of the $CD8^+$ cell-mediated response in acute viral infections was shown in studies on mice depleted of $CD8^+$ lymphocytes or mutant mice lacking $CD8^+$ cells. In these animals it was demonstrated that $CD8^+$ lymphocytes were responsible for the clearance of lymphocytic choriomeningitis virus (LCMV) and were the most dominant anti-viral response (Moskosphidis et al 1987; Fung-Leung et al 1991). The protection effected by $CD8^+$ CTL can be passively transferred. For instance, influenza specific cytotoxic clones passively transferred into naive recipients protected against viral challenge (Lin & Askonas 1981) and mediated recovery from primary pneumonia (Lukacher, Braciale & Braciale 1984). Similarly, cloned HSV-1 virus specific CTL protected mice from fatal HSV-1 infection (Sethi, Omata & Schneweis 1983) and cloned CMV specific CTL introduced into an infected mouse substantially reduced the virus load (Reddehase et al 1987).

CTL in HIV-1 Infection

HIV-1 specific CTL were first described by Plata et al (1987) using lymphocytes from the lungs of infected patients. These were class I restricted, $CD3^+CD8^+$ lymphocytes with cytolytic activity against autologous alveolar macrophages infected *in vitro* with the virus. The demonstration by this group of circulating activated CTL in freshly isolated lymphocyte preparations (primary CTL) was unusual as they are not found in other chronic viral infections (Quinnan et al 1982). It was proposed that the CTL in the lung might be directed against HIV-1 infected alveolar macrophages and were contributing to the alveolitis seen in these patients. Alveolar macrophages, which express low levels of CD4, were later found to be naturally infected with HIV-1 (Plata et al 1990) indicating that they could act as targets *in vivo*. At the same time, Walker et al (1987) demonstrated $CD3^+$ CTL in peripheral blood directed against autologous B-lymphoblastoid cell lines (BCL) infected with vaccinia vectors expressing the *gag* and *env* gene products of HIV-1. Again, primary CTL were measured without restimulation *in vitro*. Freshly isolated PBMC from 17 out of 20 $HIV\text{-}1^+$ patients were found to lyse autologous targets expressing HIV-1 *gag* (Riviere et al 1989). The response was mediated by $CD8^+$ lymphocytes and was MHC restricted. Similarly, in $HIV\text{-}1^+$ haemophiliacs $CD8^+$,

class I restricted CTL were demonstrated without preactivation (Koup et al 1989a). Primary CTL have also been found which are specific for the HIV-1 *pol* gene product reverse transcriptase (Walker et al 1988).

In asymptomatic patients primary CTL responses have also been found against BCL pulsed with peptides of gp160 (Clerici et al 1991). The effector cells are class I restricted, $CD8^+$ lymphocytes and the magnitude of the response increases after pre-incubation of CTL with the peptide.

Using a restimulation protocol in which PBMC from HIV-1^+ patients were cultured for a number of days with autologous PHA blasts, CTL were identified which were directed against both structural viral proteins such as *env* (Shepp et al 1989) and *gag* (Nixon et al 1988) and the regulatory protein *nef* (Chenciner et al 1989, Culman et al 1989). Such reactivation protocols measure memory CTL responses. Activation of PBMC with PHA or anti-CD3 has also been used to measure CTL precursor frequencies and to generate HIV-1 specific CTL clones. In HIV-1^+ individuals *env* specific CTL clones were raised which were able to lyse a murine cell line, P815, which had been doubly transfected with human HLA-A2 and HIV-1 *env* (Langlade-Demoyen et al 1988). These clones were $CD8^+$ and class I restricted but $CD4^+$ class II restricted CTL clones have also been demonstrated in humans immunised with recombinant gp160 (Orentas et al 1990).

HIV-1 specific CTL appear soon after seroconversion and remain at a high frequency during the asymptomatic stages of HIV-1 infection. However, they are not found to the same extent in patients with AIDS (Weinhold et al 1988, Hoffenbach et al 1989, Pantaleo et al 1990a) and are therefore thought to play an important role in controlling the viral load maintaining the asymptomatic stage (Walker & Plata 1990). In addition, $CD8^+$ T lymphocytes have also been shown to suppress HIV-1 replication by a non-cytolytic mechanism (Walker Moody & Stites 1986, Tsubota et a 1989) supporting the hypothesis that the $CD8^+$ subset is important in the control of HIV-1 infection.

Conversely, it has been proposed that HIV-1 specific CTL might also contribute to the pathogenesis of the disease through the lysis of autologous, uninfected $CD4^+$ cells. It is known that the association of gp120 and gp41 is unstable and quantities of free gp120 are shed into the medium (Gelderblom Reupke

& Pauli 1985). This circulating gp120 has the capacity to bind to the CD4 molecule on uninfected cells which may then become targets for CTL (Lanzavecchia et al 1988, Siliciano et al 1988)

T Cell Functional Defects in HIV-1 Infection

The very earliest descriptions of clinical AIDS included observations concerning the abnormal responses of T cells to mitogenic stimulation *in vitro*. Patients with AIDS or ARC showed marked decreases in proliferation after stimulation either with mitogen or recall antigens such as purified protein derivative (PPD) and tetanus toxoid (Table 1.1). This proliferative deficiency was measured using ^{3}HTdR uptake and confirmed using assays of colony forming cells where it was shown that the number of colonies formed after PHA stimulation was decreased in HIV-1^{+} patients (Winkelstein et al 1985, Lunardi-Iskander et al 1985, Winkelstein et al 1988). Such defects also occurred *in vivo* as AIDS patients were found to be anergic to recall responses by skin testing (Reuben et al 1983, Fernandez-Cruz et al 1988)

A summary of the range of proliferative defects described in HIV-1 infection is shown in Table 1.1. From these studies it is clear that although T cells from AIDS patients are profoundly handicapped, such defects also arise in asymptomatic individuals, including those with high CD4^{+} lymphocyte counts (Miedema et al 1988). Nevertheless, there is strong evidence that the degree of handicap increases with disease progression. For instance, responses to stimulation with mitogens such as anti-CD3 and PHA are lower in patients with symptomatic AIDS compared to HIV-1^{+} asymptomatic individuals (Winkelstein et al 1989, Hofmann et al 1989a, Gruters et al 1990). Such responses have also been found to correlate positively with absolute CD4^{+} lymphocyte counts (Antonen et al 1987, Hagler et al 1988, Winkelstein et al 1989) indicating that assays of T cell function can provide additional information concerning the progress of the disease. In addition, the low responses to anti-CD3 or pokeweed mitogen (PWM) in asymptomatic individuals have been shown to predict progression to AIDS (Schellekens et al 1990, Hofmann et al 1987).

Table 1.1 Proliferative defects identified *in vivo* using ^{3}HTdR uptake

Cell Type	Stimuli	Patient Group Ab^{+}/Asy.	ARC/AIDS
PBMC	anti-CD2		D (1)
	anti-CD3	D (15,16)	D (1,5,17)
		N (19)	
	Candida	D (12)	
	Con-A	D (12)	D (2,3,6)
		N (19)	
	CMV	D (18)	
	PHA	D (9,12,13)	D (1,2,3,4,6,8,10,13,16)
	PHA	N (19)	
	PPD	D (9)	
	PWM	D (9,12,19)	D (2,3,8)
			N (6)
	TT	D (13,19)	D (8,13)
T-Cells	Con-A	D (10)	D (10)
	MLR		N (7,11)
CD4^{+}	anti-CD3	D (14,17)	
	Con-A	D (10)	D (5,10)
	PHA		D (5)
			N (8)
	PWM		N (8)
	TT		D (8)
CD8^{+}	anti-CD3	D (14,17)	
	Con-A		D (5)
	PHA		D (5)
			N (8)
	PWM		N (8)

AB^{+} Antibody Postive (HIV-1, HTLVIII, LAV)
Asy. Asymptomatic
TT Tetanus toxoid
PPD Purified protein derivative
D Decreased ^{3}HTdR uptake compared to normal; $p < 0.05$
N Normal ^{3}HTdR uptake; $p < 0.05$

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These activation defects are unlikely to be due to a direct cytopathic effect of HIV-1 which using the most sensitive techniques including *in situ* hybridisation and PCR, can be detected in $<1\%$ of peripheral T cells (Schnittman et al 1990). In addition they are not limited to $CD4^+$ lymphocytes but also occur in $CD8^+$ T cells (Hofmann et al 1985, Ebert et al 1985). For example, both $CD4^+$ and $CD8^+$ cells from a wide range of patients respond poorly to anti-CD3 stimulation (Miedema et al 1988, Bentin et al 1989, Gruters et al 1990) and limiting dilution analyses of purified $CD4^+$ and $CD8^+$ cells from AIDS patients demonstrate a decrease in the number of clonable cells from both populations (Margolick et al 1985).

Interleukin-2 Release in HIV-1 Infection

It has been proposed that functional deficiencies in HIV-1 infection are due to an inadequate supply of IL-2 (Bell et al 1992, Winkelstein et al 1988, Ammar et al 1992). This is produced mainly by $CD4^+$ T cells and in lesser amounts by $CD8^+$ T cells in response to stimulation with specific antigen or mitogens and stimulates their growth in an autocrine fashion (Smith 1980). Interleukin 2 also acts as a paracrine factor influencing the activity of other cells such as NK cells (Henney et al 1981) and B cells (Waldemann et al 1984). Therefore a deficiency in IL-2 is likely to have far reaching effects.

There are conflicting reports concerning the amount of IL-2 released by T cells from infected individuals after activation *in vitro*. After stimulation with PHA individuals with AIDS were shown to have a 2 to 10 fold reduction in the amount of IL-2 released into the culture medium (Creemers et al 1986, Burkes et al 1987, Borzy et al 1987, Hofmann et al 1989b). However, other studies were unable to find any difference between HIV-1^+ patients and normal controls (Prince Kermani & Fahey 1984, Reuben et al 1985, Murray et al 1985, Winkelstein et al 1989) perhaps reflecting the wide variation between HIV-1^+ individuals or differences in technique. Defects in IL-2 release after stimulation with mitogen or antigen were also noted at earlier stages of the disease (Antonen et al 1987). In particular it was noted by Shearer et al (1986) that asymptomatic HIV-1^+ individuals showed a progressive decline in their ability to produce IL-2 in response to different stimuli. Patients initially lost the ability to make IL-2 in response to recall antigen, followed

by allo-antigen and finally PHA suggesting a selective depletion of T_H function in the development of AIDS. It was later shown by these authors that the loss of IL-2 production was associated with an augmented release of IL-4 which they suggested was indicative of a switch from a T_H1- to a T_H2-type of response (Clerici et al 1993), the classification system for $CD4^+$ T cell clones in the murine system (Mosmann & Coffman 1987).

There are also conflicting reports concerning the effect of exogenous IL-2 on lymphocyte function. T cells from a range of HIV-1^+ patients were shown to have normal (Alcocer-Varela et al 1985, Linette et al 1988) or lower than normal (Borzy et al 1987, Donnenberg et al 1989) responses to IL-2 alone while the addition of IL-2 to cultures stimulated with mitogen or antigen was found to increase the proliferation of cells as measured by ^{3}HTdR uptake (Ciobanu et al 1983, Ebert et al 1985, Gluckman et al 1985, Gruters et al 1990). IL-2 was also found to improve defective NK cell function in HIV-1 infection and resulted in normal LAK cell generation (Chin et al 1989, Melder et al 1990). Conversely, it was shown by Hausser et al (1984) that IL-2 had no effect on the proliferative capacity of T cells from HIV-1^+ individuals after stimulation with PHA. These differences may in part be due to the source and amount of IL-2 used which can greatly influences the response.

IL-2 Receptor Expression in HIV-1 Infection

Interleukin-2 mediates its effect through binding to its receptor. The receptor is composed of at least three membrane components; the α-chain (IL-2Rα, p55, CD25; Robb & Greene 1983), the β-chain (IL-2Rβ, p75 CD122; Sharon et al 1986, Tsudo et al 1986, Dukovich et al 1987) and the γ-chain (IL-2Rγ, p64; Takeshita et al 1990). The expression of combinations of these chains results in the generation of alternative forms of the IL-2R, each with different binding affinities for IL-2. The high affinity receptor is composed of all three chains (Takeshita et al 1990, Takeshita et al 1992, Arima et al 1992). The isolated α-chain forms the low affinity receptor (Robb & Greene 1983) and the β and γ-chains together form the intermediate affinity receptor (Dukovich et al 1987, Teshigawara et al 1987). The β-chain has the largest cytoplasmic domain and plays an important role in IL-2 induced

signalling (Smith 1988, Hatakeyama et al 1989), while IL-2Rα facilitates binding only and has no signalling role (Wang & Smith 1987). The recently described γ-chain is also thought to be critical for signal transduction (Arima et al 1992) and to be essential for ligand internalization (Takeshita et al 1992)

The constitutive expression of both IL-2Rβ and CD25 on peripheral T cells is very low but after mitogenic stimulation *in vitro* mRNA for CD25 and membrane expression of the protein is upregulated (Uchiyama Broder & Waldman 1981, Robb Greene & Rusk 1984)

In HIV-1 infection a deficient IL-2R expression has been postulated by many investigators to cause the poor proliferative response of T cells (Prince & John 1986, Greene, Bohnlein & Ballard 1989). However, there are conflicting reports concerning the expression of CD25 after stimulation *in vitro*. Using PHA as the stimulus, some investigators have reported normal percentages of $CD25^+$ cells in patients with AIDS or LAS in both $CD4^+$ and $CD8^+$ T cell subsets (Lane et al 1985, Creemers, O'Shaughnessy & Boyko 1986, Hofmann et al 1989b, Allouche et al 1990). However, using this same stimulus, others have found a reduced CD25 expression (Prince, Kermani-Arab & Fahey 1984, Gluckman et al 1985, Gupta et al 1986, Winkelstein et al 1988, Sahraoui et al 1992) occurring at all stages of the disease (Gupta et al 1986). There is also some confusion about the constitutive expression of CD25 on unstimulated lymphocytes from $HIV\text{-}1^+$ patients with both increased (Gupta 1986) and decreased (Zola et al 1991) levels reported.

There is little information concerning the alterations of IL-2Rβ chain expression during HIV-1 infection. It has been shown that infection of T cells with HIV-1 *in vitro* induces an impaired expression of both CD25 and IL-2Rβ after PHA stimulation (Sahraoui et al 1992) and that although PBL from $HIV\text{-}1^+$ patients have a reduced constitutive expression of IL-2Rβ, $CD8^+$ lymphocytes from broncho-alveolar lavage have increased expression (Zambello et al 1992). However, it is unclear how such gross changes are related to the considerable shifts in T cell subsets observed in $HIV\text{-}1^+$ patients.

T Cell Dysfunction Due to gp120/CD4 Interactions

The studies outlined above indicate that defects in antigen and mitogen driven

proliferation of HIV-1 infected individuals are not simply the result of reduced $CD4^+$ lymphocytes but are influenced by other factors including IL-2 and IL-2 receptor pathways. There is another mechanism of immunosuppression which occurs either as a direct result of infection with HIV-1 or through the interaction of the envelope glycoprotein gp120 with the CD4 molecule (Krowka et al 1988).

Direct infection of cell lines and peripheral $CD4^+$ T cells with HIV-1 has been reported to inhibit the responses of these cells to further mitogenic stimuli with anti-CD3 or anti-CD2 antibodies (Laurence et al 1989, Margolick et al 1987, Gupta & Vayuvegula 1987). The gp120 envelope glycoprotein which is released by infected cells and can be detected in the serum (Oh et al 1992) has been shown produce a similar effect. This protein suppresses T cell proliferation induced by PHA (Mann et al 1987), anti-CD3 (Oyaizu et al 1990, Weinhold et al 1989) and specific antigen (Krowka et al 1988, Gurley et al 1989, Oyaizu et al 1990, Faith et al 1992). These suppressor activities appear to be specific because only $CD4^+$ T cells were susceptible and soluble CD4 or anti-gp120 antibodies neutralized the activity (Terai et al 1991, Faith et al 1992). The binding of gp120 to the CD4 molecule was shown to give rise to increases in intracellular inositol triphosphate and Ca^{2+} (Kornfield et al 1988) indicating that the inhibitory activity may rely upon negative signalling through binding to CD4 (Corado et al 1991). In addition, it has been reported that gp120 bound to $CD4^+$ T cells primes the cells for death by apoptosis after subsequent stimulation through the TCR (Banda et al 1992).

Activation Induced T Cell Death

Mitogenic stimulation of mature peripheral T cells normally results in a positive response, ie proliferation or cytokine production, but under certain circumstances these same signals result in the death of the responding cells (Kabelitz Pohl & Pechhold 1993). Several studies have demonstrated that activation of immature thymocytes or T cell hybridomas through the TCR causes the death of the cells by a process of programmed cell death. More recently, activation induced cell death has also been shown to occur in mature T cells (Liu & Janeway 1990, Russell et al 1991, Janssen et al 1991)

Programmed cell death (PCD) is a mechanism of cell death which requires

new gene expression and which is common during embryonic development (Reviewed in Cohen 1991). Apoptosis refers to the morphology of PCD as opposed to necrosis which is the appearance of accidental death. Both mechanisms have distinct morphological differences (Searle, Kerr & Bishop 1982). In apoptosis the main target of damage is the nucleus, the DNA of which is cleaved into nucleosomal fragments through the action of an endonuclease (Wyllie 1987). This is an active process and is accompanied by a massive loss of cell volume and membrane blebbing (Thomas & Bell 1981). However, this nuclear degradation occurs whilst cell membrane integrity is maintained. Necrosis involves damage to the mitochondria resulting in the loss of the cells ability to regulate its osmotic pressure (Searle, Kerr & Bishop 1982). This ultimately leads to cell swelling and lysis. During this process the nuclear structure remains relatively intact in direct contrast to apoptosis. An important difference between the two mechanisms is the tissue damage resulting from necrosis. This occurs when the cell contents are released, inducing a local inflammatory reaction. In apoptosis the cell remains intact and is engulfed by the nearest macrophage (Duvall, Wyllie & Morris 1985; Savill et al 1990) thus removing the cell and avoiding the massive tissue damage seen in necrosis.

Genes Involved in Apoptosis

The control mechanisms for apoptosis have not yet been elucidated but there are a number of genes whose expression is directly involved in regulating cell death. For instance in the nematode *Caenorhabditis elegans* three genes; *ces-2*, *ced-3* and *ced-4* are required for cell death to occur (Ellis Jacobson & Horovitz 1991). Similarly in mammals *p53* and *c-myc* are necessary for apoptosis (Lowe et al 1993, Shi et al 1992). Expression of the proto-oncogene *c-myc* is necessary to allow cells to progress through the s-phase of the cell cycle (Bishop et al 1991). Gene and protein expression of c-myc are increased after mitogenic stimulation (Almendral et al 1988) and its enforced expression results in apoptosis of growth factor dependent cell lines upon factor deprivation (Askew et al 1991). Thus it is thought that c-Myc drives cells out of G0 and into cycle where the lack of other factors or survival signals leads to death by apoptosis. It has been proposed that cell cycle progression genes such as *myc* prime cells for two opposing fates; proliferation or apoptosis.

Apoptosis is the default pathway and must be actively suppressed to support proliferation (Evan et al 1992).

Certain genes including *ces-1* and *ced-9* in the nematode, are known to limit the extent of cell death (Ellis Jacobson & Horovitz 1991, Hengartner Ellis & Horovitz 1992). Another such gene is *bcl-2*, which has been shown to block apoptotic cell death in a variety of cell systems (Williams et al 1991, Raff et al 1992, Dancescu et al 1992, Bissonnette et al 1992).

The *bcl-2* gene was first identified in follicular B cell lymphomas at the breakpoint of the translocation between chromosome 14 and 18 (Tsujimoto et al 1985) and has subsequently been associated with other lymphoproliferative disorders (Said et al 1990, Dancescu et al 1992; Campana et al 1993). Although the protein is expressed in high levels in many malignant B and T cell lines it is absent from proliferating germinal centre B cells and cortical thymocytes, both of which are undergoing selection and are susceptible to apoptosis (Liu et al 1989, Liu et al 1991, Hockenberry et al 1991, Korsmeyer 1992). In addition, the induction of Bcl-2 expression in transgenic mice has been shown to prolong the lifespan of B and T cells, preventing clonal deletion of self-reactive clones (Siegel et al 1992) and ultimately resulting in autoimmune disease in these animals (Strasser et al 1991). Enforced Bcl-2 expression will block Myc-induced apoptosis (Bissonnette et al 1992) suggesting that the default pathway to apoptosis opened by inappropriate expression of proto-oncogenes can be blocked by survival signals provided by genes such as *bcl-2*. Thus Bcl-2 is intimately involved in the survival of lymphocytes and in its absence cells are susceptible to apoptosis.

An important receptor involved in apoptosis is the Fas/APO-1 molecule which is a surface protein and a member of the TNF receptor superfamily. When cross-linked by antibody, it induces cell death by apoptosis (Itoh et al 1991, Klas et al 1993). A clue to the role of Fas-induced apoptosis came from observations that lpr mice, which show accumulation of lymphocytes in the periphery and the production of autoantibodies (Cohen & Eisenberg 1991), have a mutation in the *fas* gene resulting in the absene of Fas protein (Watanabe-Fukunaga et al 1992). The Fas protein is expressed at high levels on double positive thymocytes and activated peripheral Tcells (Ogasawara et al 1993, Drappa et al 1993, Trauth et al 1989).

Lymphocyte Death in HIV-1 Infection

After culture *in vitro* in the absence of growth factors or mitogen, T cells from HIV-1 infected individuals show a certain vulnerability and die more quickly than cells from uninfected people. This was first described by Prince & Czaplick (1989) who found that $CD8^+$ lymphocytes co-expressing the marker CD45RO, HLA-DR or Leu8- were particularly affected. This could be partially prevented by either IL-2 or IL-4 but even in the presence of these cytokines $CD8^+$ T cells were prone to die (Prince & Jensen 1991a). Thus cells with an activated phenotype died after culture *in vitro*. This spontaneous cell death was later shown to occur by apoptosis (Meyaard et al 1992). Interestingly, similar spontaneous cell death was reported in patients with acute EBV infection (Uhehara et al 1992, Moss et al 1985) again predominantly within the activated T cell population. In HIV-1 infection cell death by apoptosis was also seen after stimulation with anti-CD3 (Meyaard et al 1992), superantigen or PWM (Groux et al 1992). Activation with anti-CD3 increased the apoptosis seen in the $CD8^+$ population but not the $CD4^+$ (Meyaard et al 1992). Conversely, Groux et al (1992), demonstrated that superantigen or PWM induced apoptosis occurred preferentially in $CD4^+$ lymphocytes and could be prevented by co-stimulation with anti-CD28.

Phenotypic Changes in T Cells from HIV-1^+ Patients

Infection with HIV-1 is associated with a range of phenotypic changes in peripheral blood T cells which could influence the function of the T lymphocyte population as a whole. The progressive decline of $CD4^+$ lymphocyte counts is a well documented feature of the infection and is one of the best markers predicting progression to AIDS (Tindall et al 1988, Phillips et al 1989, Fahey et al 1990). However, phenotypic changes within the $CD8^+$ T cell subset also occur and these are now recognized as important surrogate markers for AIDS (Levacher et al 1990, Bogner & Goebel 1991).

Both the percentage and absolute number of $CD8^+$ lymphocytes are increased during the acute stage of HIV-1 disease (CDCI; Sinicco et al 1990) and this CD8 lymphocytosis remains high throughout the asymptomatic stage (CDCII; Nicholson et al 1986, Krowka et al 1988, Giorgi et al 1989, Lang et al 1989). It was reported

by Lang et al (1989) that numbers of $CD8^+$ cells begin to fall six to twelve months prior to the development of AIDS but even in this patient group $CD8^+$ lymphocytes are higher than the HIV-1- control (Fahey et al 1984, Stites et al 1986, Ziegler-Heitbrock et al 1988). The total number of $CD8^+$ lymphocytes predicts the steep decline of $CD4^+$ cells (Munoz et al 1988) and progression to AIDS (Stites et al 1989, Anderson et al 1991).

A range of phenotypic changes have been reported within the $CD8^+$ subset. These include an increased number of cells expressing the markers HLA-DR (Nicholson et al 1984, Stites et al 1986, Krowka et al 1988, Ziegler-Heitbrock et al 1988) and CD38 (Prince et al 1985, Giorgi et al 1989) both of which are expressed on normal cells after activation. However, CD38 is also expressed on thymocytes leading to some speculation that these $CD8^+CD38^+$ cells might represent an immature population (Salazar-Gonzalez et al 1985). In addition there is an increase in the number of $CD8^+$ lymphocytes expressing CD57 (Leu 7; Stites et al 1986, Ziegler-Heitbrock et al 1988, Krowka et al 1988) and losing Leu 8 (L-selectin; Nicholson et al 1984, Giorgi et al 1987). However, these changes have also been reported to occur in HIV-1- homosexuals (Plaeger-Marshal et al 1987) and haemophiliacs (Prince et al 1985, Ziegler-Heitbrock et al 1985) suggesting they may not be related to HIV-1 infection per se. $CD8^+$ lymphocytes expressing HLA-DR, CD38 and CD57 form overlapping populations which have variously been associated with disease progression (Stites et al 1989, Levacher et al 1992, Bogner & Goebel 1991).

The function of this $CD8^+$, activated population in HIV-1 infection is not known but cells with a $CD8^+$HLA-DR^+ phenotype from HIV-1^+ individuals have been shown to have cytolytic activity in a redirected killing assay (Vanham et al 1990) and to have HIV-1 specific CTL activity (Pantaleo et al 1990a). These cells also have decreased proliferation after activation with a range of mitogens including anti-CD3 (Pantaleo et al 1990b) and die selectively after culture *in vitro* without stimulation (Prince & Czaplick 1989). Similarly, $CD8^+CD57^+$ lymphocytes from HIV-1^+ donors were also shown to have cytolytic activity in a redirected killing assay (Vanham et al 1990) but are also reported to have a suppressor role (Joly et al 1989) and to secrete suppressor factors (Quan et al 1993).

Lymph Node Involvement in HIV-1 Infection

Infection with HIV-1 is also associated with dramatic changes within lymphoid tissues. The histological changes of lymphadenitis are progressive and involve an initial expansion of the follicles (follicular hyperplasia) resulting in germinal centres with unusual shapes surrounded by a rather thin lymphocyte corona (Pileri et al 1986, Pallesen Gerstoft & Mathiesen 1987, Tenner-Racz et al 1987). This is followed at later stages by involution and fragmentation of follicular germinal centres and finally degeneration of the follicles (Schuurman et al 1985, Biberfeld et al 1987). Phagocytic macrophages accumulate in the paracotical areas. There are also signs of hypervascularization (Nakamura et al 1988) and the sinusoids show an increased frequency of polymorphs (Racz 1988). In addition, there are increased numbers of plasma cells in the medullary cords.

Follicular dendritic cells (FDC) form a network within the germinal centres and have a crucial role in the regulation of the humoral immune response by trapping and retaining antigen in the form of immune complexes (Gerdes & Stein 1982). In early HIV-1 infection, HIV-1 virus particles complexed with immunoglobulin or complement accumulate in the germinal centres and are trapped within the expanded FDC network (Racz et al 1985, Tenner-Racz et al 1986, Cameron et al 1987, Fox et al 1990). However, as the disease progresses the FDC's degenerate and there is less trapping. It is postulated that the loss of FDC might contribute to the pathogenesis of HIV-1 by interfering with the maintenance of memory or responses to new antigenic challenge.

The mechanisms of GC involution is not known. Using *in situ* hybridisation Speigel et al (1992) found HIV-RNA in the majority of FDC in a pattern which was consistent with active infection rather than just virus trapping on the cell surface. Therefore, active infection may play a role in FDC depletion. Alternatively, $CD8^+$ lymphocytes are found in unusually high numbers in the germinal centres during HIV-1 infection and it has been suggested that these are CTL effectors which are directed against the FDC (Laman et al 1989, Devergne et al 1991). This influx of $CD8^+$ cells also occurs in the paracortical areas (Janossy et al 1985, Wood et al 1986, Brask et al 1987). The majority of these $CD8^+$ lymphocytes express the $CD45RO^+$ antigen and are therefore primed (Racz et al 1990, Janossy et al 1991)

Comparison of Chronic HIV-1 Infection with Acute Viral Infections

The acute stage of HIV-1 infection is similar to the mononucleosis-like syndromes induced by other viruses eg EBV and CMV. The symptoms of all of these are immunopathological in that it is the immune response to the virus, rather than the virus itself which causes the symptoms. However, while other viral infections resolve and viral replication is controlled by the immune response, HIV-1 is released throughout the course of the disease and is therefore a chronic or persistent infection. The acute stage of viral infections share some similarities with chronic HIV-1. For example $CD8^+$ lymphocytosis, lymphadenitis and the appearance of activated cells in the circulation. A comparative study between these two groups might therefore provide useful information concerning the fundamental differences that on one hand lead to viral clearance and immune memory but on the other, complete destruction of the immune system and ultimately death.

AIMS

Infection with HIV-1 results in a progressive decline in immune function which is unlikely to be due solely to the loss of $CD4^+$ T cells. Qualitative defects in T cell function both *in vivo* and *in vitro* have been reported and are likely to be of crucial importance to the pathogenesis of the disease. In this study such qualitative defects in $CD4^+$ and $CD8^+$ T lymphocytes from peripheral blood were investigated using assays of T cell function *in vitro*. As HIV-1 is a chronic viral infection, responses were compared to those in individuals suffering acute viral infections (AVI) to determine if defects were unique to HIV-1 or were common to other viral diseases. The broad aims of the study were as follows:

(i) To develop an assay of T cell function *in vitro* that could easily be used to measure responses of peripheral blood T cell subsets in a routine laboratory setting and to use this assay to compare blastogenic responses to mitogenic stimuli in different T cell subsets and HIV-1^+ patient groups.

(ii) By investigating $CD45RA^+$ and $CD45RO^+$ T cell subsets, to determine if losses of immunological memory were particularly linked to losses of, or defects within, the $CD45RO^+$ population.

(iii) To determine the role, if any, of IL-2/IL-2R in qualitative defects in T cell function both by investigating the effects of exogenous IL-2 and by measuring IL-2R expression after activation.

(iv) As activation can, under certain circumstances, result in cell death, the next aim was to determine if this was an important factor in HIV-1 infection. The mechanism of cell death would be investigated as well as the role of Bcl-2, a protein known to be involved in the prevention of cell death.

(v) Efficient proliferation involves not only TcR engagement but also co-stimulatory signals provided by other receptor:ligand interactions. One such signal is provided through CD28 on the T cell. The next aim was to determine if co-stimulation through CD28 could restore defective T

cell function and to investigate any change in expression of this molecule in HIV-1 infection.

(vi) As both HIV-1 and the acute stages of other viral infections are associated with a $CD8^+$ lymphocytosis, one aim of the study was to measure the cytolytic function of the $CD8^+$ T cells and to determine if this was directed against the HIV-1 virus. As there are a number of phenotypic changes within the $CD8^+$ subset it was also our aim to define more clearly the phenotype of CTL precursor and effector cells.

(vii) The majority mature T cells are located within the secondary lymphoid tissue. Therefore, the aims listed above were also investigated in lymphoid tissue to determine if this would provide further information concerning the pathogenesis of HIV-1.

CHAPTER 2

METHODS

This chapter describes a number of general procedures that were used throughout the study. Methods pertaining to particular chapters are given in detail at the beginning of each individual chapter.

A Lymphocyte Separation Techniques

Isolation of PBMC

Venous blood was taken into preservative free heparin and diluted 1:1 with Hanks Balanced Salt Solution (Hanks BSS, Gibco Ltd, Glasgow, Scotland). The blood was layered onto Lymphoprep (Nycomed Ltd, Oslo, Norway) to give a final ratio of 4:3, blood: lymphoprep and centrifuged at 400 x g for 25 minutes. The PBMC at the interface were taken off and the cells washed twice with Hanks BSS at 160 x g for 10 minutes to remove contaminating platelets.

Preparation of lymph node and tonsil suspensions

Lymph nodes were obtained from HIV-1^+ individuals with suspected lymphoma and normal tonsils from patients after tonsillectomy. The tissue was gently teased apart with forceps and a single cell suspension prepared by aspiration through a pasteur pipette. The cell suspension was layered onto lymphoprep as described to remove contaminating red cells.

Lymphocyte enumeration and viable cell counts

Trypan Blue at a final concentration of 0.08% in saline was used to determine lymphocyte viability after isolation. Cells obtained from HIV-1 infected patients were routinely fixed with 8% formalin in PBS before removal from the safety cabinet to the microscope. The cell:formalin:trypan blue ratio used, 1:1:2, was found not to effect cell viability as measured by trypan blue uptake. The cell concentration was determined using an improved neubauer counting chamber (BDH Ltd, Dagenham, Essex) under light microscopy.

Preparation of conditioned medium

PBMC were isolated as described and adjusted to 2 x 10^6/ml in RPMI-1640 (Gibco Ltd) supplemented with 10% foetal calf serum (FCS), 100IU/ml penicillin and 100μg/ml streptomycin (all purchased from Gibco Ltd) and 2mM L-glutamine (ICN Biomedicals Ltd, High Wycombe, Bucks.). The cells were stimulated with 5μg/ml PHA (Welcome Diagnostics, Dartford, Essex) and cultured in 250cm^2 tissue culture flasks for 4 hours at 37°C in a humidified CO_2 incubator. After this initial short culture period the cells were harvested and washed repeatedly to remove the PHA. The cells were recultured in supplemented RPMI-1640 without PHA for a further 2 days when the supernatant was recovered. This was passed through a 0.22μm millepore filter and stored at —20°C until use.

Preparation of T lymphocytes

Sheep Red Blood Cells (SRBC) bind to human T lymphocytes via the CD2 antigen on the lymphocyte surface. This phenomenon was exploited to separate $CD2^+$ cells from the heterogeneous population. Isolated PBMC were adjusted to 2 x 10^6/ml in RPMI-1640 medium supplemented with 10% FCS. The SRBC (TCS Ltd, Botolph Clayton, Bucks.) in alsevers, an anti-coagulant, were washed three times with HBSS by centrifugation at 400g for 10 minutes. The SRBC were resuspended in 10ml of serum free RPMI-1640 and 300μl of 1 unit/ml neuraminidase (Sigma Ltd, Poole, Dorset) added. The red cells were incubated for 1 hour at 37°C, washed twice in HBSS and resuspended to 10% in RPMI-1640. One millilitre of 10% neuraminidase treated SRBC was added to 10ml of adjusted PBMC and incubated for 30 minutes at 37°C. The SRBC/PBMC mixture was pelleted and incubated for a further 2 hours on ice. The pellet was then resuspended gently and 13ml of lymphoprep layered underneath. After centrifugation at 400 x g for 25 minutes the non-T cell fraction (B cells, monocytes) settled at the interface while the T cell/SRBC rosettes and free SRBC were located in the pellet. The non-T cells were removed and the lymphoprep poured off leaving the pellet. To remove the red cells, 10ml of Hofmanns lysis buffer (0.82% Ammonium chloride, 0.37mg/100ml EDTA and 0.1g/100ml Potassium carbonate) was added to each tube and left for a couple of minutes until the red cells had lysed and the solution become transparent. The

tubes were then topped up with HBSS and the cells washed twice.

Complement lysis

Mouse monoclonal antibodies eg Leu11b (CD16) and RFT8μ (CD8) were used together with rabbit complement to deplete cells bearing these antigens from the heterogeneous PBMC. All antibodies were titrated before use to determine saturating concentrations. The antibody, normally in the form of culture supernatant or ascites, was added to the cell pellet and mixed well. This was incubated at room temperature for 15 minutes and washed twice with serum free RPMI-1640. Thirty day old rabbit serum (Serological Reagents Ltd, East Grinstead, Sussex) was used as a source of complement to lyse the antibody coated cells. The amount required to lyse the cells was titrated out for each new batch of complement but was normally in the region of 200μl for every ten million lymphocytes. Complement was added to the cell suspension at a final ratio of 1:2, complement:cell suspension. The tubes were incubated for 45 minutes at 37°C, washed twice with HBSS and the depletion procedure repeated with another aliquot of complement. In every experiment control depletions in which cells were incubated with complement in the absence of antibody were run simultaneously and used as complement controls in the functional experiments.

To check the efficiency of the depletion procedure an aliquot of lymphocytes was removed before and after the procedure and the expression of the antigen investigated.

Cell separations using magnetic beads

Dynabeads (Dynal, Oslo, Norway) are magnetic polystyrene beads which, when coated with antibody are used for immunomagnetic separations. The beads were normally coated with purified goat anti-mouse immunoglobulin (GAM-Ig) and used to remove the unwanted cell population. Negative selection rather than positive was used to prevent the possible interference of normal lymphocyte responses by antibody. Dynabeads were either conjugated in house with GAM-Ig following the procedure recommended by the manufacturer or were purchased already conjugated from the company.

Separation procedure The volume of dynabeads required to remove the desired population was titrated for each new batch of either beads or GAM-Ig but was generally in the region of 200μl for every 10^7 cells. PBMC were incubated with saturating amounts of antibody for 15 minutes at 20°C and washed twice with HBSS. Dynabeads were washed five times with HBSS using a magnetic particle concentrator (MPC, Dynal) and resuspended to their original volume in HBSS. The beads were added to the cell pellet and centrifuged briefly to aid cell:bead contact. The dynabead/cell suspension was incubated for 30 minutes at 20°C and then resuspended in 5ml of HBSS. The MPC was used to remove cell:bead rosettes and free dynabeads. The supernatant was centrifuged to recover the unbound cells and the procedure was repeated. After depletion, an aliquot of cells was removed to check the efficiency of the procedure. As a control PBMC without antibody were simultaneously subjected to identical depletion procedures.

Positive selection using dynabeads

In order to obtain extremely pure $CD4^+$ or $CD8^+$ T cell subsets quickly, positive selection procedures were used. Dynabeads conjugated to either anti-CD4 or anti-CD8 were added to PBMC at a cell:bead ratio of 3:1 and incubated for 30 minutes at 4°C with rotation. The cell:bead rosettes were recovered using the MPC. To uncouple the cells from the beads the rosettes were incubated with one unit of detach-a-beads (Dynal Ltd) for 45 minutes at room temperature with agitation. The detached beads were removed using the MPC and the free lymphocytes washed prior to use.

Preparations of cytospins

Normal PBMC, isolated by density gradient centrifugation as previously described were adjusted to 1 x 10^6/ml in HBSS and cytospins prepared using a Cytospin 2 (Shandon Scientific Ltd, Runcorn, Cheshire) from two drops of the cell suspension by centrifugation at 400rpm (20 x g) for four minutes. The slides were removed from the rotor, air dried rapidly and left at room temperature overnight. The following morning the cells were fixed by immersion in acetone (BDH Ltd.) and the slides wrapped in clingfilm prior to storage at —40°C. When dealing with

PBMC from HIV-1 infected individuals cell smears were prepared rather than cytospins. The PBMC were transferred to a 1.5ml eppendorf tube and pelleted by centrifugation. The supernatant was drawn off using a fine tipped pasteur pipette and the cells transferred onto the slide. The cell smear was immediately dried under hot air and the slides fixed and stored as previously described.

Preparation of cryostat sections

Small tissue blocks (5 x 5 x 3mm) were snap frozen in liquid nitrogen and stored at —70°C. These were transferred to a Frigocut 2800 cryostat (Reichert-Jung, Germany) and 4μm section cut onto glass slides and allowed to dry overnight at room temperature. The following day the slides were fixed for 30 minutes in acetone, air dried and stored at —40°C until use.

B Immunophenotyping of Cell Suspensions for FACScan Analysis

Fig. 2.1 shows a table of the monoclonal antibodies used in study. The majority of these were mouse monoclonals prepared in house. Polyclonal, affinity purified, goat anti-mouse (GAM) secondary antibodies conjugated with either Fluorescein isothiocyanate (FITC), R-Phycoerythrin (PE) or biotin were purchased from Southern Biotechnology Associates, Europath Ltd, Stratton, Cornwall). These were used in the indirect staining procedures. In three colour studies on the FACScan a third fluorochrome either Tandem (SBA) or Tricolor (Bradsure Biologicals Ltd, Shepshed, Loughborough) conjugated to streptavidin was used together with biotin conjugated monoclonal antibodies.

Direct immunofluorescence

Direct immunofluoresence (IF) is a single step staining procedure in which fluorochrome conjugated antibodies eg CD4-PE are added directly to the cells. *Procedure*: The PBMC were adjusted to 1 x 10^6/ml in PBS-A (PBS, 0.2% BSA and 0.2% Sodium azide) and 100μl dispensed into a 3ml FACScan test tube (Becton Dickinson, Cowley, Oxford) together with pre-titrated, optimal concentrations of the directly conjugated antibody. The contents of the tube were mixed by flicking and incubated at room temperature for 10 minutes. The tube was topped up with PBSA

Table 2.1 Monoclonal antibodies used in this study[a]

CD Number	Name	Isotype	Alternative Names	Molecular Structure	Main Expression	Proposed Function	Source
CD2	GT2ID7 OKT11	IgG1 IgG1	E-rosette receptor, T11, LFA-2	50kD	T Cells, NK Cells,	Adhesion molecule T cell activation	Prof PCL Beverley[b]
CD3	OKT3 T10B9 Mem57	IgG2a IgM IgG2a	T3, Leu4	5 chains 16,20,25-28kD	T Cells	Signal Transduction	ATTC[c] Dr S Brown[d] Dr J Horejsi[e]
CD4	RFT4 Edu-2	IgG1 IgG2a	T4, Leu3	50kD	MHC class II restricted T cells	Signal Transduction	Royal Free[f] Cymbus Biosciences[g]
CD8	RFT8γ RFT8μ	IgG1 IgM	T8, Leu2	2 chains $\alpha\beta$ 34kD, $\alpha\alpha$ or $\alpha\beta$ dimer	MHC Class I restricted T cells	Signal Transduction	Royal Free
CD16	Leu11b	IgM	FcRγIII	50-70kD	NK Cells, Granulocytes	Low affinity Fcγ receptor, ADCC	Prof JR Thompson[h]
CD25	RFT5γ1 RFT5γ2	IgG1 IgG2a	TAC, p55 IL-2Rα, low affinity IL-2R	55kD	Activated T & B cells, activated macrophages	Complexes with p75 to form high affinity receptor	Royal Free Royal Free
CD28	Kolt2 L293	IgG1 IgG1	Tp44	44kD	T cells Most $CD4^+$ & subset of $CD8^+$	Co-stimulation Ligand for B7/BB1 (CD80)	Dr K Sagawa[i] Becton Dickinson[j]
CD38	RFT10μ	IgM	T10	45kD	Germinal centre B cells, plasma cells, activated T cells.	Signal transduction? Adherence to endothelium	Royal Free
CD45RA	SN130	IgG1	CD45R, high MWt form of CD45	220kD	"Naive" T cells, B cells	Role in signal transduction (Tyrosine phosphatase)	Royal Free
CD45RO	UCHL1	IgG2a	Low MWt form of CD45	180kD	Primed T cells, monocytes	As above	Prof PCL Beverley
CD56	NKH1A	IgM	Leu19,NCAM NKH1	135 & 220kD heterodimer	NK cells	Homotypic Adhesion	Coulter[k]
CD57	HNK1	IgM	Leu7	110kD	NK cells, Subset of T cells	Unknown	ATCC
CD122	Tu27 MIKβ1	IgG1 IgG2a	p75, IL-2Rβ intermediate IL-2R	75kD	LGL, $CD16^+$ NK cells, some $CD8^+$ T cells	Complexes with p55 to form high affinity receptor	Prof Sugamura[l] Eurogenetics[m]

Table 2.1 Continued

CD Number	Name	Isotype	Alternative Names	Molecular Structure	Main Expression	Proposed Function	Source
	bcl-2	IgG1		25kD	Small B cells in LN corona, GC blasts are negative, many cells in T area positive	Inhibition of apoptosis	Dako Ltd[n]
	TIA-1	IgG1		15kD	Subset of peripheral T cells with cytolytic function	Target cell killing by CTL	Coulter
	PA1	IgG2b	Anti-perforin (PFP)	70kD	NK cells CTL	As above	Prof B Dupont[o]
	Na2.1	IgG1	Anti-HLA-A2				Prof A McMichael[p]

a The information in this table was taken from 3rd, 4th and 5th International Workshops on Human Leukocyte Differentiation Antigens.
b Prof. P.C.L. Beverley, University College, London
c American Tissue Typing Culture Collection, Rockville, Maryland, USA
d Dr S Brown, University of Kentucky, Lexington, Kentucky, USA
e Dr J Horejsi, Charles University, Prague
f Produced by the Department of Immunology, Royal Free Hospital, London
g Cymbus Bioscience Ltd, Southampton, UK
h Prof J.R. Thompson, University of Kentucky, Lexington, Kentucky, USA
i Dr K. Sagawa, Kurume University, Kurume, Japan
j Becton Dickinson UK Ltd, Cowley, Oxford
k Coulter Immunology, Luton, Beds.
l Prof K. Sugamura, Tohoku University, Sendai, Japan
m Eurogenetics Ltd, Teddington, Middlesex
n Dako Ltd, High Wycombe, Bucks.
o Prof B Dupont, Memorial Sloan Kettering Cancer Center, New York, New York
p Prof A McMichael, John Radcliffe Hospital, Oxford

and centrifuged at 200 x g for 7 minutes. The cells were washed in this way twice and then fixed with 100μl of 4% formalin in PBS and stored at 4°C prior to FACScan analysis.

Indirect immunofluorescence

This is a two step procedure in which unconjugated antibodies in the form of either culture supernatant, ascites or purified immunoglobulin are used together with a GAM fluorochrome conjugated second layer.

Procedure: PBMC, prepared and adjusted as before, were incubated with the unconjugated antibody for 10 minutes at room temperature. After the second wash, the pellet was resuspended and the conjugated GAM-Ig added. In single colour analyses the GAM-Ig used was normally FITC conjugated and was not isotype specific. The cells were incubated with the GAM-Ig for 10 minutes and then washed and fixed as before.

Two colour immunofluorescence

Two colour IF studies allow the investigation of two antigens simultaneously. This was achieved using two fluorochromes, FITC and PE that can be excited at the same wavelength but which emit at different wavelengths. By using a combination of filters and compensation controls both fluorochromes can be visualised on the FACScan at the same time. The simplest way to perform two colour studies is to use direct IF with antibodies coupled to different fluorochromes eg CD3-FITC with CD4-PE. This method is particularly useful when the antibodies used are of the same isotype. Alternatively, an indirect method can be used which exploits the isotype of the primary antibody eg CD8 (IgM) plus PE conjugated GAM-IgM together with CD45RO (IgG2a) plus FITC conjugated GAM-IgG. When using this method the primary antibodies were added together ie CD8 and CD45RO and incubated and washed as before. The two fluorochrome conjugated second layers were then added, also together and the cells were incubated, washed and fixed as previously described. A combination of direct and indirect immunofluorescence can also be used eg Leullb (IgM, CD16) plus FITC conjugated GAM-IgM together with PE conjugated SN130 (IgG1, CD45RA). In this case the directly conjugated

antibody was added at the same time as the GAM.

Three colour immunofluorescence

Three colour studies use a third fluorochrome which is excited at the same wavelength as FITC and PE but which emits in the far red. The third fluorochrome, either tandem or tricolor, was coupled to streptavidin and was used in an indirect staining procedure with biotinylated primary antibodies. Broadly similar principles apply to three colour staining procedures as described for two colour. Antibodies were either directly conjugated eg CD3-FITC, CD4-PE plus a third biotin conjugated antibody which reacted with the strep-tandem eg CD8-biotin, or the isotype of the primary antibodies were exploited using isotype specific fluorochrome conjugated GAM eg RFT8μ (CD8) plus GAM-IgM-FITC, SN130 (CD45RA, IgG1) plus GAM-IgG1-PE and biotin conjugated UCHL1 (CD45RO, IgG2a). For triple colour IF any indirect staining procedures were carried out first and the directly conjugated antibodies added together with the strep-tandem.

Immunophenotyping using the whole blood method

Single, double and triple colour IF studies were also performed on whole blood. This is a more accurate method of immunophenotyping as cells are lost during the ficoll separation procedure. It is also quicker and more cost effective than using separated PBMC.

Procedure: All staining procedures were as previously described for PBMC except that 100μl of whole blood was used instead of PBMC. Generally slightly more antibody was required when using whole blood. This was added in a larger volume, 20μl per sample, to facilitate proper mixing. After the final wash and prior to fixation, the RBC were lysed using 2ml of 1 in 10 Facs lysing solution (Becton-Dickinson) in H_2O. The cells were incubated with the lysing solution for 10 minutes at room temperature and then spun gently (700rpm) for 5 minutes to recover the white blood cells. These were washed twice with PBSA and fixed in the normal way.

Controls for immunophenotyping

The controls were incorporated to determine the degree of background IF due to non-specific binding of the antibodies and thus to locate the negative population during data analysis

Indirect immunofluorescence: The control consisted of either an irrelevant antibody of the same isotype as the test antibody eg RFAL10 (CD10) or normal mouse immunoglobulin together with GAM-fluorochrome conjugated second layer.

Direct immunofluorescence: For this method the controls incorporated were either normal mouse immunoglobulin conjugated to FITC or PE and used at the same concentration as the test conjugate or an irrelevant directly conjugated antibody eg RFAL10.

Double and triple colour immunofluorescence: For double and triple colour IF studies on the FACScan it was necessary to stain each of the parameters separately in order to set up the compensation levels of the machine for each combination. Once these had been determined it was not necessary to repeat this for every run unless a new combination or new batch of antibody was being tested. Controls were essentially those for direct and indirect IF except that a biotin conjugated normal mouse immunoglobulin was incorporated as a control for the biotinylated reagents.

Immunofluorescence staining of tissue sections and cytospins

For single or double IF studies GAM-conjugated FITC or TRITC reagents (SBA) were used and the slides viewed in a Zeiss epifluorescence microscope, equipped with selective filters for both of these fluorochromes.

Procedure: Microscope slides were removed from the —70°C freezer and allowed to warm to room temperature before unwrapping. The position of the tissue section or cytospin was ringed with a diamond marker and the intervening space painted with water repellant (15% Dimethyl-polysiloxane in Iso-propanol plus 1% concentrated Sulphuric acid). Once the water repellant had dried, the slides were wetted in PBS and were not allowed to dry out throughout the staining procedure. The dilution of monoclonal antibody was determined for each antibody used but was generally in the region of 5-20μl of culture supernatant plus 30μl of PBS per section. The slides were incubated with antibody for 1 hour at room temperature and

washed twice in a Coplin jar containing PBS for 5 minutes. The fluorescent conjugated GAM second layer was then added and the slides incubated and washed as before. After the final wash the slides were mounted in Citifluor (Citifluor Ltd, London, UK). Negative controls consisting of either normal mouse serum or fluorescent conjugate alone were run simultaneously as background controls.

C Statistical Analyses

Two tests were used to determine if mean values in patient groups were statistically significantly different from the normal control. The first, the Students t test, is a parametric test that compares the differences between means of two independent samples, of less than or equal to 30. A prerequisite for using this analysis is that both sets of data are approximately normally distributed. The formula used to calculate "t" is different depending if the variances of the two populations are equal or unequal. This test can be used for larger sample numbers, provided "Z" distribution is used instead of "t". Where sample numbers were small (<6) or the data had a skewed distribution, a non-parametric analysis of the data was performed. In such cases, the data are presented as median values rather than arithmetic means. These data were analysed using the Mann-Whitney U test, in which ranked data are compared. To determine if two variables were connected eg $CD4^+$ lymphocyte count and $CD4^+$ proliferation, linear regression analyses were performed. This measures the strength of a linear relationship and reflects the consistency of the effect that a change in one variable has on the other. All tests were performed using CSTAT computer software. Probability values (p) of <0.05 for two tailed tests were considered statistically significant.

D Subjects Investigated

The HIV-1 seropositive cohort investigated attended outpatient clinics at the Royal Free Hospital, London between January 1989 and December 1992. This cohort was composed of mainly male homosexuals (85%), mean age 35 years, age range 19-61 years. Subjects were grouped according to CDC classification as previously outlined (Chapter 1). The HIV-1 seronegative group were healthy laboratory personnel with a mean age of 31 years, range 21-60 years. The

serological status of the individuals was confirmed at the Department of Virology at the hospital using a Wellcozyme HIV recombinant assay (Wellcome Diagnostics) in combination with an HIV-1 enzyme immunoassay (Abbott Diagnostics, Maidenhead, England) and Serodia particle agglutination test (Mast Diagnostics, Bootle, England). The AVI patient group was composed of HIV-1$^-$ laboratory personnel with upper respiratory tract infections and also patients with acute EBV or VZV infections attending the infectious diseases unit, Coppetts Wood Hospital, London.

CHAPTER 3

PROLIFERATIVE DEFECTS IN HIV-1$^+$ AND AVI PATIENTS

Introduction

Defects in the ability of lymphocytes from HIV-1+ patients to respond to antigen and mitogens *in vitro* have been widely reported (Table 1.1) and are thought to be of great importance in the pathogenesis of the disease. These observations are mainly based on decreases in uptake of ^{3}HTdR into the DNA of the proliferating cells and are therefore not fully quantitative in terms of the number or phenotype of the responding cells and give no information concerning cell viability. Lymphocyte death after short-term culture *in vitro* has been reported in HIV-1 infection and might play a crucial role in the defective activation responses.

The first aim of this chapter was to investigate more closely defective activation responses in CD4$^+$ and CD8$^+$ T cell subsets from peripheral blood of HIV-1 infected individuals, particularly with reference to cell viability. The second aim was to develop a technique to quantify proliferative responses of T cell subsets which could be transferred to a clinical setting for the routine investigation of T cell function in HIV-1 infection. To this end flow cytometric and microscopic methods were used to accurately enumerate viable cells and lymphoblasts developing after activation *in vitro*. When combined with IF studies the response of CD4$^+$ and CD8$^+$ lymphocytes and were investigated without the need for protracted purification procedures.

Methods

Stimulation of PBMC

PBMC were isolated as previously described in chapter 2 and adjusted to 1 x 10^6/ml in RPMI-1640 supplemented medium. All mitogens used in the study were previously titrated on PBMC from HIV-1$^-$ controls using ^{3}HTdR uptake as a measure of proliferation. The cells were plated out in 200μl aliquots in flat bottom, 96 well, tissue culture plates in the presence of optimal concentrations of mitogen (PHA 1.0μg/ml; anti-CD3 0.5μg/ml; PWM 10μg/ml). Cultures were also routinely supplemented with 1.0ng rIL-2 (10units/ml; Sandoz, Basel, Switzerland) and 30% PHA-conditioned medium. Control cultures in which cells were cultured in the

absence of mitogen or additional growth factors were also set up. Cultures were incubated at 37°C in a humidified CO_2 incubator.

Disaggregation of PHA stimulated cells

PHA binds to sugar residues on the surface of lymphocytes and acts as an agglutinin causing aggregation of cells into clusters. The sugar N-Acetylgalactosamine (NAG) binds PHA and can be used competitively to remove PHA from the surface of the lymphocyte and so aid disaggregation.

Procedure The PHA activated cells were recovered by centrifugation and the pellet resuspended in 100mg/ml NAG (Sigma, Poole, Dorset). The lymphocytes were incubated with the sugar solution for 20 minutes at 37°C and then washed to remove the PHA and sugar. Unactivated cells underwent identical procedures and were used as the controls.

Quantitation of lymphocyte activation and viability

(i) Tritiated thymidine uptake

This method is widely used to quantify DNA synthesis after lymphocyte activation. Tritiated thymidine (^{3}HTdR) is added to the cultures and is incorporated into the replicating DNA. This procedure does not provide any information on cell viability.

Procedure Lymphocyte cultures were set up as described and 16 hours prior to harvesting 25KBq of ^{3}HTdR (2Ci/mM; Amersham Plc, Amersham, Bucks) was added to each well. Control unactivated cells were also pulsed to give a measure of the background stimulation. The cells were collected onto filter paper using an automatic cell harvester (Skatron, Lierbyan, Norway). The filter paper disks were air dried and transferred to scintillation vials along with 2 ml of scintillant (NBS Biologicals, Hatfield, Herts). The radioactivity was measured on a Beta counter (LKB Ltd, Croydon, UK) and the results expressed as counts per minute (cpm).

(ii) Measurement of viable cells and lymphoblasts by microscopic examination

Before a cell divides it increases in size. This change in size can easily be seen by microscopic examination. An improved neubauer counting chamber was used to

count the number of cells and lymphoblasts. Cells were viewed under light microscopy using trypan blue exclusion to determine viability. This technique had the advantage that dead cells and debris were easy to distinguish from live cells however small changes in cell size were difficult to quantify.

Enumeration of lymphoblasts by flow cytometry

The changes in cell size which occur after activation can easily be visualised using the FSC and SSC profiles on the FACScan (Fig. 3.1a). However, because the FACScan does not give a quantitative measurement of cell numbers, a method was standardised which utilized reference beads to accurately enumerate lymphoblasts. This method relied on the proportion changes in lymphocytes:beads which occurred when known numbers of cells were mixed with fixed numbers of beads.

Procedure The FSC and SSC settings on the FACScan were adjusted to allow the lymphoblasts to be cleanly gated separate from the small lymphocytes and cell debris. Beads of 7.2μm diameter (FCSC Reference Standards, Becton-Dickinson) were diluted to $5x10^5$/ml in PBS and 100μl added to known numbers of PBMC also in 100μl aliquots. This mixture was run on the FACScan and analysis gates drawn around the cell and bead populations. The ratio of cells:beads was plotted against the lymphocyte count to construct a standard curve (Fig. 3.1b). After culture of PBMC in 96 well plates, the entire contents of each well was transferred to a FACScan tube and 100μl of beads added. The number of lymphoblasts was then calculated from the standard curve.

Lymphocyte counts using the CytoronAbsolute

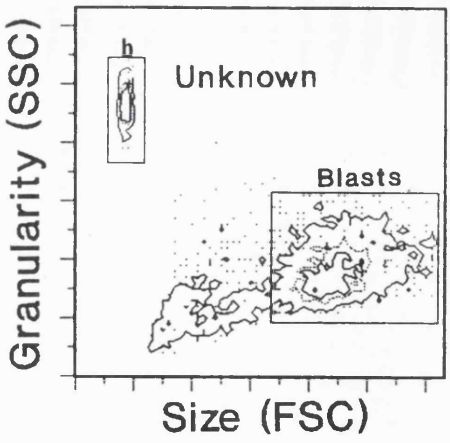
The acquisition of a Cytoron*Absolute* (Ortho Diagnostics Ltd, High Wycombe, Bucks) enabled the accurate enumeration of both small lymphocytes and lymphoblasts. This flow cytometer directly enumerates cells. This is a time based process as compared to the FACScan which counts events. The use of this flow cytometer bypassed the need for bead standards. After culture with mitogen, cells were again harvested from each well and the volume was adjusted to 2ml with 1% paraformaldehyde in PBS. These were run through the Cytoron*Absolute* and were then analysed using preset gates for small viable cells or lymphoblasts (Fig. 3.2a).

Figure 3.1 Enumeration of lymphoblasts using the FACScan

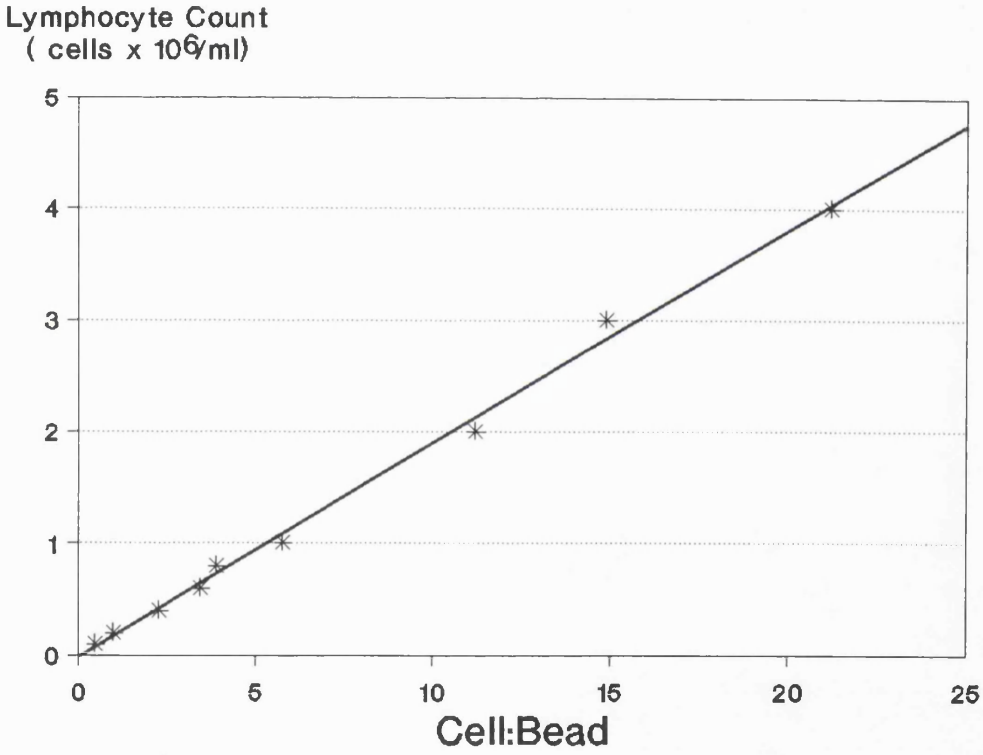
After activation *in vitro* cells were mixed with reference beads and run on the FACScan (A). Analysis gates were drawn around the beads (b) and lymphoblasts and the ratio of events, cells:beads, used to calculate the cell count from a previously constructed calibration curve (B).

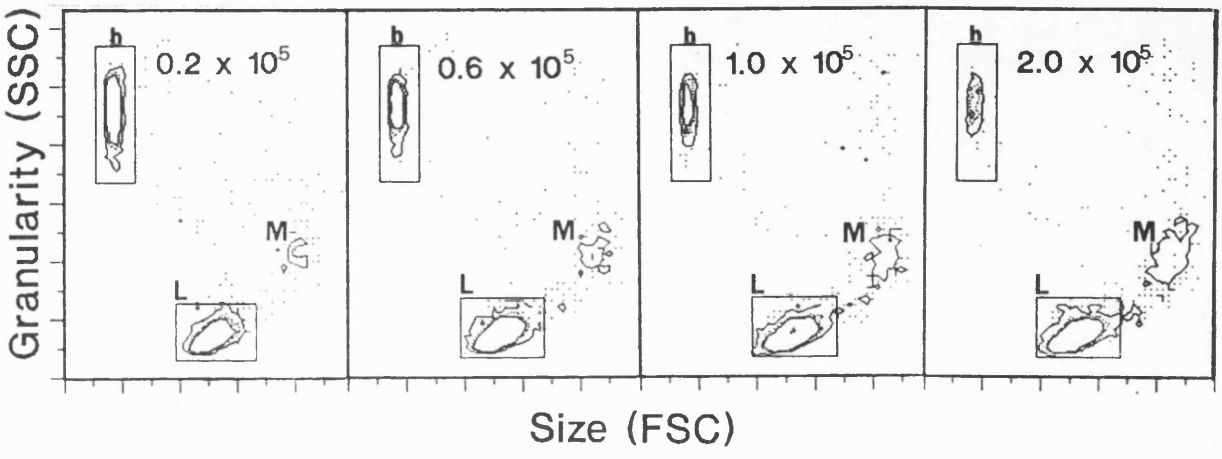
b reference beads, L lymphocytes (resting), M monocytes

(A)


h
Unknown
Blasts
Granularity (SSC)
Size (FSC)

(B)


Lymphocyte Count
(cells x 10⁶/ml)
5
4
3
2
1
0
0
5
10
15
20
25
Cell:Bead


Granularity (SSC)
h
0.2 x 10⁵
M
L
h
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M
L
h
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h
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M
L
Size (FSC)

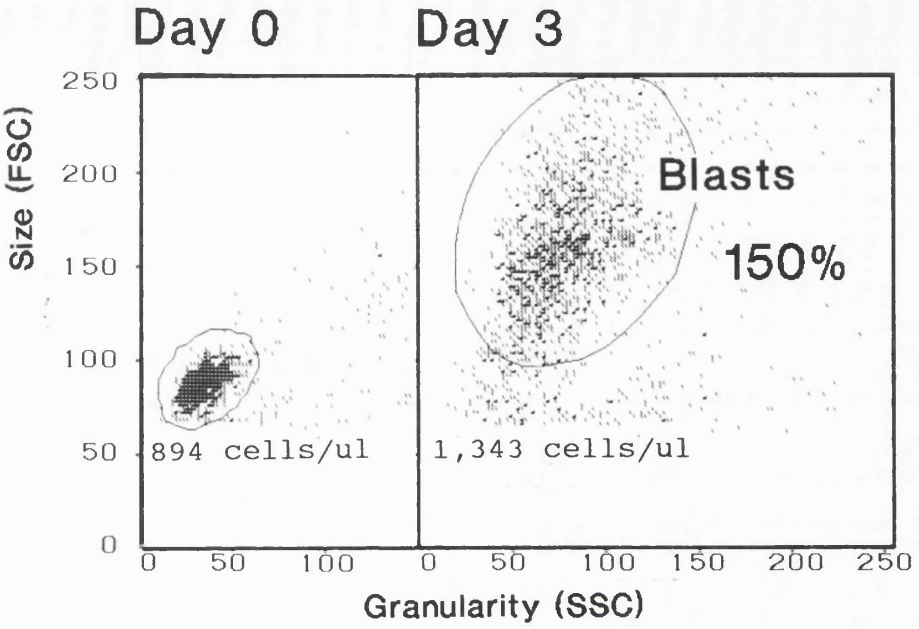


Figure 3.2 Enumeration of lymphoblasts using the Cytoron*Absolute*

The Cytoron*Absolute* is a flow cytometer which accurately counts cells gated on the basis of forward (FSC) and side (SSC) scatter profiles and fluorescence. Using this cytometer, cells were analysed before and after activation *in vitro* and the percentage of lymphoblasts determined in relation to the original input.

This could also be combined with fluorescent markers to obtain accurate counts of lymphocyte subsets. To achieve this, directly conjugated antibodies were added to the cells for 10 minutes prior to the addition of the paraformaldehyde. This technique was useful as it allowed the enumeration of both small lymphocytes and lymphoblasts.

Results

A comparison of ^{3}HTdR incorporation and lymphoblast development for the enumeration of lymphocyte activation

The number of lymphoblasts measured by flow cytometry was compared to ^{3}HTdR incorporation to determine if DNA synthesis correlated with blast development. PBMC from 10 HIV-1$^+$ donors were stimulated with PHA and the lymphoblast percentage plotted against the ^{3}HTdR counts for each individual (Fig. 3.3). PHA was chosen as the stimulus because it is a strong mitogen that transforms the majority of T cells into large lymphoblasts that are easily gated by flow cytometry. The time course for PHA stimulation was previously determined using PBMC from HIV-1$^-$ individuals, with maximal lymphoblasts and ^{3}HTdR incorporation occurring after 3 days activation. The results show a highly significant correlation between the two sets of data ($r=0.927$; $p<0.001$) indicating that an increase in cell size correlates well with activation induced DNA synthesis as measured by ^{3}HTdR incorporation. Thus a flow cytometric method can be used to measure lymphocyte activation responses *in vitro*.

The development of lymphoblasts after mitogenic stimulation

In the next part of the study, the proliferative response of PBMC from a range of HIV-1$^+$ donors and HIV-1$^-$ controls to three different mitogens; anti-CD3, PHA and PWM were compared using the flow cytometric technique for the enumeration of lymphoblasts. These mitogens were chosen since responses to anti-CD3 and PWM have previously been shown by other investigators to predict progression to AIDS and PHA induced activation also declines with disease progression. The number of CD4$^+$ and CD8$^+$ lymphoblasts present after 3 days stimulation was determined in relation to the original input of resting cells (Fig.

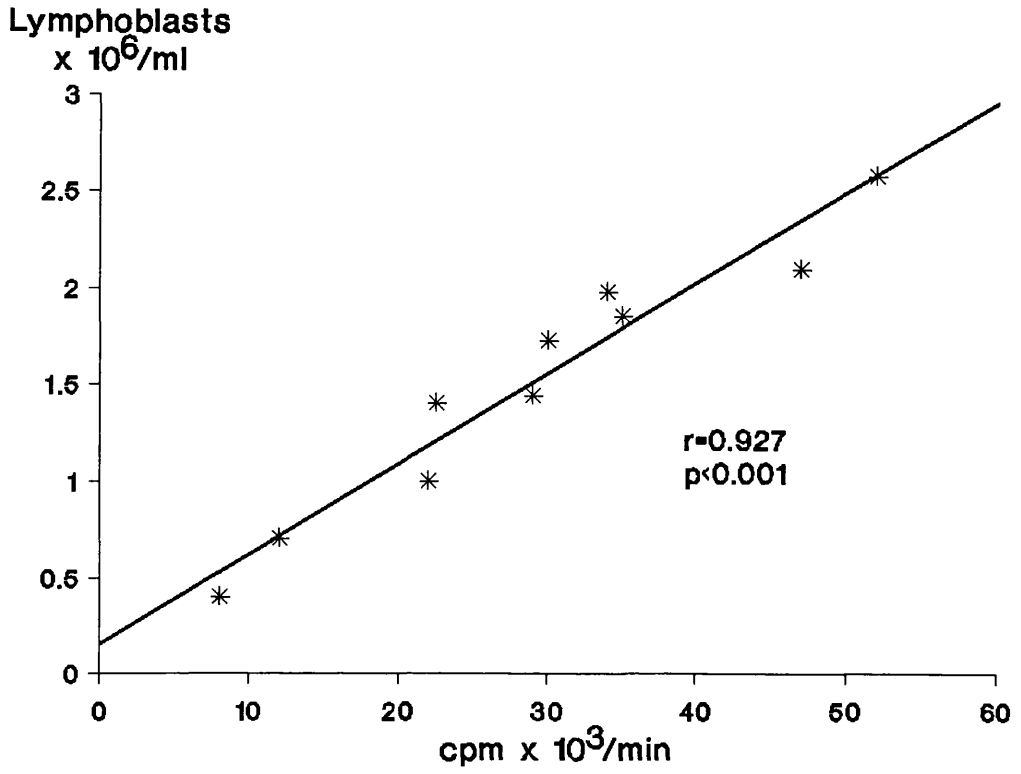


Figure 3.3 The correlation between ^{3}HTdR uptake and lymphoblast recovery

PBMC from 10 HIV-1$^+$ individuals were activated with PHA and the responses compared using ^{3}HTdR uptake and lymphoblast recovery measured by flow cytometry. The data are compared using regression analysis.

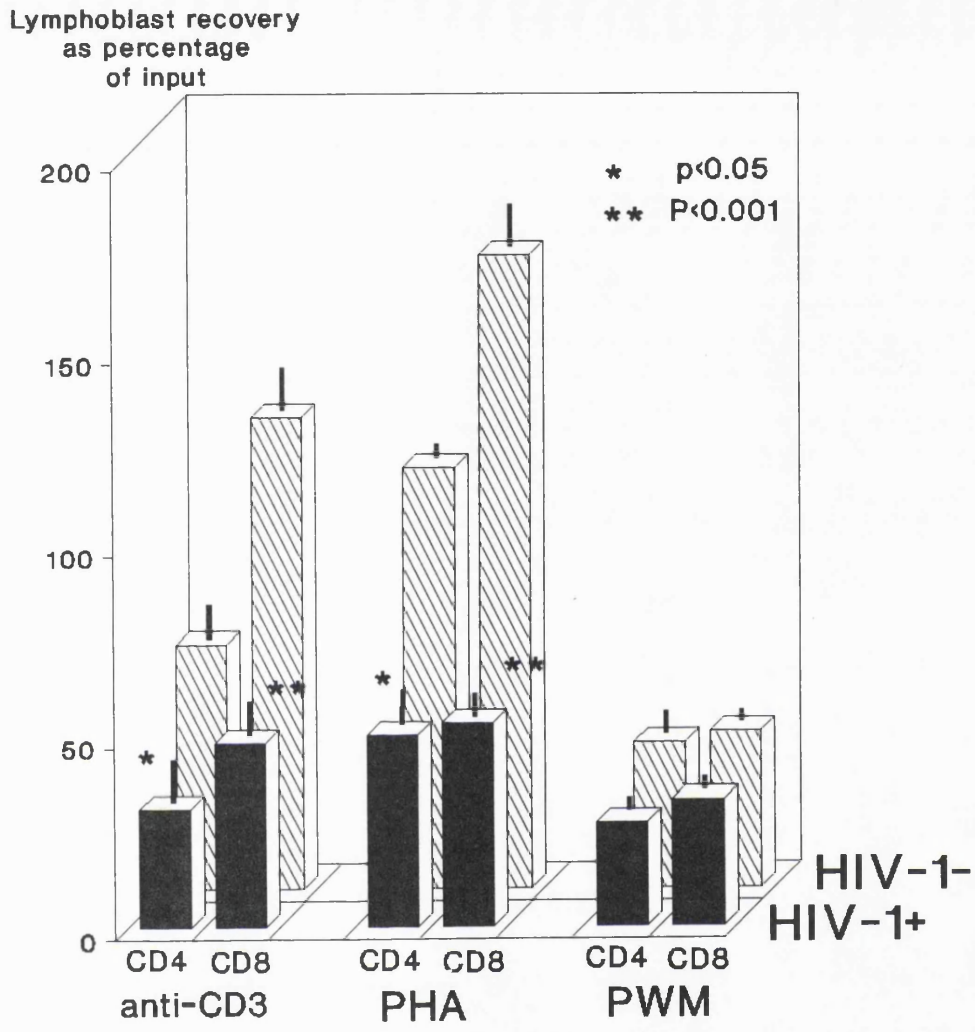


Figure 3.4 The percentage of $CD4^+$ and $CD8^+$ lymphoblasts recovered after 3 days stimulation with anti-CD3, PHA or PWM.

PBMC from 15 HIV-1^+ and 10 HIV-1^- individuals were investigated by flow cytometry for the development of lymphoblasts after stimulation. The results are shown as the mean lymphoblast recovery in relation to input of $CD4^+$ or $CD8^+$ cells on day 0. Mean values for $CD4^+$ and $CD8^+$ T cell subsets from HIV-1^+ individuals were compared to the control using the students t-test.

3.4). All cultures were supplemented with rIL-2. Using this assay a profound proliferative defect was observed in HIV-1^+ individuals using either anti-CD3 or PHA.

In HIV-1^- individuals, activation with either anti-CD3 or PHA induced the transformation of a large proportion of both $CD4^+$ and $CD8^+$ lymphocytes. However, there was evidence that the $CD8^+$ population were preferentially stimulated by these mitogens (anti-CD3 CD4 64±8.1%, CD8 123±15.9%; PHA CD4 110±4.3%, CD8 165±11.9%). In HIV-1^+ donors, the proportion of both $CD4^+$ and $CD8^+$ lymphoblasts developing after activation with anti-CD3 or PHA was significantly reduced compared to the control group (Fig. 3.4). However, the $CD8^+$ subset was proportionally more handicapped than the $CD4^+$ when compared to the responses of the HIV-1^- group, due to the preferential stimulation of the $CD8^+$ subset in normal individuals.

Stimulation of PBMC with PWM resulted in the development of far fewer lymphoblasts compared to either anti-CD3 or PHA. In normal individuals both $CD4^+$ and $CD8^+$ cells responded equally well ($CD4^+$ 38±2.8%; $CD8^+$ 41±4.6%) and this was not significantly reduced in HIV-1 infection ($CD4^+$ 27±5.5%; $CD8^+$ 33±3.3)

Large scale study of activation defects in HIV-1 infection using the flow cytometric technique

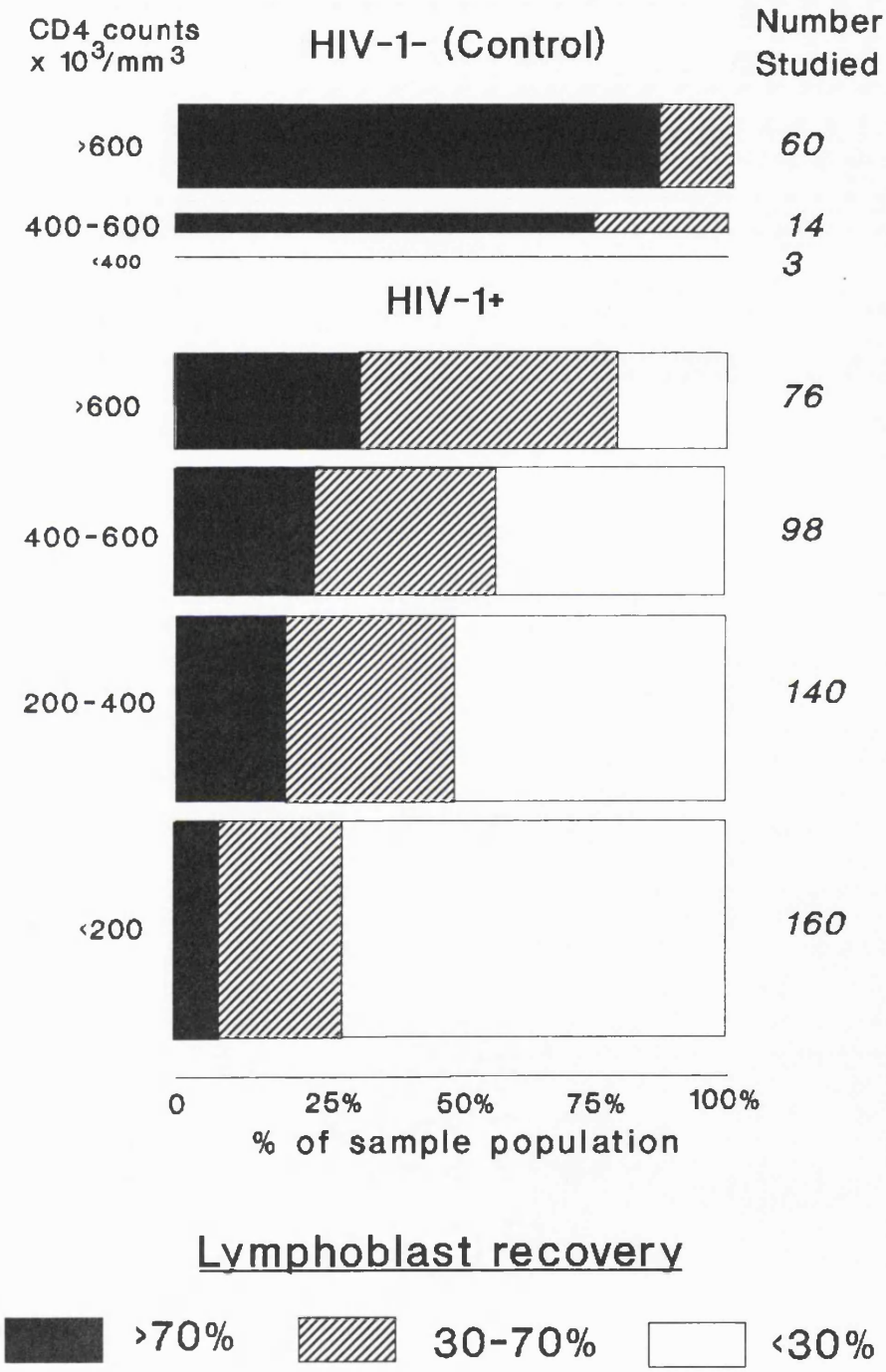
Because of the weak stimulatory properties of PWM, this was thought to be unsuitable for a large flow cytometric based study. However, both anti-CD3 and PHA induced lymphoblasts which could easily be enumerated using this technique. The HIV-1^+ patients investigated previously showed defective responses to both of these mitogens, although the degree of handicap was greater after PHA activation. As anti-CD3 stimulation is more reliant on adherent cells than PHA the latter was chosen as the preferred mitogen.

In this study 551 individuals were stimulated with PHA and the number of T lymphoblasts developing in the cultures used as a measure of the activation response. This test was incorporated by the NHS staff into the HIV routine service. The results of this quantitative analysis (Fig. 3.5) are expressed in relation to $CD4^+$

Figure 3.5 Lymphoblasts recovered from PHA stimulated cultures from 77 HIV-1$^-$ and 474 HIV-1$^+$ individuals.

The percentages shown are blasts per lymphocyte input; >70%, 30-70% and < 30%. The subjects are grouped according to the absolute CD4$^+$ lymphocyte count in peripheral blood. The width bars are proportional to the number of people in each group (shown on the right).

PHA stimulation



Lymphoblast recovery

>70% 30-70% <30%

lymphocyte counts and reveal a significantly poorer blast recovery even in HIV-1^+ individuals whose $CD4^+$ lymphocyte count is within the normal range ($>600/mm^3$). Only 31% of these individuals have a blast recovery >70% of input compared to 82% in HIV-1^- control group while 21% of patients already have very poor blast transformation responses, <30%. This study reveals an obvious deterioration in the blastogenic response with disease progression. The numbers of patients showing <30% blast recovery was increased as $CD4^+$ lymphocyte counts fell from 400-600 (40%) to 200-400 (48%) and below 200 (65%; Fig. 3.5).

In 215 HIV-1^+ patients and 57 controls, cultures were also assessed for the activation response of $CD4^+$ and $CD8^+$ lymphocyte subsets (Fig. 3.6). The results here are grouped according to the CDC classification system and reveal a poorer lymphoblast recovery within the $CD8^+$ population compared to the $CD4^+$ population. In the control group the $CD4^+$ lymphoblast recovery was 118%. This was decreased in the CDCII group (78%) but near normal levels (103%) of $CD4^+$ lymphoblasts developed in the CDCIII individuals. At the same time the number of $CD8^+$ lymphoblasts dropped from 95% in the control group to 37% and 40% in the CDCII and CDCIII groups respectively. The lymphoblast recovery in the CDCIV patients (18-20%) reflects the activity of the $CD8^+$ population as these late stage patients have few remaining $CD4^+$ lymphocytes.

Decrease in viable lymphocytes after short-term culture In Vitro

As the flow cytometric method provides no information about lymphocyte viability, a microscopic study using trypan blue exclusion was undertaken to investigate changes in viability after culture. In this study lymphocytes from control, HIV-1^+ and AVI individuals were compared after culture in the presence or absence of anti-CD3. This mitogen binds to the CD3 component of the TcR complex and therefore mimics antigen, providing a more physiological stimulus than PHA. Anti-CD3 stimulated cultures were supplemented with 1ng/ml rIL-2 and 30% PHA-conditioned medium (Chapter 2) to allow for any cytokine deficiencies due to low $CD4^+$ lymphocyte counts. Samples of cultured cells were removed daily and the proportion of viable $CD4^+$ and $CD8^+$ lymphocytes determined by IF using either CD4 or CD8-PE. Cell viability was measured by trypan blue exclusion (Chapter 2).

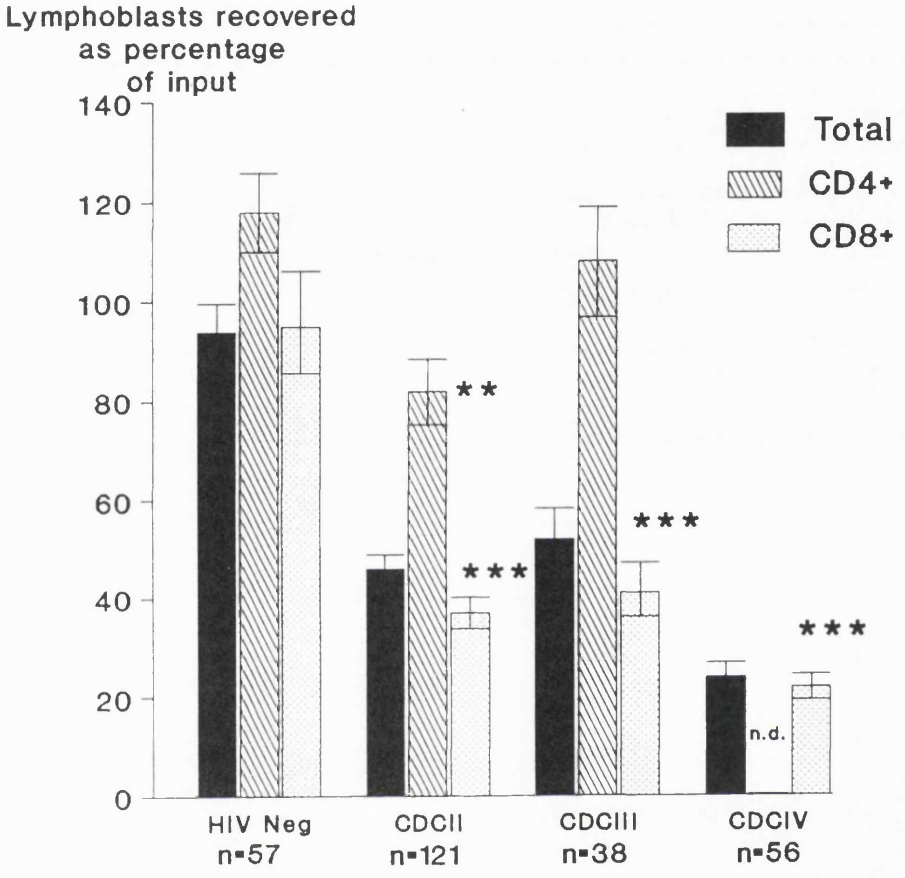


Figure 3.6 Lymphoblasts recovered after 3 days stimulation with PHA.

The percentages shown are $CD3^+$, $CD4^+$ and $CD8^+$ lymphoblasts assessed within the CDC disease stages shown. The bars represent the mean ± s.e.m.; n.d. not determined due to the small numbers of $CD4^+$ lymphocytes. The data for $CD4^+$ and $CD8^+$ lymphoblast recovery in the HIV-1 infected individuals was compared to the control group using the Students t-test; ** $p < 0.005$, *** $p < 0.001$

(i) Culture Without Stimulation

After short-term culture in medium alone, the number of viable lymphocytes from normal individuals was only slightly reduced and after 3 days, 75±2.8% of the original cell input remained viable (Fig. 3.7). Thus in the control group only 25% of lymphocytes died spontaneously during the three day culture period. Spontaneous cell death was more pronounced in the HIV-1^+ patients and by day 3 significant differences were found between the number of cells recovered from HIV-1^- control and HIV-1^+ CDCIII & IV individuals (% of input; CDCIII 58±6.8%, CDCIV 49±5.2%; $p<0.03$ and $p<0.001$ respectively; Table 3.1). As previously reported, spontaneous cell death was increased in patients with acute viral infections compared to the HIV-1^- group (% of input; control 75±2.8%, AVI 47±5.1%; $p<0.001$) and was comparable to the levels found in the HIV-1^+, CDCIV patients (49±5.2%).

(ii) Anti-CD3 Activation

Stimulation of lymphocytes from HIV-1^+ patients with anti-CD3 significantly increased the degree of lymphocyte death (Fig. 3.7). In the control group, activation over the short 3 day culture period did not cause any significant change in cell numbers and by day 3, 103% of the original cell input remained viable. In HIV-1 infected individuals however, activation induced a dramatic decrease in the number of viable cells recovered. A significant cell loss was apparent in all CDC categories after only 24 hours in culture, (% of input; CDCII 72.6%, CDCIII 73.6% and CDCIV 59.5%). Thereafter, the number of cells declined steadily until by day 3 only 41.5%, 44.3% and 21.4% of the original input of cells from CDCII,III & IV patients respectively, remained viable. This cell death was significant in all CDC categories compared to the HIV-1^- group after only 2 days in culture (CDCII $p<0.001$, CDCIII $p<0.006$, CDCIV $p<0.001$; Table 3.1) and became more pronounced on day 3 ($p<0.001$). Although cell death occurred even in the CDCII patients, the degree of handicap increased with disease progression (CDCII&CDCIII > CDCIV; $p<0.03$). It is important to note that activation significantly increased the degree of cell death in both CDCII and CDCIV stages of HIV-1 infection compared to the unstimulated cultures (CDCII $p<0.013$; CDCIV $p<0.001$; Fig. 3.7). Thus, in addition to spontaneous cell death, a proportion of the

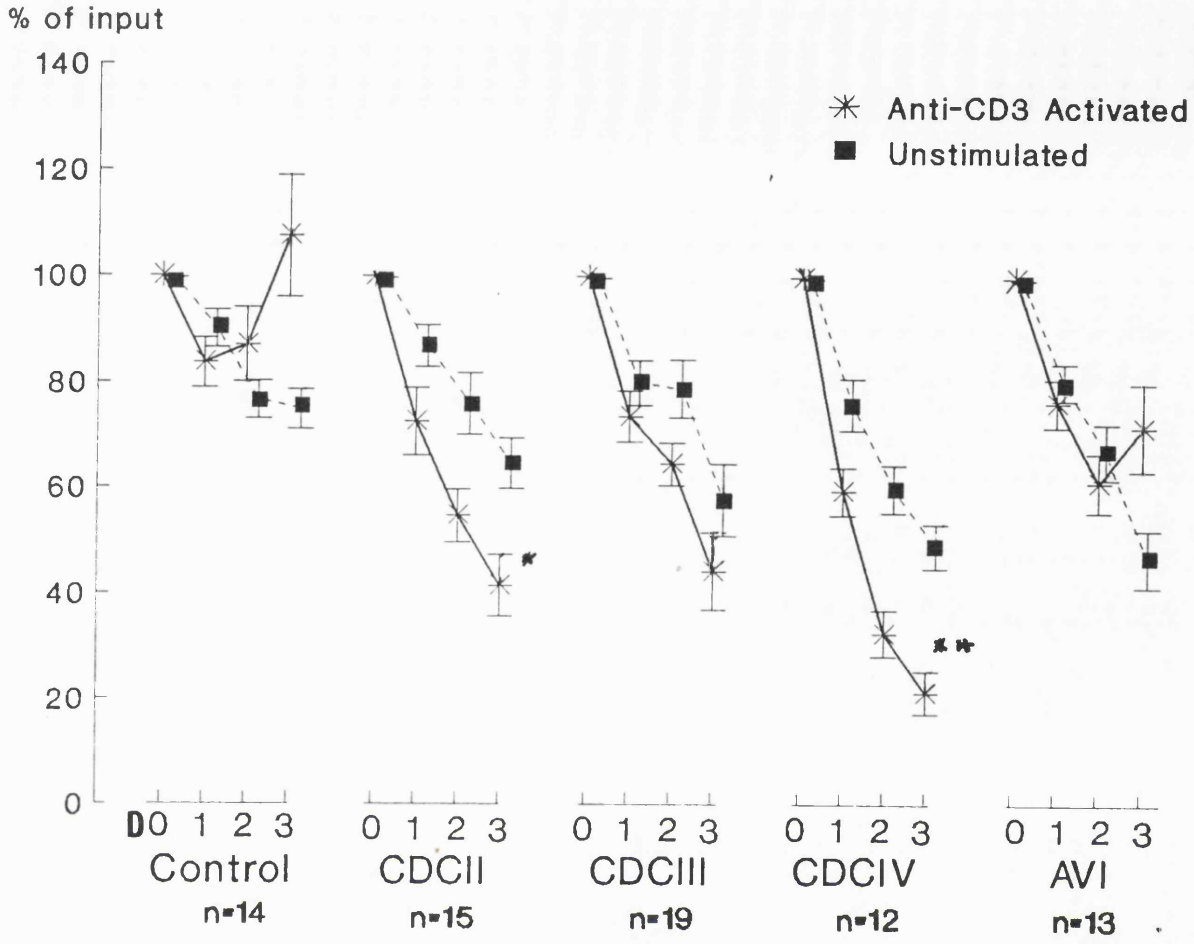


Figure 3.7 Time course for numbers of viable lymphocytes present after culture in the presence or absence of anti-CD3.

PBMC were cultured for the time periods shown and the numbers counted by microscopic examination using trypan blue exclusion to determine viability. The results are shown as the percentage of the initial input on day 0. The mean ± SEM are plotted. The mean values in the patient groups are compared to the HIV-1⁻ control in Table 3.1

Viable cells remaining on day 3 with or without stimulation were compared using the paired students t test; * $p<0.01$, ** $p<0.001$

Table 3.1: Comparison of mean values obtained after stimulation with anti-CD3[a]

	Patient Groups	Time in Culture Day1	Day2	Day3
Unstimulated lymphocytes	CDCII	ns	ns	ns
	CDCIII	ns	ns	0.03[b]
	CDCIV	0.023	0.01	0.001
	AVI	ns	ns	0.001
Anti-CD3 stimulated lymphocytes	CDCII	ns	0.001	0.001
	CDCIII	ns	0.006	0.001
	CDCIV	0.001	0.001	0.001
	AVI	ns	0.011	0.023
$CD4^+$ lymphocytes	CDCII	0.014	0.008	0.029
	CDCIII	ns	ns	ns
	CDCIV	0.035	0.002	0.001
	AVI	0.048	0.013	ns
$CD8^+$ lymphocytes	CDCII	ns	0.013	0.001
	CDCIII	ns	ns	0.003
	CDCIV	ns	0.001	0.001
	AVI	ns	ns	ns

[a] The mean number of viable cells in the test groups were compared to the corresponding value in the HIV-1^- control group using the Students t-Test.

[b] Significance level at which the null hypothesis was rejected

ns Not significant

cells die in response to a signal that would normally induce proliferation.

In AVI patients an increase in cell death after activation was not apparent. Although the number of lymphocytes recovered after 1-2 days stimulation declined in parallel with the unstimulated cells, by day 3 the cell number had risen to $72 \pm 8.3\%$ of input. This suggests that a proportion of the lymphocytes from these individuals will die spontaneously but that residual cells are able to respond normally to anti-CD3.

The name we have given to the increase in cell death which occurs in HIV-1^+ individuals after activation *in vitro* is activation associated lymphocyte death (AALD; Janossy, Borthwick, Lomnitzer et al 1993).

Comparison of AALD in $CD4^+$ and $CD8^+$ lymphocytes

To determine if AALD occurred in both $CD4^+$ and $CD8^+$ T lymphocytes, cultures from HIV-1^- control, HIV-1^+ and AVI individuals were investigated daily for proportional changes in these subsets (Fig. 3.8). Both $CD4^+$ and $CD8^+$ lymphocytes from the control group responded to the anti-CD3 stimulus and proliferated (day 3: CD4 $107 \pm 13.8\%$; CD8 $124 \pm 14.7\%$). However, there was a significant loss of cell viability in both the $CD4^+$ and $CD8^+$ populations from HIV-1^+ donors compared to the equivalent cells in the control group (day3: $CD4^+$ $p < 0.035$; $CD8^+$ $p < 0.003$; Table 3.1). The only exception occurred in the HIV-1^+, CDCIII donors where the $CD4^+$ lymphocyte population was less handicapped. Interestingly, in the AVI patients, the proliferation described previously in whole PBL was due to a preferential response within the $CD8^+$ subset (day 3: $CD4^+$ 55.4%; $CD8^+$ 124%).

AALD after activation with PHA

To determine if PHA also induced AALD in HIV-1^+ patients, lymphocytes from 10 HIV-1^+, CDCII patients were activated with PHA in the presence of rIL-2 and 30% conditioned medium as described above. In these short term cultures PHA induced a similar degree of proliferation in normal individuals when compared to anti-CD3 (day 3: PHA 115%; anti-CD3 107%). Using PHA, AALD was again observed in the HIV-1^+ cultures (day 3 $63.4 \pm 11.5\%$; $p < 0.02$) and there was some

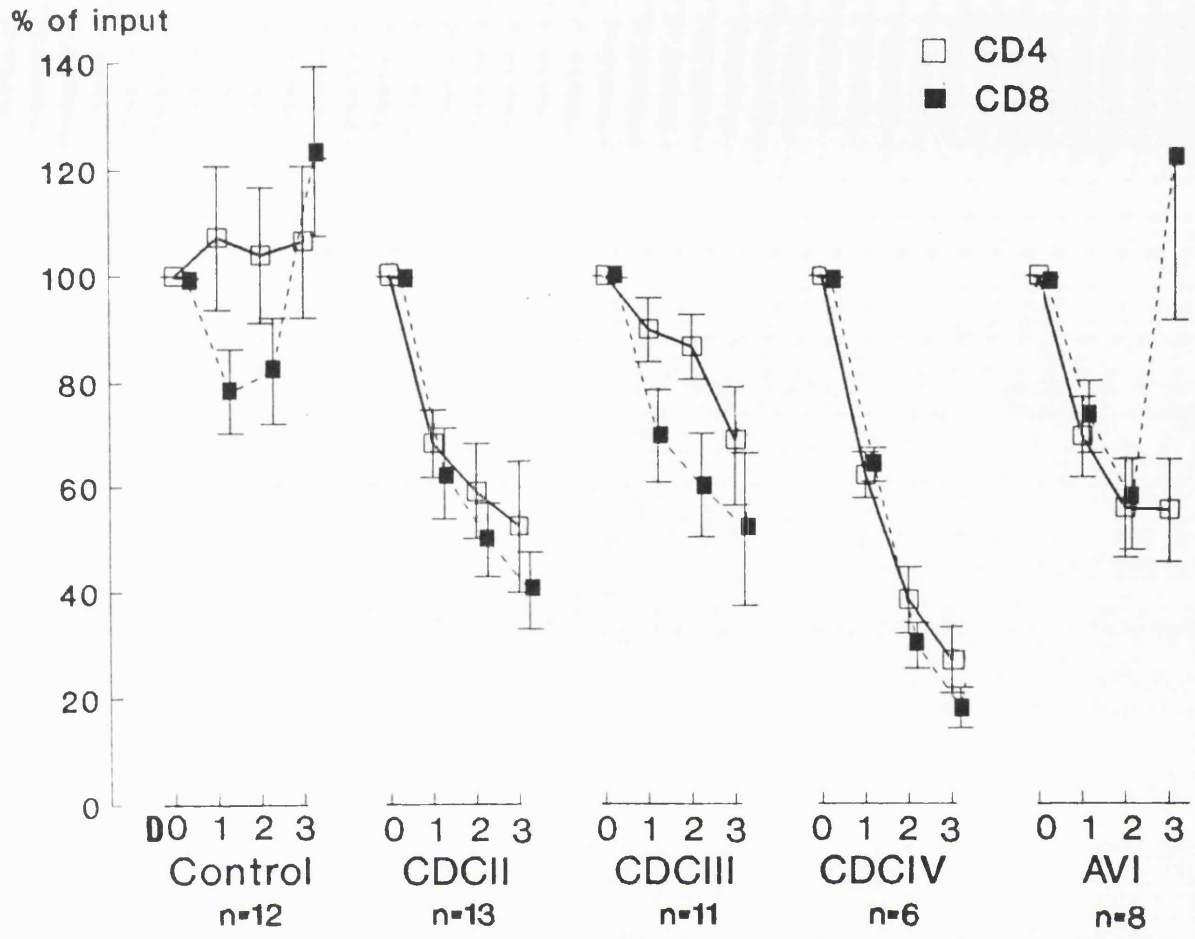


Figure 3.8 Time course for the numbers of $CD4^+$ and $CD8^+$ cells recovered from anti-CD3 stimulated cultures.

PBMC were cultured for the time periods shown and the number of cells counted by microscopic examination using trypan blue exclusion to determine viability. The proportion of $CD4^+$ and $CD8^+$ lymphocytes was measured daily by flow cytometric analysis using CD4-PE/CD8-FITC double IF. The results are shown as the percentage of the initial input on day 0. The mean ± SEM are plotted. The mean values in the patients groups are compared to the HIV-1^- control in Table 3.1

evidence that $CD8^+$ T cells were more affected by AALD than the $CD4^+$ subset (% of input; $CD4^+$ 75±19.5, $CD8^+$ 59±17.5).

To confirm that both $CD4^+$ and $CD8^+$ lymphocytes from HIV-1^+ individuals were affected by AALD, purified populations prepared by negative selection of E^+ cells (Chapter 2), were stimulated with PHA plus rIL-2 in the presence of 10% autologous non-T cells (Fig. 3.9). The data shown is from representative HIV-1^+, CDCII and CDCIV individuals and one normal control. As expected, the normal individual showed no evidence of spontaneous cell death in either lymphocyte subset and both of the isolated populations responded well to the stimulus. In the HIV-1^+, CDCII individual AALD was greatest in the $CD8^+$ subset (Day 3, % of input; CD4 91%, CD8 45%). However, in symptomatic AIDS (CDCIV), both $CD4^+$ and $CD8^+$ subsets showed evidence of AALD (Day 3, % of input; CD4 37%, CD8 35%). This supports the data in Fig. 3.6 showing that $CD4^+$ cells are less susceptible to AALD early in HIV-1 disease. However, when similar experiments were carried out using anti-CD3 as the stimulus, both $CD4^+$ and $CD8^+$ subset from a range of patients were handicapped (eg HIV-1^+, CDCII, $CD4^+$ 48%, $CD8^+$ 20%) indicating that the stimulus used can effect the degree of AALD in the $CD4^+$ subset.

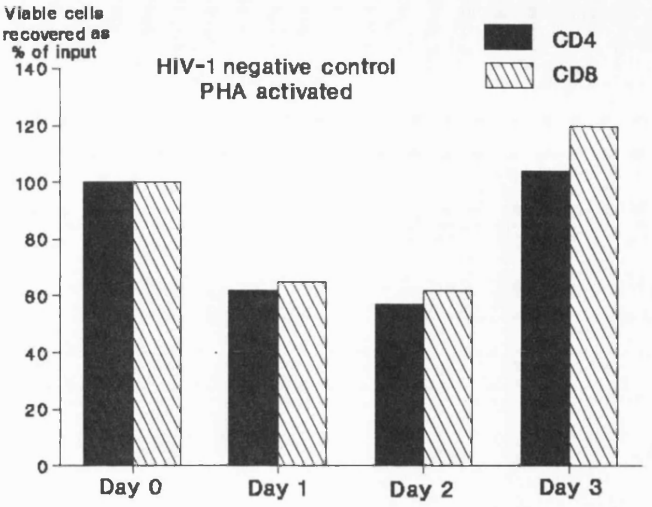
Exogenous IL-2 does not prevent AALD

As the defective proliferative responses described above occurred even in the presence of exogenous IL-2 and PHA-conditioned medium, it was unlikely that a deficiency in IL-2 alone was responsible for the effect. To confirm this, lymphocytes from HIV-1^- controls, HIV-1^+ individuals with high $CD4^+$ lymphocyte counts ($>400/mm^3$) and AVI patients were cultured alone or stimulated with either IL-2, anti-CD3 or anti-CD3 plus IL-2 (Table 3.2). This group of HIV-1^+ individuals were chosen to ensure that any proliferative deficiency was not simply due to low numbers of $CD4^+$ lymphocytes. In the HIV-1^- control group, the presence of IL-2 alone did not significantly alter cell viability after 3 days in culture but it did have a mild enhancing effect on the response to anti-CD3. In HIV-1^+ individuals, IL-2 reduced the degree of spontaneous cell death (% cell recovery; unstimulated 57%, IL-2 70%) but did not reverse the AALD phenomenon (% cell recovery; anti-CD3

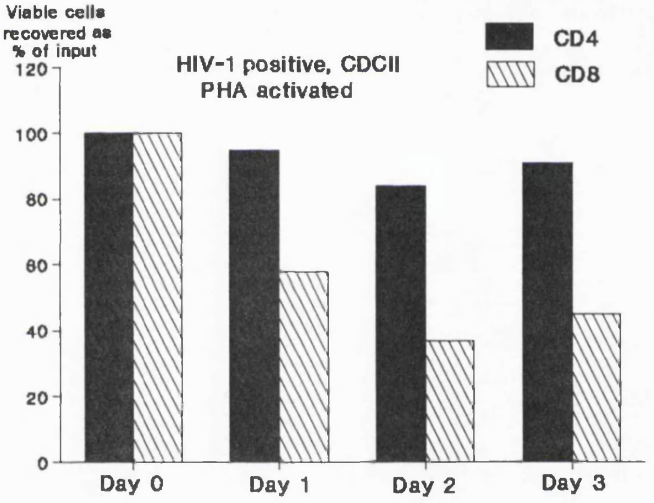
Figure 3.9 The survival of purified $CD4^+$ and $CD8^+$ T cell subsets after 3 days culture in the presence or absence of PHA.

T cell subsets were purified from E^+ T cells by negative depletion procedures and cultured either alone or together with 10% autologous non-T cells rIL-2 (1ng/ml) and PHA. The numbers of viable lymphocytes were enumerated daily using microscopy and trypan blue exclusion. The data are shown as a percentage of the initial input of $CD4^+$ or $CD8^+$ cells at the start of the culture.The results from one representative HIV-1^+ CDCII (A), CDCIV (B) and HIV-1^- (C) individual are shown.

(A)


Viable cells recovered as % of input
HIV-1 negative control
PHA activated
CD4
CD8
140
120
100
80
60
40
20
0
Day 0
Day 1
Day 2
Day 3

(B)


Viable cells recovered as % of input
HIV-1 positive, CDCII
PHA activated
CD4
CD8
120
100
80
60
40
20
0
Day 0
Day 1
Day 2
Day 3

(C)

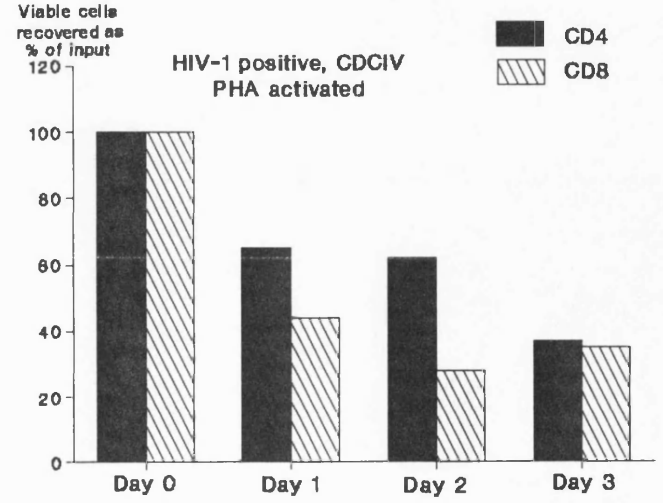

Viable cells recovered as % of input
HIV-1 positive, CDCIV
PHA activated
CD4
CD8
120
100
80
60
40
20
0
Day 0
Day 1
Day 2
Day 3

Table 3.2 The effect of rIL-2 on lymphocyte survival after culture *in vitro*[a]

	Control	IL-2	anti-CD3	anti-CD3 + rIL-2
HIV-1^{-}[b]	73±2.2[c]	78±5.8	103±9.8	112±10.7
HIV-1^{+}	57±3.3	70±8.0	30±4.1	27±4.9
AVI	48±5.2	66±3.1	41±5.0	40±5.6

[a] Lymphocytes were cultured in the presence or absence of rIL-2 or anti-CD3 for 3 days and the number of viable cells enumerated.
[b] Five individuals in each group were investigated.
[c] The results are viable cells recovered as percentage of input; mean ± sem

30%, anti-CD3 + IL-2 27%; Table 3.2). Similarly, the addition of IL-2 improved the survival of lymphocytes from AVI patients in these short term cultures but had no effect on anti-CD3 induced stimulation. These results suggest that IL-2 has a pronounced effect on the survival characteristics of unstimulated cells which may require the presence of the growth factor to live. However, AALD occurs even in the presence of IL-2 indicating that this cytokine is not the limiting factor.

The correlation between AALD and the absolute $CD4^+$ lymphocyte count

To determine if the activation defect correlated with disease progression, lymphoblast recovery after activation with anti-CD3 or PHA was compared to the absolute $CD4^+$ lymphocyte count (Table 3.3). Using regression analyses, the response of both $CD4^+$ and $CD8^+$ lymphocyte subsets to anti-CD3 stimulation correlated well with the $CD4^+$ lymphocyte count ($p<0.05$). However, when PHA was used as the stimulus, a significant correlation was seen only in the response of $CD8^+$ cells ($p<0.001$). The response of this subset to PHA stimulation was also found to correlate well with the rate of decline of $CD4^+$ lymphocyte counts in individual patients ie the faster the $CD4^+$ lymphocytes declined, the more pronounced was the handicap in the $CD8^+$ subset after stimulation (data not shown).

AALD occurs even in the presence of normal cells

The next experiment was designed to more clearly establish the importance of the culture conditions in AALD. In this assay PBMC from HIV-1^- and HIV-1^+ individuals were activated with PHA simultaneously in the same well. The donors were identified in these mixed cultures by their differential expression of HLA-A2, measured in double labelling combinations with CD4 and CD8. As the activation stimulus given by PHA is stronger, and the response faster, than seen in a mixed lymphocyte reaction (MLR), the PHA response effectively masks the allogeneic reaction. The results from one representative experiment are shown in Fig. 3.10. Although the original input of the two donors was the same, there was a rapid decline in the total number of lymphocytes from the HIV-1^+ donor until by day 5 only 36% of the original number remained viable. In contrast, the number of lymphocytes from the HIV-1^- donor had risen to 164% of input. Similarly, the

Table 3.3 Linear regression analyses of activation data compared to $CD4^+$ lymphocyte count[a]

Mitogen Used	Total Lymphocytes	$CD4^+$ Lymphocytes	$CD8^+$ Lymphocytes
anti-CD3 (n=57)	-0.298[b] *(0.030)*	-0.286 *(0.040)*	-0.272 *(0.050)*
PHA (n=66)	-0.523 *(0.001)*	*ns*[c]	-0.634 *(0.001)*

[a] Linear regression analyses were performed to determine if defective proliferation responses correlated with $CD4^+$ lymphocyte counts.

[b] The regression coefficient "r" is shown with the significance in brackets

[c] ns, not statistically significant

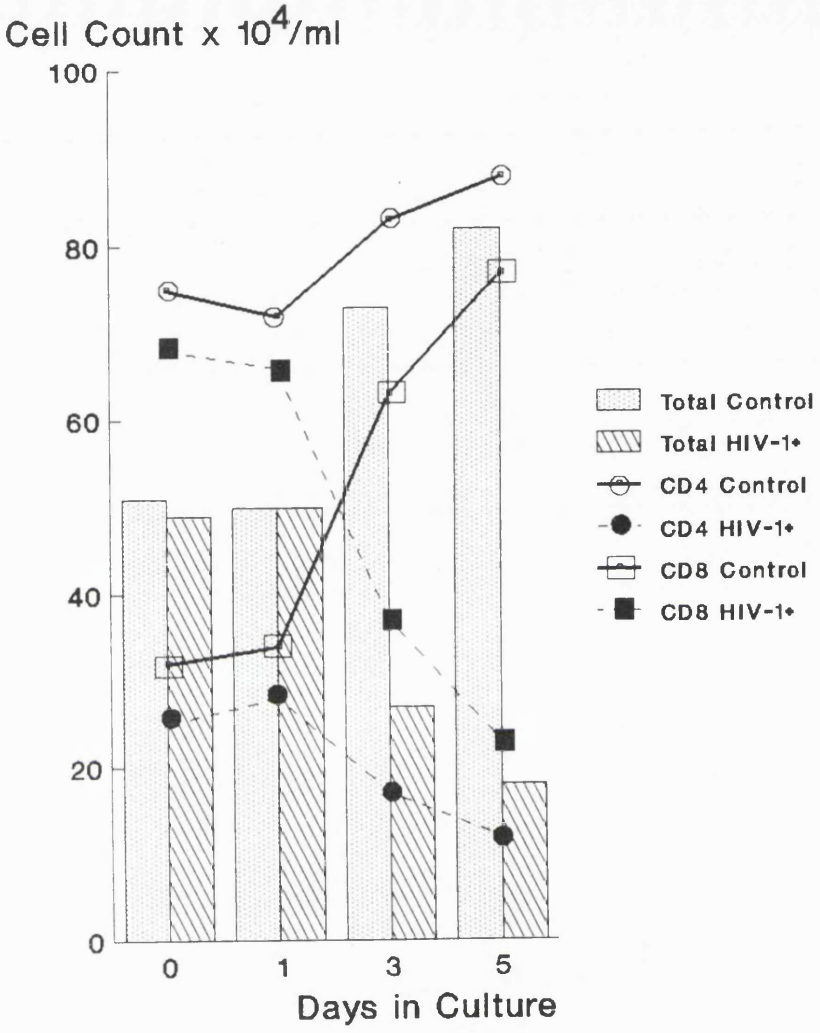


Figure 3.10 Representative mixing experiment using HLA-A2 disparate lymphocytes from HIV-1$^-$ (HLA-A2$^-$) and HIV-1$^+$ (HLA-A2$^+$) donors.

Lymphocytes were set up in equal numbers (50%) and incubated in the presence of PHA plus rIL-2 for the time periods shown. The total number of viable lymphocytes and the number for each individual were determined by microscopic examination in combination with FACScan analysis.

number of $CD4^+$ and $CD8^+$ lymphocytes from the HIV-1^+ donor declined ($CD4^+$; 48% and $CD8^+$; 34%) while the normal cells proliferated ($CD4^+$; 117% and $CD8^+$; 240%). This experiment indicates that differences in growth patterns between the cells are not attributable to culture conditions but are inherent within the cells.

AALD also occurs in lymphoid tissue

In three HIV-1^+ individuals it was possible to measure activation responses in cell suspensions prepared from biopsied lymph nodes. All three individuals were diagnosed as PGL (CDCIII). Two of the patients showed very poor activation responses after stimulation with PHA (T cells recovered on day 3 as % of input; patient 1 40%, patient 2 22%) while the third responded well (patient 3 99.9%). In the latter case, the addition of rIL-2 greatly improved lymphocyte recovery (control unstimulated 17.3%; IL-2 alone 20.4%; PHA 61.1%; PHA IL-2 99.9%)

In one patient it was possible to directly compare the activation responses of $CD4^+$ and $CD8^+$ subsets in lymph node and peripheral blood. These were found to be comparable (CD4 blood 40%, lymph node 46%; CD8 blood 54%, lymph node 35%) indicating that T cells in lymphoid tissue also have defective activation responses.

Discussion

The inability of lymphocytes from HIV-1^+ individuals to respond to antigenic stimuli is thought to play a crucial role in the pathogenesis of AIDS. The failure to respond normally not only to the HIV-1 virus but also new or pre-existing pathogens ultimately causing the disease condition. In this chapter these defective responses were further investigated using the T cell mitogens PHA and anti-CD3. It was found that peripheral blood T cells from HIV-1 infected individuals die after short term culture *in vitro*. Thus, many of the proliferative defects reported in HIV-1 infection may be due to lymphocyte death.

Two types of lymphocyte death were noted; spontaneous death and AALD. The spontaneous death occurred when lymphocytes were cultured in the absence of any stimuli or growth factors and was most prevalent in AVI patients and those with

AIDS. Previous studies have also shown that PBL from HIV-1 infected individuals die rapidly after culture in the absence of any activation stimuli (Prince & Jensen 1991a, Gougeon et al 1992). Such spontaneous cell death has also been reported in patients with acute EBV (Moss et al 1985, Uehara et al 1992). In both cases cell death was detected primarily in the $CD8^+$ lymphocyte subset. In contrast, cell death after activation was limited to HIV-1^+ individuals and was of a greater magnitude than spontaneous death. Such activation induced death in HIV-1 infection was also reported by Meyaard et al (1992) after anti-CD3 stimulation and by Groux et al (1992) after stimulation with the bacterial superantigen, staphylococcus enterotoxin B (SEB). Stimulation with anti-CD3 induced cell death in both $CD4^+$ and $CD8^+$ subsets although in patients with high $CD4^+$ lymphocyte counts the $CD8^+$ subset were more handicapped (Meyaard et al 1992). Conversely, using SEB activation-induced death occurred only in the $CD4^+$ subset. In this study, using flow cytometric and microscopic techniques, it was found that both $CD4^+$ and $CD8^+$ T cell subsets were susceptible to AALD induced by anti-CD3 or PHA, however the degree of handicap was greatest in the $CD8^+$ subset, particularly when PHA was used as the stimulus. Thus the PHA response of CD4+ cells from asymptomatic patients was less affected by AALD than the $CD8^+$ subset. However, in the later stages of HIV-1 disease, the $CD4^+$ subset became more vulnerable. This is in agreement with other studies which have shown the relative maintenance of PHA responses early in HIV-1 disease (Groux et al 1992).

It has been postulated that apoptosis of $CD4^+$ lymphocytes provides one mechanism for immunosuppression in HIV-1 infection. This has been shown to occur both as a direct result of infection with HIV-1, and after stimulation of uninfected $CD4^+$ T cells with viral gp120 on their surface (Banda et al 1992). Similarly, spontaneous cell death in HIV-1 and EBV infection occurs by apoptosis (Gougeon et al 1992; Uehara et al 1992) and it was reported that the cell death induced by anti-CD3 or by the superantigen SEB also occurred by apoptosis (Groux et al 1992, Meyaard et al 1992). This implies that apoptosis may act in $CD4^+$ and $CD8^+$ T cell populations in HIV-1 infection and is involved in the functional deficiencies of both populations.

Not all of the lymphocytes underwent AALD; those that transformed

proliferated normally and could be used to generate cell lines. This indicates that a particular subset within the $CD4^+$ and $CD8^+$ populations is vulnerable to AALD. In support of this, it has been shown by Pantaleo et al (1990a,b) that $CD8^+$,$HLA\text{-}DR^+$ lymphocytes from HIV-1 infected individuals have a particularly poor clonogenic potential after stimulation with anti-CD3 or PHA. Similarly, in normal individuals, cells which express CD57 do not transform after mitogenic stimulation (Campana Coustan-Smith & Janossy 1988). Cells of this phenotype are expanded in HIV-1 infection (Stites et al 1986, Ziegler-Heitbrock et al 1988) and may contribute to the AALD phenomenon. Therefore, changes in the composition of peripheral T cells might greatly effect AALD.

The reason why activation results in death rather than proliferation is not known although it has been demonstrated in other systems that both processes are intimately linked (Kabelitz Pohl & Pechhold 1993). Many transformed lymphoid cell lines and bone-marrow derived cells have strict growth factor requirements for culture *in vitro*. For instance, the cytotoxic T cell line CTLL proliferates in response to IL-2 but will die rapidly in the absence of the cytokine. Therefore, growth factor deprivation provides one means whereby cells die rather than proliferate. In $HIV\text{-}1^+$ infection, the depletion of $CD4^+$ lymphocytes results in a deficit of IL-2 which adversely effect activation responses *in vitro*. A number of studies on $HIV\text{-}1^+$ patients have shown that when cells are stimulated with specific antigen (Wahren et al 1987) or mitogens (Ciobanu et al 1983, Lifson et al 1984, Gluckman et al 1985, Gruters et al 1990) poor proliferative responses could be enhanced by the addition of exogenous IL-2. However, IL-2 did not completely reverse the defect and responses did not return to normal levels. In this study, cultures were routinely supplemented with PHA conditioned medium and rIL-2 but defective blastogenic responses were found even in individuals with high $CD4^+$ counts. To further eliminate a cytokine deficiency as the cause of AALD, PBMC from normal and $HIV\text{-}1^+$ individuals were co-cultured and activated with PHA in the same well. In this way the T cells from both individuals were exposed to identical culture conditions and antigen presenting cells (APC). Under these conditions, only the $HIV\text{-}1^+$ donor showed any evidence of AALD, suggesting that the defect is inherent within each cell and does not result from a cytokine or APC deficiency.

The activation of T cells requires not only interactions between the TCR and MHC but also involves co-stimulatory signals provided by accessory molecules on APC (Meuller Jenkins & Schwartz 1989). Several pairs of receptor:ligand interactions have been identified as co-stimulatory for activation via the TCR. These include B7/BB1:CD28, CD58(LFA-3):CD2, CD54(ICAM-1):LFA-1 and CD72:CD5 (Linsley et al 1991; Van Seventer et al 1990, Van Seventer et al 1991, Imboden et al 1990). Among these, co-stimulation through CD28 provides the strongest accessory signal described so far. Therefore, defects in accessory molecule/ligand interactions might account for AALD.

CHAPTER 4

THE ACTIVATION RESPONSE OF PRIMED AND UNPRIMED LYMPHOCYTES

Introduction

Recall responses to soluble antigens are a feature of primed/memory T cells. Such recall responses are defective in HIV-1 infection both *in vivo*, as manifest by a loss of skin test responsiveness to a range of commonly encountered pathogens (Reuben et al 1983, Fernandez-Cruz et al 1988) and *in vitro* as assessed by antigen stimulated proliferation and IL-2 production (Lane et al 1985; Hofmann et al 1985; Prince et al 1986; Clerici et al 1989a,b; Quinti et al 1991). This is one of the earliest defects in T cell function and can occur before $CD4^+$ lymphocytes fall below $400/mm^3$ but while cells still retain responses to other stimuli such as PHA and alloantigens (Lane et al 1985, Miedema et al 1988, Clerici et al 1989b). The loss of recall responses has led to a general hypothesis that HIV-1 infection causes early T memory cell defects and that this is an important component in the pathogenesis of the disease (Clerici 1989b, Miedema Tersmette & Van Lier 1990, Shearer & Clerici 1990).

Although there is no definitive marker for memory T cells, it has been suggested that differential expression of two of the isoforms of the leucocyte common antigen (LCA, CD45) can split human T cells into unprimed ($CD45RA^+$) and primed ($CD45RO^+$) populations (Beverley 1987, Akbar et al 1988). This has been proposed for a number of reasons: Firstly and perhaps most importantly, proliferative responses to recall antigens *in vitro* are retained exclusively within $CD45RO^+$ T cell population (Merkenschlager et al 1988, Beverley, Merkenschlager & Terry 1988) while primary T cell proliferative responses are restricted to $CD45RA^+$ T cells (Plebanski et al 1992). Secondly, the $CD45RO^+$ subset performs a number of effector functions either exclusively or more efficiently than the reciprocal $CD45RA^+$ cells. For instance, $CD4^+CD45RO^+$ cells provide help for specific antibody production while those which express the CD45RA do not (Morimoto et al 1985a, Beverley Terry & Pickford 1986). Similarly, although allo-specific $CD8^+$ CTL can be generated from both $CD45RA^+$ and $CD45RO^+$ populations (Merkenschlager & Beverley 1989, Yamashita & Clement 1989, Akbar

et al 1990) the most efficient CTL effector cells are $CD8^+CD45RO^+$ (Yamashita & Clement 1989, Akbar et al 1990). Also, EBV specific CTL precursors are predominantly found within the $CD8^+CD45RO^+$ subset (Merkenschlager & Beverley 1989). In view of their functional activity it is therefore not surprising that $CD45RO^+$ T cells express more adhesion molecules than the reciprocal subset (Sanders et al 1988, Buckle & Hogg 1990) and have a much wider cytokine profile (Reviewed in Akbar, Salmon & Janossy 1991). These findings together with the low levels of CD25 on $CD45RO^+$ lymphocytes (Wallace & Beverley 1990) suggest that $CD45RO^+$ cells might represent an activated population. Thirdly, although CD45RA and CD45RO are reciprocally expressed on resting T cells, after activation with mitogens such as PHA (Byrne, Butler & Cooper 1988; Akbar et al 1988; Sanders et al 1989) or with allo-antigen (Akbar et al 1988) $CD45RA^+$ T lymphocytes lose expression of this antigen and acquire CD45RO while activated $CD45RO^+$ cells retain this phenotype. Finally, CD45RO is low or absent on T cells from cord blood (Bofill et al 1994) but increases with age (Hayward Lee & Beverley 1989, Gabriel Schmitt & Kinder 1993), a feature expected of a primed cell marker. Although there is some speculation that $CD45RO^+$ T cells can revert to a $CD45RA^+$ phenotype (Brod et al 1989; Bell & Sparshott 1990; Warren & Skipsey 1991; Michie et al 1992) the expression of either CD45RA or CD45RO on T cells remains a reliable method of distinguishing between unprimed and recently activated lymphocyte populations.

Studies on the expression of CD45RA and CD45RO on T cells in HIV-1 inefection have shown an expansion of the $CD8^+CD45RO^+$ lymphocyte subset in the peripheral blood of infants (Froebel et al 1991, Borkowsky et al 1992) and adults (Prince & Jensen 1991b). Similar investigations on lymph nodes from HIV-1 infected individuals also show an accumulation of $CD8^+CD45RO^+$ lymphocytes within the paracortical areas and infiltrating the germinal centres (Racz et al 1990; Janossy et al 1991).

In this chapter the reported loss of memory responses in HIV-1 infection was investigated in two ways. Firstly, the presence of primed cells, defined by CD45RO, in the $CD4^+$ and $CD8^+$ subsets was measured by flow cytometry to determine if HIV-1 infection resulted in a depletion of memory cells. Secondly, the ability of

both primed and unprimed cells to respond to mitogenic stimulation was investigated to determine if these subsets were functioning normally.

Results

The accumulation of $CD8^+CD45RO^+$ lymphocytes in HIV-1^+ and AVI patients

Initial flow cytometric studies were performed to determine the proportion of $CD4^+$ and $CD8^+$ lymphocytes expressing CD45RA and CD45RO. In normal individuals CD45RA and CD45RO are expressed on $CD4^+$ T cells at a ratio of approximately 1:1. Although HIV-1 infection caused a depletion of $CD4^+$ lymphocytes the remaining cells showed no significant differences in the proportions of CD45RA and CD45RO in the $CD4^+$ T cell subset compared to the seronegative control group (Control: CD45RA 43 ± 5.7, CD45RO 49 ± 4.4, $n=10$; HIV-1^+: CD45RA 38.8 ± 4.8, CD45RO 40.3 ± 3.6, $n=20$). Therefore, HIV-1 infection does not result in a preferential depletion of $CD4^+CD45RO^+$, primed lymphocytes. In contrast, patients with acute viral infections showed a shift towards a $CD45RO^+$ phenotype (CD45RA 27.5 ± 4.5, CD45RO 72.5 ± 4.5; $n=7$)

Cells falling within a normal lymphocyte gate on the FACScan, have a tri-modal distribution of CD8 with a clearly distinguishable $CD8^{high}$, $CD8^{low}$ and $CD8^-$ populations. In both normal and HIV-1^+ individuals, the majority of $CD8^+$ lymphocytes were $CD8^{high}$. The minor $CD8^{low}$ cells were $CD3^-$, expressed the NK associated markers CD56, CD16 or CD57 and were $CD45RA^+$ (Fig. 4.1). Although this population was not expanded in HIV-1 infection a small population of $CD3^+CD8^{low}$ cells were found in a number of HIV-1^+ and AVI patients and therefore all $CD8^+$ cells were included in the phenotypic study.

The total percentage of $CD8^+$ lymphocytes in peripheral blood was increased compared to the seronegative control in each of the patient groups investigated ($p<0.001$, Table 4.1). In the HIV-1 seropositive donors this was due both to a loss of $CD4^+$ lymphocytes and a rise in the absolute number of $CD8^+$ cells. The greatest increase in $CD8^+$ lymphocytes was found in the AVI patients who showed a massive rise of 700% over the control group ($p<0.001$) but significant increases were also seen in each of the HIV-1^+, CDC categories ($p<0.005$ & $p<0.05$; Table 4.1). The $CD8^+$ lymphocytes from seronegative controls expressed CD45RA and

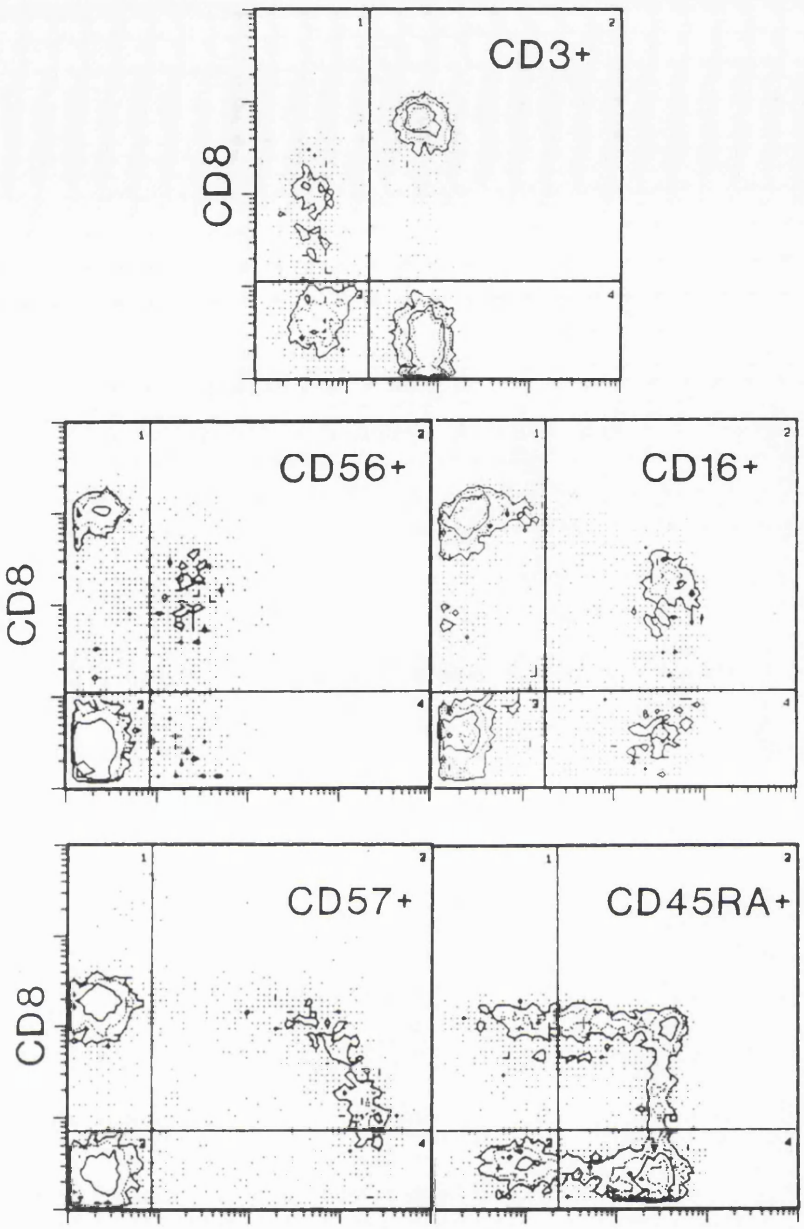


Figure 4.1 FACScan profile of normal $CD8^+$ lymphocytes

$CD8^+$ lymphocytes (y-axis) were stained in double combination with CD3 (A), the NK markers CD56 (B), CD16 (C) and CD57 (D) and CD45RA (E). Note that the $CD8^{low}$ population is mainly $CD3^-$ and expresses NK cell markers.

Table 4.1 Changes in $CD8^+$ lymphocyte populations during HIV-1 infection[a]

Patient Groups	n	Percentage of lymphocytes[b]			Total absolute counts/mm^3		
		Total $CD8^+$	$CD8^+$ $CD45RA^+$	$CD8^+$ $CD45RO^+$	Total $CD8^+$	$CD8^+$ $CD45RA^+$	$CD8^+$ $CD45RO^+$
Normal Control	14	28.4 ± 1.6[c]	72.5 ± 3.4	27.1 ± 3.2	581 ± 65	412 ± 57	169 ± 24
HIV-1^+CDCII	11	$46.3 \pm 2.4^{****}$	$53.7 \pm 4.0^{***}$	$41.6 \pm 4.2^{**}$	$755 \pm 61^{*}$	406 ± 45	$305 \pm 35^{***}$
CDCIII	11	$46.4 \pm 4.9^{****}$	63.4 ± 7.2	25.3 ± 5.6	$850 \pm 123^{***}$	$602 \pm 66^{*}$	264 ± 73
CDCIV	11	$67.2 \pm 2.5^{****}$	$45.1 \pm 4.7^{****}$	$48.1 \pm 4.7^{****}$	$898 \pm 216^{***}$	340 ± 70	$468 \pm 129^{*}$
AVI	11	$48.8 \pm 5.1^{****}$	$37.0 \pm 6.3^{****}$	$64.7 \pm 6.9^{****}$	$4{,}018 \pm 1720$	851 ± 396	$3{,}237 \pm 1{,}418$

[a] The expression of CD45RA and CD45RO on CD8+ lymphocytes was investigated by two colour immunofluorescence.

[b] The percentage of $CD8^+CD45RA^+$ and $CD8^+CD45RO^+$ lymphocyte populations are depicted in Figure 4.3

[c] Mean $\pm$ SD; mean values were compared to the normal control using the Students t-Test; * $p<0.05$, ** $p<0.01$, *** $p<0.005$ and **** $p<0.05$

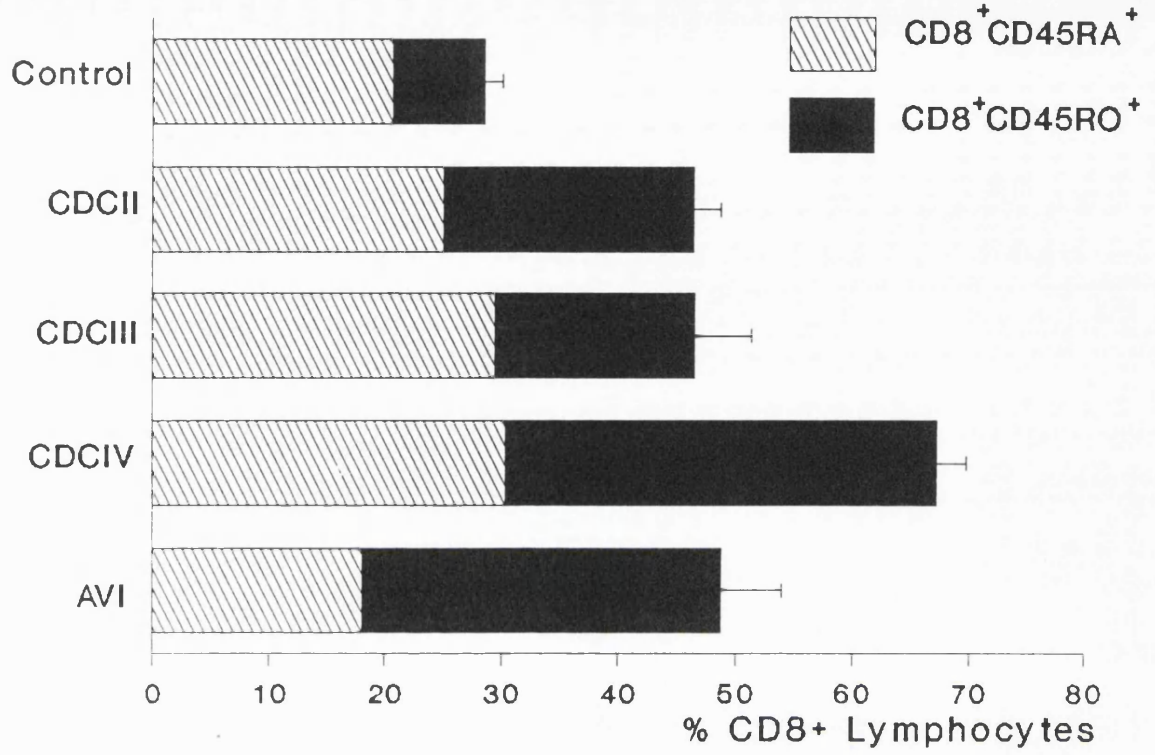


Figure 4.2 CD45RA and CD45RO expression within the $CD8^+$ subset

PBMC from 14 normal individuals, 33 HIV-1^+ and 11 AVI patients were investigated by two colour indirect IF using the combinations CD8μ plus GAM IgM-PE and either CD45RA or CD45RO plus GAM IgG-FITC. During data acquisition a live gate was set up around the lymphocytic, $CD8^+$ population and the percentage of $CD45RA^+$ or $CD45RO^+$ cells within this determined. The total length of the bars represents the mean percentage of $CD8^+$ cells $\pm$ sem, with the relative percentages of $CD45RA^+$ (hatched) and $CD45RO^+$ (filled) shown within.

CD45RO in a ratio of 3:1. In all virally infected patients this ratio was reduced due to an increase in $CD8^+CD45RO^+$ lymphocytes (Fig. 4.2). The absolute numbers of $CD8^+CD45RA^+$ lymphocytes in HIV-1^+, CDCII/IV patients were similar to those found in the control group but the number of $CD8^+CD45RO^+$ cells was significantly increased ($p<0.005$, $p<0.05$), indicating that this population is expanded in HIV-1 infection. Similarly, the $CD8^+$ lymphocytosis seen in the AVI group was due almost exclusively to a rise in the absolute number of $CD8^+CD45RO^+$ cells. Therefore, the increase in $CD8^+$ lymphocytes seen in both the acute and chronic stages of viral infection are due primarily to an expansion of the $CD8^+CD45RO^+$ population while the $CD8^+CD45RA^+$ remains relatively unaltered.

The predominant proliferative defects of $CD45RO^+$ lymphocytes from HIV-1^+ patients

Purified $CD45RA^+$ or $CD45RO^+$ cells prepared by negative selection from E^+ T cells as described (Chapter 2) were stimulated with anti-CD3 Mab plus rIL-2 in the presence of 10% autologous non-T-cells and the number of viable $CD4^+$ and $CD8^+$ lymphocytes determined daily by flow cytometric analysis (Fig. 4.3). The responses of HIV-1^+, CDCII individuals and normal seronegative controls were compared to determine if either of the $CD45RA^+$ or $CD45RO^+$ populations were handicapped early in the disease. In HIV-1^- individuals, cell recovery from both $CD45RA^+$ and $CD45RO^+$ cultures was high (>80%) and little difference was found between the response of either subset. However, in HIV-1^+ individuals differences were found between the responses of the $CD45RA^+$ and $CD45RO^+$ lymphocytes. In the $CD45RA^+$ purified population the percentage of viable cells decreased to 55% after 2 days in culture but recovered to 90% by day 4, indicating that these cells were actively proliferating. In contrast, by day 2 60% of the $CD45RO^+$ cells had died and by day 3-4 only 20% of the initial input was recovered. When examined more closely, it was clear that more lymphoblasts were found in the $CD45RA^+$ cultures from HIV-1^+ patients compared to the $CD45RO^+$. This is illustrated in Fig. 4.4 which shows an example of the FACScan profile obtained from $CD45RA^+$ or $CD45RO^+$ cells after stimulation with anti-CD3 for 3

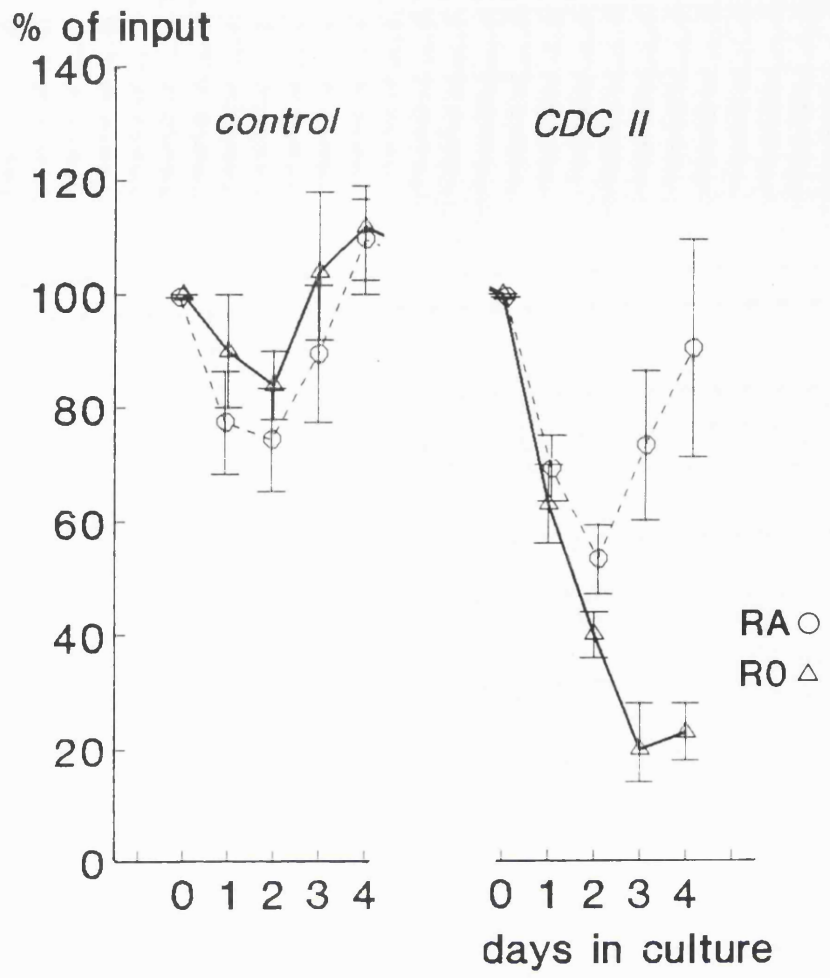


Figure 4.3 Time course of the numbers of CD45RA$^+$ and CD45RO$^+$ cells recovered from anti-CD3 stimulated cultures.

Purified CD45RA$^+$ and CD45RO$^+$ T cells were prepared from E$^+$ T cells by negative selection and stimulated with anti-CD3 plus rIL-2 in the presence of 10% autologous non-T cells. The number of viable cells was determined at the time points shown. The data is shown as the mean percentage of cells recovered in relation to the initial input of CD45RA$^+$ or CD45RO$^+$ on day 0 for 10 normal and 20 HIV-1$^+$, CDCII individuals.

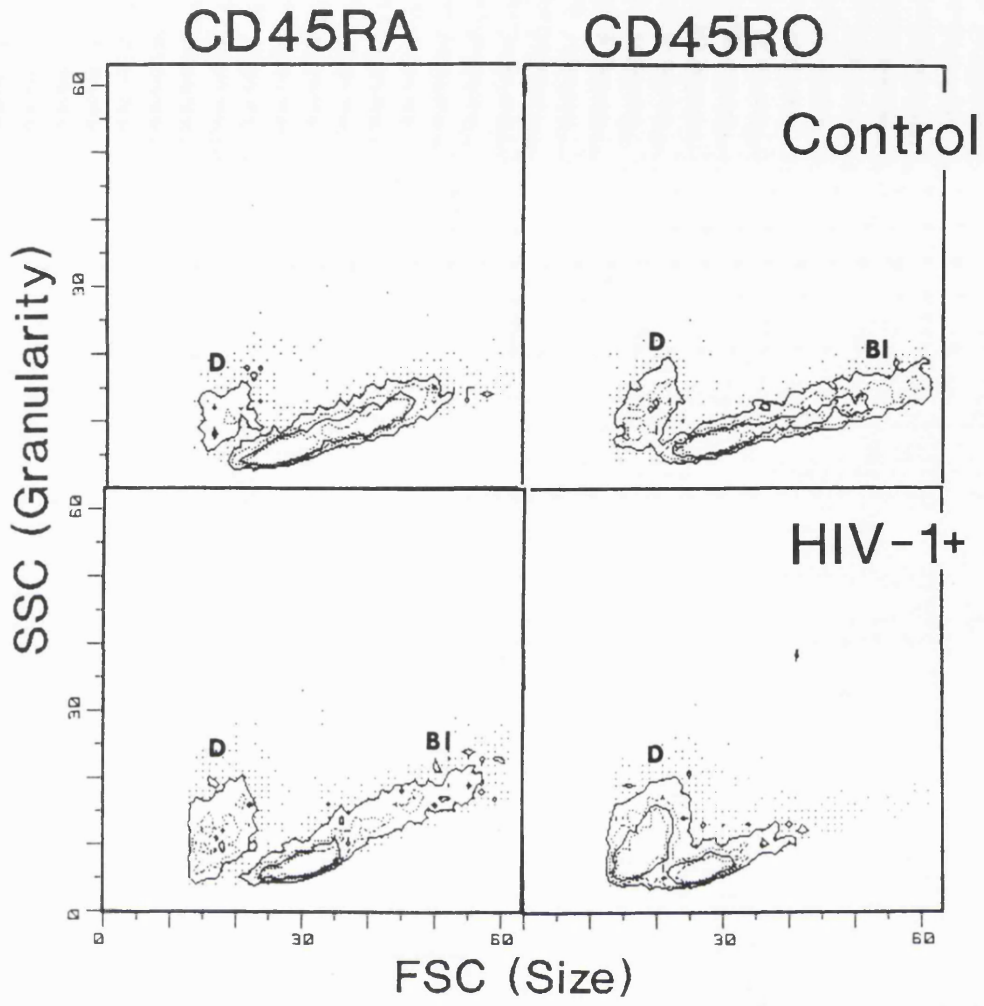


Figure 4.4 The appearance of CD45RA$^+$ and CD45RO$^+$ cells after stimulation

FACScan profile of the appearance of CD45RA$^+$ and CD45RO+ lymphocytes from a representative normal and HIV-1$^+$ patients after stimulation with anti-CD3 for 3 days. The forward scatter (FSC; size; x-axis) and side scatter (SSC; granularity; y-axis) profiles of purified CD45RA$^+$ and CD45RO$^+$ lymphocytes are shown after culture with anti-CD3 Mab. Large lymphoblasts (bl) are seen in both populations from normal individuals and the CD45RA$^+$ population from the CD45RA$^+$ cells from the HIV-1$^+$ patient. These are absent in the CD45RO$^+$ population and are replaced by dead cells and debris (d).

days. In the seronegative individual both subsets responded to the stimulus and transformed into lymphoblasts. However, in the HIV-1 infected patient more lymphoblasts (bl) were found in the $CD45RA^+$ culture and larger amounts of dead cells (d) and cell debris, but few blasts, were seen in the $CD45RO^+$.

When $CD4^+$ and $CD8^+$ lymphocytes were compared, all four populations in HIV-1^+ patients were found to be affected by AALD although the degree of handicap was greatest in the $CD8^+CD45RO^+$ cells, as shown in Fig. 4.5 in a representative HIV-1^+ patient and seronegative control. Anti-CD3 preferentially stimulated the $CD8^+$ cells in the control and proliferation was greatest in the $CD8^+CD45RO^+$ cells (250%), numerically the smallest subset. A loss of viability was seen in each of the four subsets in the HIV-1^+ individual but the greatest proportional loss was found in the $CD8^+CD45RO^+$ population (57%). This pattern of cell loss was found consistently in all the HIV-1^+ patients investigated (n=10), although the degree of AALD in each of the subsets varied considerably between individuals.

The preferential defect in $CD45RO^+$ lymphocytes also occurs in unseparated PBMC

The activation response of primed and unprimed lymphocytes was also examined in unseparated PBMC in order to ensure that the findings described previously were not due to cytokine deficiencies which might occur when $CD45RA^+$ or $CD45RO^+$ subsets are cultured alone. Mononuclear cells from a range of HIV-1^+ patients and seronegative controls were triple stained with the following directly conjugated reagents, CD3-FITC; CD45RA-PE and CD45RO-Biotin plus streptavidin-tandem, prior to stimulation and after 3 days in culture with PHA plus rIL-2. During data analysis, the $CD3^+$ cells were gated and the percentage of $CD45RA^+$ or $CD45RO^+$ T cells and lymphoblasts determined. Although CD3 is modulated from the cell surface early after lymphocyte activation, by day 3 it had reappeared, enabling the use of this triple combination to investigate the phenotype of the lymphoblasts. During activation $CD45RA^+$ lymphocytes switch to $CD45RO^+$, passing through a transient $CD45RA^{low}CD45RO^{low}$ stage. After 3 days activation the full CD45RA to CD45RO change has not yet occurred but cells

expressing the $CD45RA^{low}CD45RO^{low}$ phenotype were taken to have arisen from the $CD45RA^+$ population.

The same trend previously described in the purified populations was also found in the unseparated cultures. In the seronegative donors, both $CD45RA^+$ and $CD45RO^+$ cells responded to the stimulus and transformed into lymphoblasts (Table 4.2). In each of the HIV-1, CDC categories, the number of $CD45RO^+$ lymphoblasts recovered was lower than the number of $CD45RA^+$. This was most evident in the asymptomatic (CDCII) patients where the pattern of responses was similar to that found using anti-CD3 in that the $CD45RA^+$ lymphocytes produced only a slightly lower response compared to the HIV-1^- individuals (70% of normal) while the $CD45RO^+$ population was much more severely impaired (37% of normal). This dichotomy became less apparent in the more advanced stages of HIV-1 disease, CDCIII/IV, when both $CD45RA^+$ and $CD45RO^+$ populations were equally affected by AALD.

Discussion

The early loss of memory responses is a well documented feature of infection with HIV-1 and is thought to have important implications in the pathogenesis of the disease (Miedema Tersmatte & van Lier 1990; Shearer & Clerici 1990). However, no study has yet identified the lymphocyte subsets affected nor determined how such responses change over the course of the disease. In this chapter primed and unprimed lymphocytes, identified by expression of CD45RO and CD45RA respectively, were isolated and examined for their ability to respond to stimulation with anti-CD3 in cultures supplemented with rIL-2. In the asymptomatic patients tested the $CD45RO^+$ cells preferentially died after stimulation indicating that primed cells are more vulnerable.

In normal individuals $CD45RA^+$ and $CD45RO^+$ T cell subsets have different requirements for optimal stimulation. Unprimed, $CD45RA^+$ cells have a more stringent dependence on accessory signals in the form of monocytes, IL-1 or anti-CD28 (Horgan et al 1990). For instance, $CD4^+CD45RA^+$ cells respond to anti-CD3 or anti-CD2 Mabs only in the presence of a second signal provided by monocytes or exogenous IL-2 while $CD4^+CD45RO^+$ cells do not (Matsuyama et al

Table 4.2 The response of $CD45RA^+$ and $CD45RO^+$ lymphocytes to stimulation with PHA in unseparated cultures[a]

	n	$CD3^+CD45RA^{+b}$	$CD3^+CD45RO^+$
$HIV\text{-}1^-$ Control	9	119.5 ± 20.8^c	94.6 ± 11.0
$HIV\text{-}1^+$ CDCII	19	81.8 ± 10.8	$35.1 \pm 5.4^{***d}$
CDCIII	7	46.1 ± 21.1	25.8 ± 7.7
CDCIV	10	44.4 ± 9.6	23.8 ± 4.7

a PBMC were isolated and stimulated with PHA plus rIL-2.

b The number of $CD3^+CD45RA^+$ and $CD3^+CD45RO^+$ cells were determined prior to stimulation and after 3 days in culture using the triple combination CD3-FITC, CD45RA-PE and CD45RO-biotin plus streptavivdin-Tricolour

c The numbers shown are the percentage of lymphcytes recovered in relation to the initial input of $CD3^+CD45RA^+$ or $CD3^+CD45RO^+$ cells; mean $\pm$ sem

d The data in each patient group were compared using the paired Students t-Test to determine if there was a difference in the response of either subset; *** $p < 0.001$

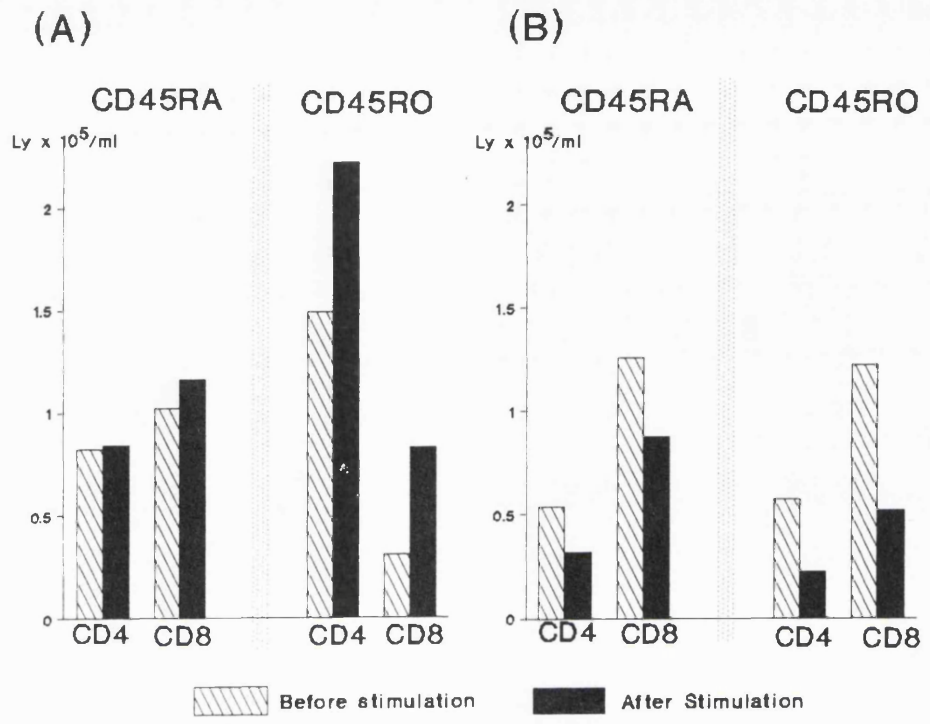


Figure 4.5 T cell subsets recovered after stimulation of purified CD45RA$^+$ and CD45RO$^+$ T cells from a representative normal (A) and HIV-1$^+$ (B) individual.

Purified CD45RA$^+$ and CD45RO$^+$ populations were investigated for the relative contribution of CD4$^+$ and CD8$^+$ T cell subsets before and after 3 days activation. The bars show the absolute number of lymphocytes before and after culture with anti-CD3 Mab plus rIL-2 for 3 days. The proportion of CD4$^+$ and CD8$^+$ lymphocytes in the cultures was determined by two colour IF using CD4-PE and CD8-FITC.

1988, Byrne Butler & Cooper 1988, Byrne et al 1989). Also the magnitude of the proliferative response induced by anti-CD3 is greater in $CD45RO^+$ T cells (Sanders et al 1989, Schnittman et al 1990b). Conversely, $CD45RA^+$ cells respond preferentially to stimulation with PHA (Matsuyama et al 1988; Salmon et al 1988, Schnittman et al 1990b, Wallace & Beverley 1990). Differences between subset responses may also depend upon the degree of stimulation. Optimal amounts of anti-CD3 Mab have been reported to stimulate CTL function in both $CD8^+CD45RA^+$ and $CD8^+CD45RO^+$ populations while sub-optimal amounts only stimulate the $CD8^+CD45RO^+$ population although the responses can be equalized by the presence of rIL-2 (de Jong et al 1991).

To minimise these subset variations, purified $CD45RA^+$ and $CD45RO^+$ populations were cultured in the presence of autologous macrophages and rIl-2 but the $CD45RO^+$ lymphocytes from HIV-1^+ patients remained less viable than the $CD45RA^+$ cells. Activation with either anti-CD3 Mab or PHA as well as activation of unseparated PBMC resulted in the same defective response in the $CD45RO^+$ subset, confirming that these cells are particularly handicapped and that this is not a function of the differential activation requirements of the subset *per se*.

Using conventional ^{3}HTdR incorporation assays it has been demonstrated that $CD4^+CD45RA^+$ cells from HIV-1^+ patients proliferate normally after stimulation with PHA or Con A but that responses of $CD4^+CD45RO^+$ cells to anti-CD3 and tetanus toxoid are impaired (Schnittman et al 1990b). We can now confirm this defect in $CD4^+CD45RO^+$ cells and extend this to include the $CD8^+CD45RO^+$ population using either anti-CD3 Mab or PHA as the stimulus. Furthermore, this failure to proliferate is due to the death of the responding $CD45RO^+$ cells.

Studies *in vitro* have shown that $CD45RO^+CD4^+$ lymphocytes have a higher viral burden than the unprimed subset and preferentially replicate HIV-1 after PHA stimulation (Cayota et al 1990, Schnittman et al 1990b). Therefore, one reason for the vulnerability of the $CD4^+CD45RO^+$ population may be infection with HIV-1. However, studies in asymptomatic individuals have shown that HIV-1 virus replication occurs in both $CD4^+CD45RA^+$ and $CD4^+CD45RO^-$ subsets after anti-CD3 stimulation and is therefore a function of the activation signal employed (Cayota et al 1993). Also, using in situ hybridisation techniques, comparatively few

virus positive cells can be identified (1 in 2,000 to 1 in 350; Lewis et al 1990) and these alone are unlikely to account entirely for the degree of handicap observed.

The acute stage of a range of viral infections, including HIV-1, is associated with an expansion of $CD8^+$ lymphocytes which express the activation markers HLA-DR and CD45RO (Reinherz et al 1980, Crawford et al 1981, Thomas et al 1982, Maher et al 1985, Tindall et al 1988, Miyawaki et al 1991). While in the acute condition this $CD8^+$ lymphocytosis disappears with disease resolution, in chronic HIV-1^+ patients it remains high (Nicholson et al 1984, Zeigler-Heitbrock et al 1985, Prince et al 1985, Nicholson et al 1986) and has been shown to predict progression to AIDS (Lang et al 1989). A $CD8^+$ lymphocytosis was also observed in both HIV-1^+ and AVI patients in this study. This was found to be due entirely to the expansion of the $CD45RO^+$ subset. Thus we can confirm previously reported data on the inreased expression of CD45RO whithin the $CD8^+$ T cell subset in HIV-1 infection. Additionally, in contrast to the $CD8^+$ population, $CD4^+$ lymphocytes from HIV-1^+ patients show no alteration in CD45RA or CD45RO expression.

In direct contrast, using CD29 as a marker for memory (Morimoto et al 1985) some investigators have reported a loss of memory T cells in HIV-1 infection (van Noesel et al 1990, Gruters et al 1990). This is apparently due primarily to the selective loss of $CD4^+CD29^+$ cells in the asymptomatic stages of the disease (de Paoli et al 1988, Fletcher et al 1989, Gruters et al 1991, Klimas et al 1991) while in the later stages both $CD4^+CD45RA^+$ and $CD4^+CD29^+$ cells decline (de Paoli et al 1988, de Martini et al 1988, Gruters et al 1991). However, recent data suggests that while $CD4^+$ T lymphocytes decline, there is no selective loss of phenotypically defined memory cells (Chou et al 1994). Also, no change was noted in the number of $CD8^+CD29^+$ cells (de Paoli et al 1988, Gruters et al 1991). As the distribution of CD45RO and CD29 do not completely overlap (Sohen et al 1990) and some $CD45RA^+$ cells express CD29 (Okumura et al 1993), statements involving the loss of memory cells in HIV-1 infection can be misleading. What does seem clear from this study is that cells with a primed phenotype do exist in HIV-1^+ patients and are in fact expanded within the $CD8^+$ subset. However, these cells are particularly vulnerable and die after short-term culture *in vitro*.

CHAPTER 5

INTERLEUKIN-2 RECEPTOR EXPRESSION IN HIV-1$^+$ AND AVI PATIENTS

Introduction

In chapter 3 it was demonstrated that AALD occurs despite the presence of exogenous IL-2 in the culture medium, indicating that a deficiency in this cytokine alone cannot account for the phenomenon. The possibility remains however, that cells lack the second signal normally provided through IL-2, due to a defective IL-2 receptor expression which may already be detectable in resting lymphocytes.

The IL-2Rα (CD25) and IL-2Rβ are differentially expressed on subsets of unstimulated PBMC. Large granular lymphocytes (LGL) or NK cells defined by the marker NKH-1 (Leu 19, CD56) or CD16 express IL-2Rβ (Tsudo et al 1987, Siegel et al 1987, Ohashi et al 1989, Voss et al 1990, Nishikawa et al 1990) estimated by binding of ^{125}I-labelled IL-2 at 540-1,630 sites per cell (Ohashi et al 1989) and by immunofluorescence at 12,000 sites per cell (Voss et al 1990). The IL-2Rβ chain is also found on a subpopulation of CD8$^+$ lymphocytes at a slightly lower density, estimated by binding of IL-2 at 180-410 sites per cell (Ohashi et al 1989, Taga et al 1991). In contrast, CD25 is found on CD4$^+$ lymphocytes and B cells but is absent from resting CD8 or NK cells (Smith 1980, Ohashi et al 1989, Taga et al 1991). Both CD25 and IL-2Rβ are preferentially expressed on CD45RO$^+$ lymphocytes within the CD4$^+$ and CD8$^+$ subsets respectively (Wallace & Beverley 1990, Taga et al 1991, Akbar et al 1991).

After stimulation *in vitro* with mitogens such as PHA or anti-CD3, mRNA for CD25 and membrane expression of the protein is upregulated. A CD25 expression of 59,100$\pm$10,800 sites per cell is reached after 72 hours (Uchiyama Broder & Waldman 1981) returning to normal levels 2,500$\pm$600 by day 10 (Robb Greene & Rusk 1984). Mitogenic stimulation also increases the surface expression of IL-2Rβ but to a far lesser extent that CD25 (Akbar et al 1991).

The aim of this chapter was to determine if HIV-1$^+$ individuals showed any changes in IL-2R expression that might help to explain AALD. To this end, quantitative IF techniques were used to compare IL-2R expression in T cell subsets from patient groups, while resting and after activation *in vitro*.

Methods

Flow cytometric methods were used to enumerate IL-2R expression on T cell subsets. As both direct and indirect IF techniques could be applicable, both methods were compared using commercially available reagents to enumerate cell surface antigenic sites.

Quantitative analysis of indirect immunofluorescence staining

The number of molecules of CD25 present on the cell surface was measured by indirect immunofluorescence using QIFIKIT beads (Biocytex Genie Cellulaire, Marseille, France). In this technique cells labelled with the primary antibody, RFT5 (CD25, IgG2a) and beads bearing defined quantities of the mouse monoclonal 5T1 (CD5, IgG2a) are stained with the same GAM-FITC conjugate second layer. The intensity of the staining on the beads is then used to construct a standard curve (Fig. 5.1) from which the unknown can be calculated.

Procedure: Eight bead preparations were provided which differed according to the number of CD5 molecules on their surface. The beads were resuspended by vortex mixing and 100μl removed into separate tubes. These were washed once with PBS-A and then incubated for 10 minutes with the FITC-labelled second layer (GAM-FITC, SBA) at saturating concentrations. The beads were then washed and fixed with 1% paraformaldehyde in PBS prior to flow cytometric analysis. The MFI of each bead standard was determined and plotted against the number of CD5 molecules per cell (Fig.5.1). PBMC stained with saturating concentrations of both CD25 and GAM-FITC were then investigated for CD25 expression.

Provided care is taken that cytometer settings are constant between samples, the same standard curve can be used on different occasions for the enumeration of a variety of antigens. However, it is important when using this technique that saturating concentrations of all reagents are used otherwise the results obtained are inaccurate.

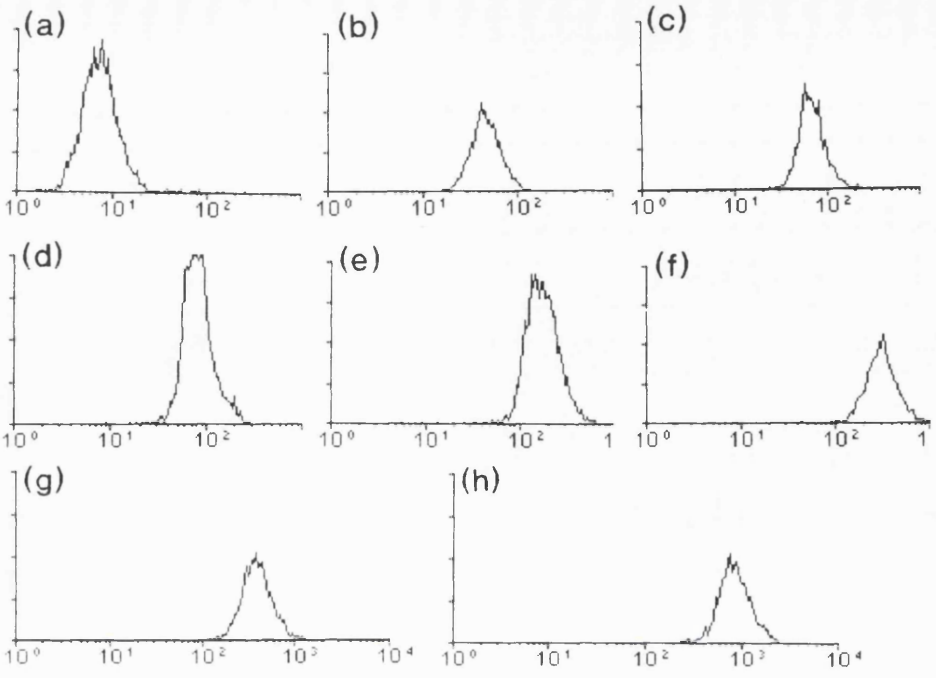
Quantitative analysis of direct immunofluorescence staining

CD25 staining was also quantified on lymphocytes and T cell subsets directly using Simply Cellular Microbeads (Flow Cytometry Standards Corporation, Leiden,

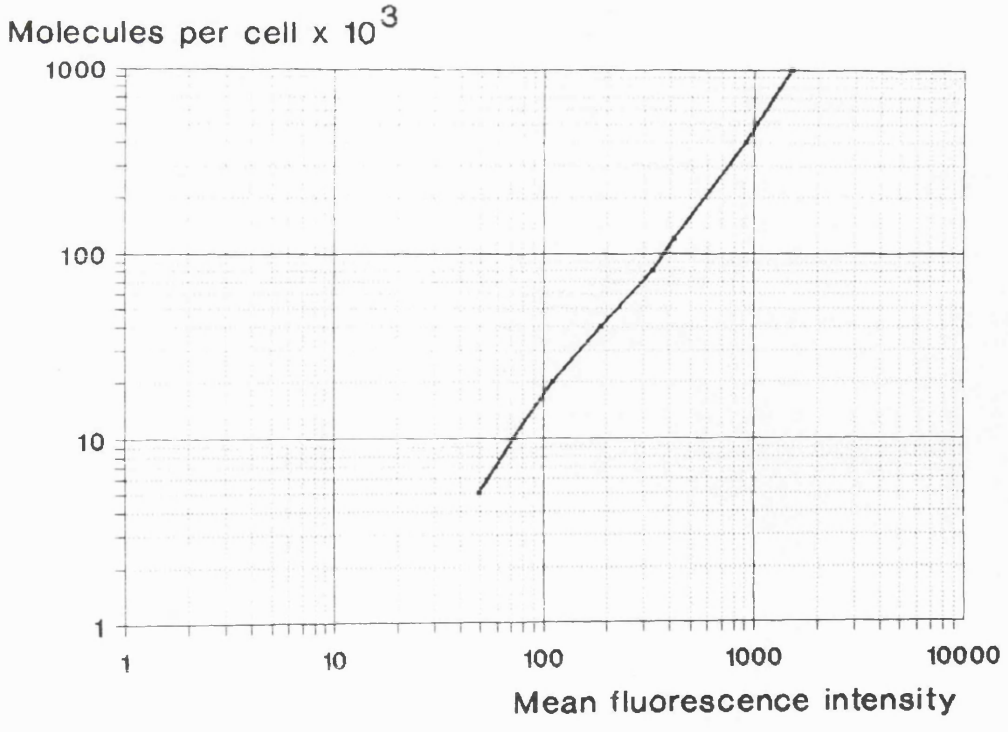
Figure 5.1 QIFIKIT beads used to construct a standard curve

In order to enumerate the molecules of CD25 per cell using indirect immunofluorescence, QIFIKIT beads were stained with saturating amounts of GAM-FITC conjugate. The beads, which are coated with varying amounts of a mouse monoclonal antibody were then run of the FACS*can* Part I; (a) bead control, no antibody, (b) 5 x 10^3, (c) 10 x 10^3, (d) 20 x 10^3, (e) 40 x 10^3, (f) 80 x 10^3, (g) 160 x 10^3, (h) 400 x 10^3. The mean fluorescence intensity (MFI) of the bead standards was then used to construct a standard curve, Part II

Part I



Part II



The Netherlands). The simply cellular beads have four calibrated binding capacities of GAM-IgG on their surface together with a negative bead control (Fig. 5.2). These are provided together in the same tube. When mixed with a directly FITC-conjugated Mab the MFI of the beads can again be used to construct a standard curve. In this case, an analysis program is provided which constructs the standard curve and calculates unknown values based upon the data provided by the cytometer.
Procedure: The beads were resuspended by vortex mixing and two drops (100μl) added to a 5ml test tube. A saturating concentration of CD25-FITC Mab was added to the beads and incubated for 10 minutes at room temperature. The beads were washed once with PBSA and fixed with 1% paraformaldehyde prior to analysis on the FACScan. The MFI of each bead peak was determined and fed into the analysis program. Because of the different scatter profiles of the beads and lymphocytes, PBMC and beads were stained in separate tubes.

Again, provided care was taken to ensure uniform cytometer settings, the same standard curve could be used to measure CD25 expression on different occasions.

Results

Quantitative analysis of CD25 expression on normal T cells before and after stimulation in vitro

The QIFI and simply cellular methods were used to compare CD25 expression on normal lymphocytes after stimulation with PHA and anti-CD3. The QIFI method could not be used to quantify anti-CD3 induced CD25 expression because the presence of the anti-CD3 antibody interfered with the second layer staining. Both methods revealed a weak constitutive expression of CD25 on resting cells (4-10 x 10^3 MPC; Table 5.1), equivalent to the levels reported previously. The expression increased with time after stimulation with either PHA or anti-CD3, but expression of CD25 was greater after stimulation with PHA regardless of the quantitation method employed. This suggests that the number of CD25 molecules on the cell surface may be a function of the strength of the activation stimulus. Alternatively, a difference in the relative size of the lymphoblasts induced by PHA might account for the discrepancy.

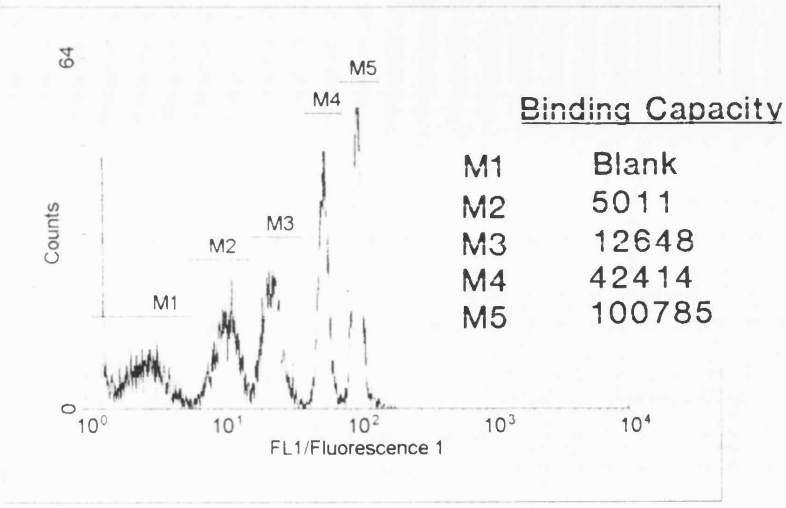


Figure 5.2 Simply cellular microbeads used for the quantitation of direct immunofluorescence

The microbeads were incubated with a saturating concentration of CD25-FITC conjugate. A standard curve was then constructed based upon the relative binding capacity and MFI for each bead.

Table 5.1 Quantitation of CD25 expression on PBMC after stimulation[a]

Stimulus		Time in culture (hours) 0	24	48	72
anti-CD3	**Simply Cellular**	4.1[b]	4.8	18.8	65.3
PHA	**Simply Cellular**	4.1	9.9	56.8	96.5
	QIFI	10.0	7.0	28.0	78.0

[a] The simply cellular and QIFI methods were used to measure CD25 expression on PBMC from normal individuals after stimulation with anti-CD3 or PHA for the time periods shown.

[b] The numbers represent CD25 molecules per cell x 10^3 calculated from the standard curve

Although both quantitation methods gave comparable results, the simply cellular method had certain advantages; (1) because it employed directly conjugated reagents it could be used to measure CD25 expression after stimulation with anti-CD3, (2) it could easily be adapted for double and triple immunofluorescence studies, therefore allowing the investigation of CD25 expression in T cell subsets and (3) the fluorescence of the negative control beads was lower allowing the quantitation of weak immunofluorescent staining. Therefore, where possible the simply cellular quantitation method was used. However, indirect IF techniques are more sensitive than direct. Therefore when measuring weak constitutive expression of CD25 and IL-2Rβ indirect techniques were used.

IL-2R expression on resting lymphocytes

A comparison of CD25 and IL-2Rβ on resting PBMC from HIV-1$^-$ control, HIV-1$^+$ and AVI patients revealed an apparent loss of constitutive CD25 expression in HIV-1 infection (Fig 5.3). The percentage of CD25$^+$ cells was reduced in CDCII, asymptomatic individuals (control 14.8$\pm$2.2%; CDCII 8.6$\pm$1.6%; $p<0.004$) and was further decreased in the symptomatic, CDCIV patients (6.9$\pm$1.6%; $p<0.001$). However, the intensity of CD25 expression on those cells which retained the antigen was unaltered. In addition, although CD25 was reduced, IL-2Rβ levels were found to be equivalent to normal (control 18.8$\pm$1.6%; CDCII 13.3$\pm$3.2%; CDCIV 17.1$\pm$3.2%). The acute *in vivo* stimulation in the AVI patients did not result in an enhanced CD25 expression (18.3$\pm$4.2%) nor was any significant change noted in the levels of IL-2Rβ (13.2$\pm$5.9%).

As any virus specific changes in IL-2R expression could be masked by proportional alterations in T cell subsets, it was necessary to clearly define the cell subsets which normally constitutively express IL-2R and relate this to the equivalent populations in HIV-1$^+$ patients. Therefore, the expression of CD25 and IL-2Rβ on NK cells and T cells from seronegative individuals were investigated using two colour IF, combining FITC labelled CD4, CD8, CD28 or CD16 with an indirect PE staining for the IL-2 receptor chains (Fig. 5.4). The majority of CD25 staining in seronegative individuals was found on CD4$^+$ lymphocytes (24%), while little was detected on either CD8$^+$ T cells or CD16$^+$ NK cells. As the majority of CD4$^+$

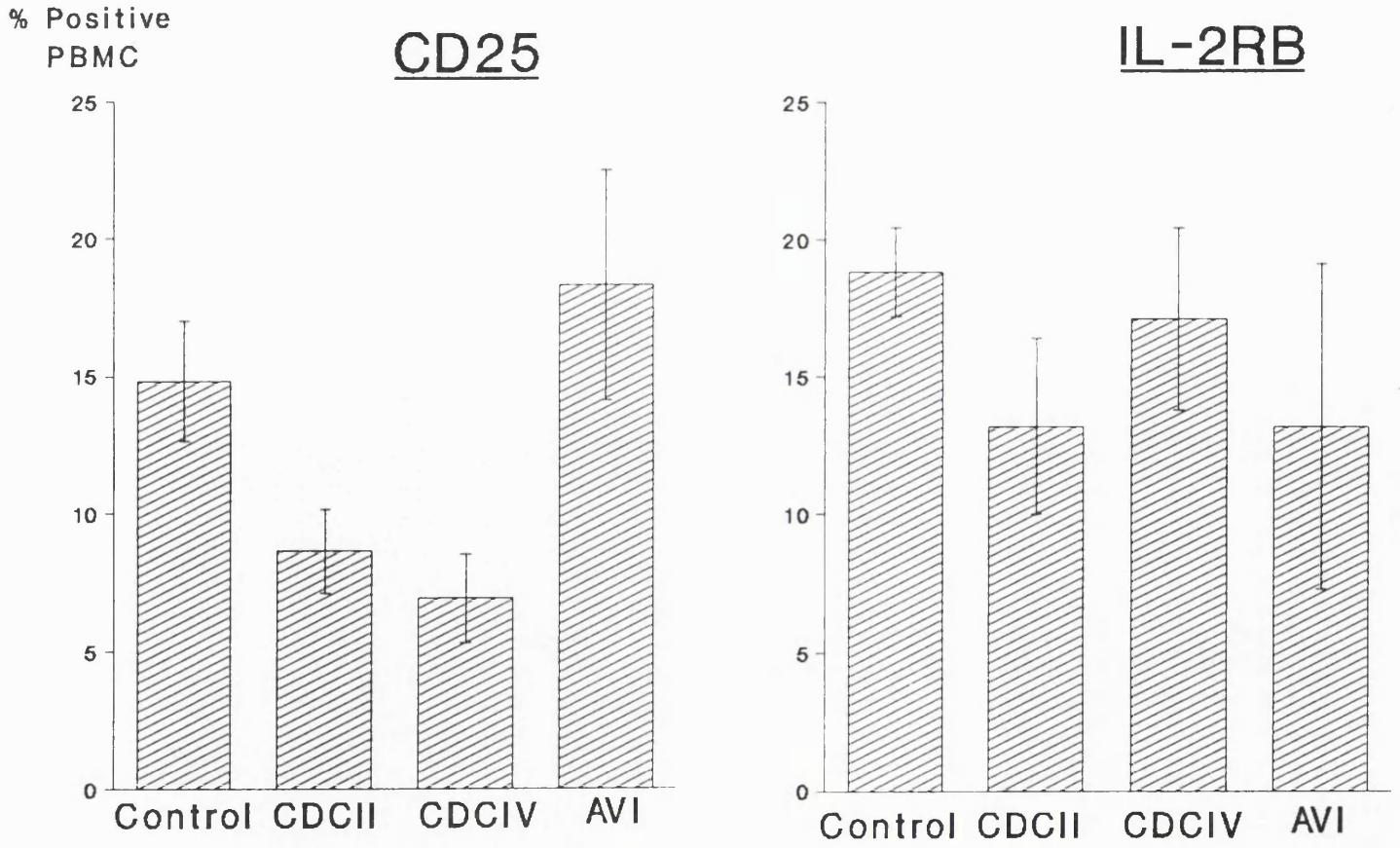
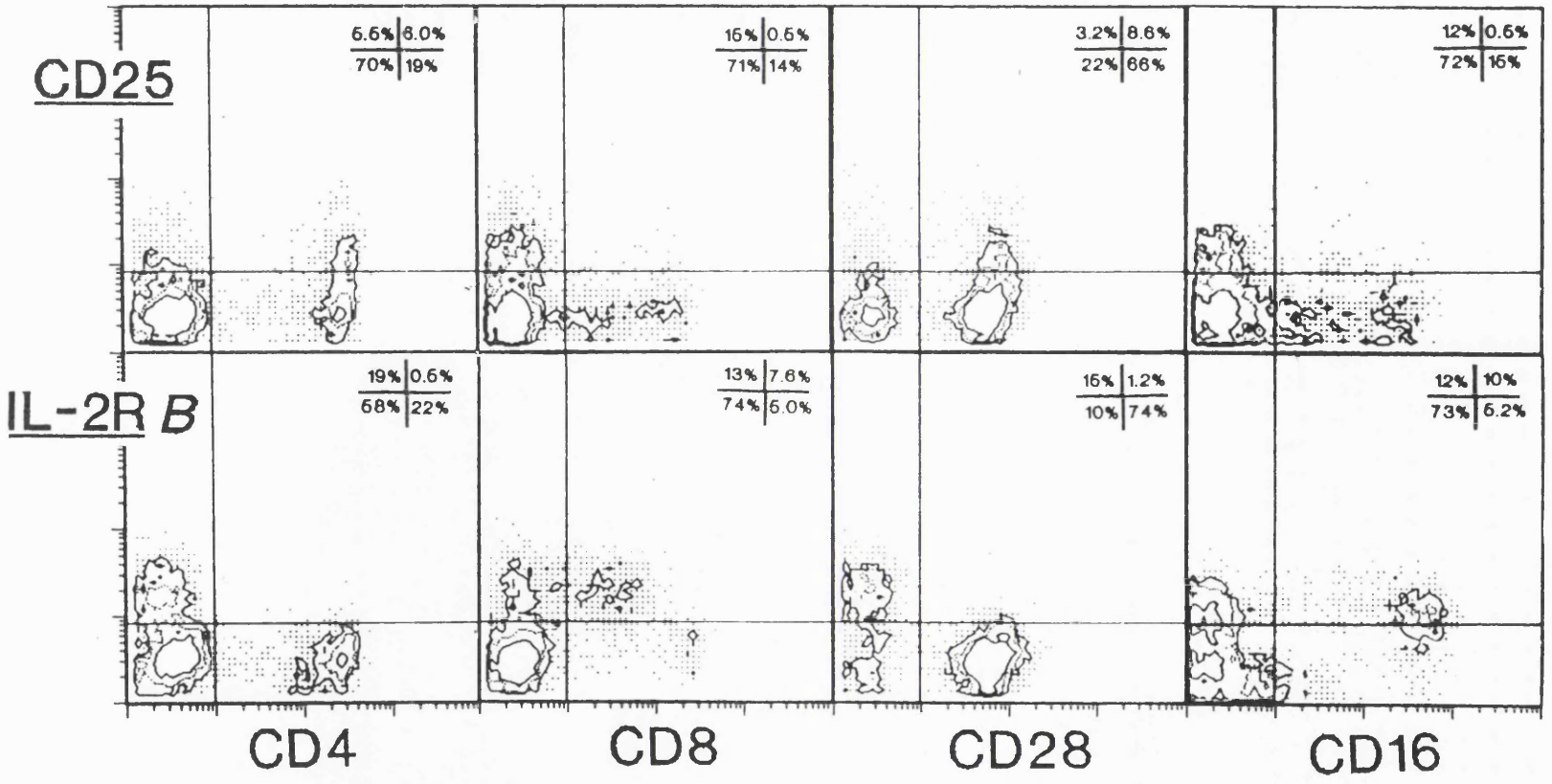


Figure 5.3 The expression of CD25 and IL-2Rβ on PBMC from normal and patient groups

PBMC from 10 HIV-1$^-$ controls, 23 HIV-1$^+$ (CDCII 13; CDCIV 10) and 8 AVI patients were investigated by indirect IM using anti-CD25 (RFT5γ; IgG1) or anti-IL-2Rβ (Tu27; IgG1) together with GAM IgG-PE. The bars represent the mean $\pm$ sem of the data.

Figure 5.4 The expression of CD25 and IL-2Rβ on normal T cell subsets

PBMC from HIV-1$^-$ individuals were investigated by two colour IF for the presence of CD25 or IL-2Rβ on lymphocyte subsets. An indirect staining procedure using anti-CD25 or anti-IL-2Rβ plus GAM IgG-PE was combined with direct IF with FITC labelled reagents against CD4, CD8, CD28 and CD16. During data acquisition an analysis gate was set up around the lymphocytic population based upon FSC and SSC characteristics and 5000 events collected. The results from one representative individual are shown. The numbers shown indicate the percentage of cells within each quadrant.


CD25
IL-2R B
6.6% 6.0%
70% 19%
15% 0.5%
71% 14%
3.2% 8.6%
22% 66%
12% 0.6%
72% 15%
19% 0.6%
68% 22%
13% 7.6%
74% 5.0%
15% 1.2%
10% 74%
12% 10%
73% 5.2%
CD4
CD8
CD28
CD16

lymphocytes express CD28, this accounts for the positivity of this subset. In contrast, IL-2Rβ was mainly seen on $CD16^+$ NK cells (62%) and intermediate staining $CD8^+$ lymphocytes ($CD8^{dim}$, $CD28^-$) but was largely absent from $CD4^+$ and $CD28^+$ cells.

The distribution of CD25 and IL-2Rβ on $CD45RO^+$ T lymphocytes was investigated in purified $CD45RO^+$ and $CD45RA^+$ T cell subsets. These were prepared by negative selection from T cells isolated and depleted of $CD16^+$ NK cells (Chapter 2). The expression of CD25 was mainly confined to the $CD4^+CD45RO^+$ subset (10.4%) while IL-2Rβ was restricted to $CD8^+CD45RO^+$ cells (35.5%; Fig. 5.5). After removal of NK cells, which retain the $CD45RA^+$ phenotype, there was no detectable IL-2 receptor on the $CD45RA^+$ population. In summary, the constitutive expression of CD25 is restricted to the $CD4^+CD45RO^+$ T cell subset. In whole PBMC, IL-2Rβ is mainly found on NK cells but is also found on a proportion of $CD8^+CD45RO^+$ lymphocytes.

The expression of CD25 and IL-2Rβ on T cell subsets from HIV-1$^+$ and AVI patients

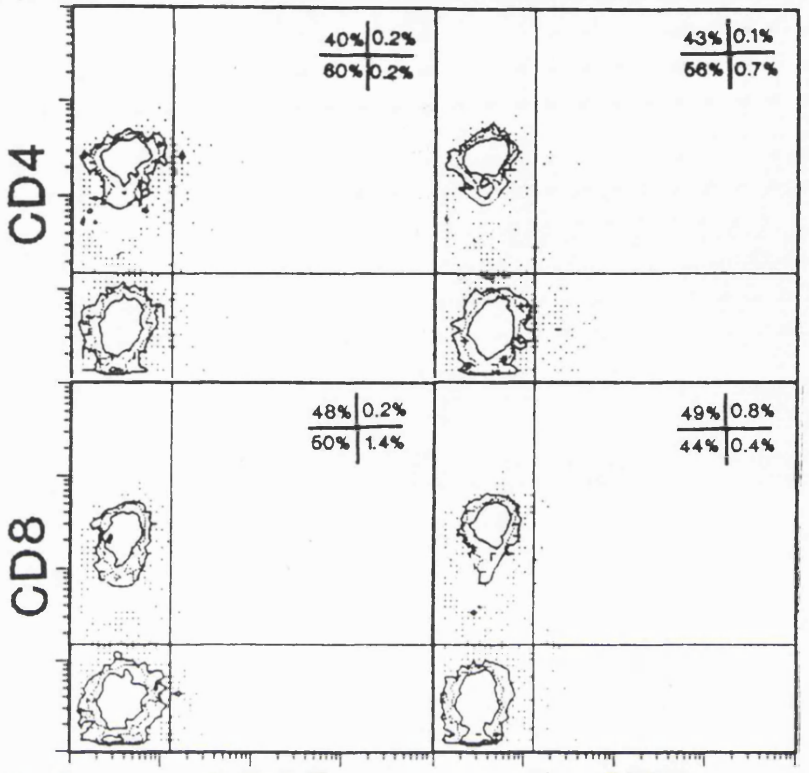
In the following part of the study equivalent T cell subsets known to constitutively express IL-2R in seronegative individuals were compared in HIV-1^+ and AVI patients. Purified T cells depleted of CD16 were investigated for CD25 and IL-2Rβ by indirect IF in combination with direct IF for the subset markers CD4, CD8, CD28, CD45RO and CD45RA (Table 5.2). Within the $CD4^+$ T cell subset the percentage of $CD25^+$ cells was reduced by more than two thirds in both HIV-1^+ CDCII and CDCIV patients ($p<0.001$) suggesting that as well as $CD4^+$ lymphocyte depletion, changes within the subset also cause a loss of CD25 expression. Other significant losses in CD25 were seen in the $CD45RO^+$ subsets where CD25 was again reduced by two thirds ($p<0.001$) and in the $CD28^+$ subset from HIV-1^+, CDCIV patients ($p<0.025$). Conversely, there was an increased IL-2Rβ expression on $CD8^+$ lymphocytes from AIDS patients ($p<0.05$) and on the $CD45RO^+$ subset from both HIV-1^+ groups when compared to the normal control ($p<0.001$).

As CD25 and IL-2Rβ are preferentially expressed on $CD45RO^+$ lymphocytes within the $CD4^+$ and $CD8^+$ subsets respectively it was necessary to investigate if

Figure 5.5 The expression of CD25 and IL-2Rβ on $CD45RA^+$ and $CD45RO^+$ T cell subsets

$CD45RA^+$ (A) and $CD45RO^+$ (B) T cells prepared by negative depletion procedures were investigated for the presence of IL-2R. An indirect staining procedure using anti-CD25 or anti-IL-2Rβ plus GAM-IgG-FITC (y-axis) was combined with direct IF with PE labelled anti-CD4 or CD8 (x-axis). During data acquisition an analysis gate was set up around the lymphocytic population and 5,000 events collected. The numbers shown indicate the percentage of cells within each quadrant.

CD45RA



CD45RO

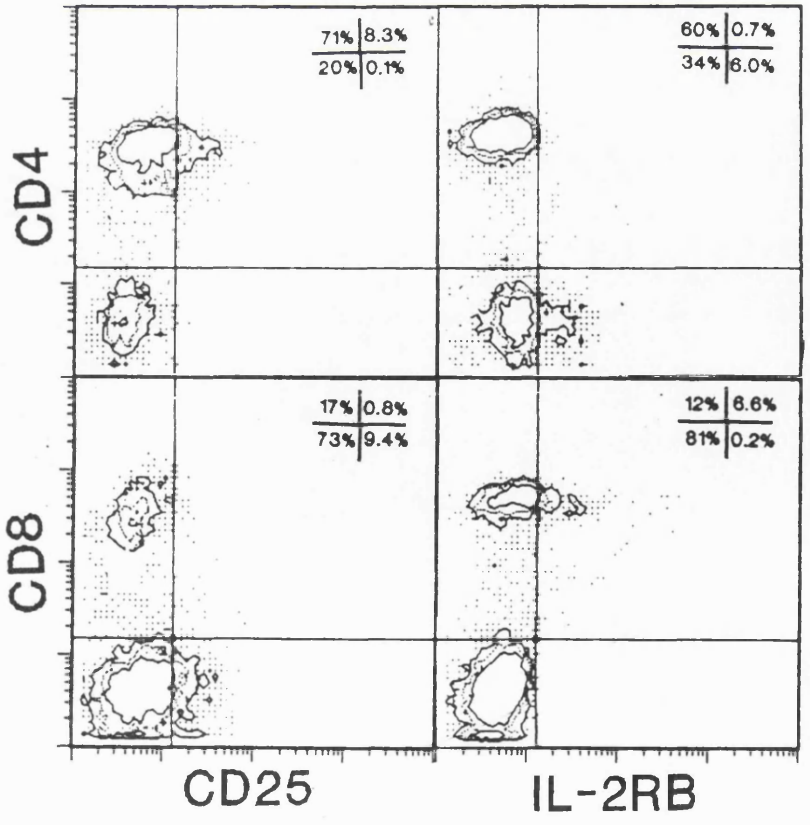


Table 5.2 IL-2 receptor expression on T cell subsets[a]

	CD4[b]	CD8	CD28	CD45RA	CD45RO
CD25					
HIV-1$^-$ Control[c]	23.4±2.4[d]	2.5±0.5	18.7±2.7	9.0±1.3	21.3±0.6
HIV-1$^+$ CDCII	7.9±2.5***	0.6±0.4*	18.7±8.5	8.9±3.8	6.3±2.0***
CDCIV	7.9±1.7***	1.9±0.6	9.2±2.9**	4.5±1.4**	5.7±1.9***
AVI	29.5±10.5	13.2±5.9*	ND	ND	ND
IL-2Rβ					
HIV-1$^-$ Control	1.3±0.2	7.5±2.9	1.9±0.8	1.2±0.5	5.1±0.9
HIV-1$^+$ CDCII	1.2±2.0	8.2±1.7	8.3±2.5**	0.8±1.0	15.9±0.9***
CDCIV	1.0±0.6	13.5±1.1*	8.4±1.7***	0.4±0.6	15.7±2.8***
AVI	1.2±0.5	12.8±2.5	ND	ND	ND

a The expression of CD25 and IL-2Rβ was determined on E^+ T cells depleted of $CD16^+$ NK cells. The T cell subsets were identified directly using FITC conjugated reagents.

b During data acquisition the FITC positive cells were gated and 5,000 events collected.

c A minimum of 7 individuals in each group were investigated.

d The data shown is the percentage of PE postive cells within the gated population (mean±SEM). These were compared to the control values using the Students T-test; * $p<0.05$, ** $p<0.025$, *** $p<0.001$

ND Not determined

the comparable subsets in HIV-1 infected patients were defective in IL-2R expression. To achieve this, E^+NK^- T cells were further depleted of $CD45RA^+$ cells using magnetic dynabeads leaving a >95% $CD45RO^+$ population. This was investigated by two colour IF for CD25 and IL-2Rβ expression on $CD4^+$ and $CD8^+$ cells (Table 5.3; Fig. 5.6). In HIV-1^- individuals, 27% of $CD4^+CD45RO^+$ cells were found to express CD25. This was reduced in HIV-1 infection (CDCII 16%; CDCIV 19%) indicating a small preferential loss of the $CD4^+CD45RO^+CD25^+$ lymphocyte subset. A modest decrease in CD25 expression on $CD8^+CD45RO^+$ cells was also seen in patients compared to the control group (control 9%, CDCII 2%; CDCIV 3%). However, levels of IL-2Rβ expression on $CD8^+CD45RO^+$ T cells were unchanged (control 13%; CDCII 11%; CDCIV 12%).

In conclusion therefore, to a large extent the reduced constitutive expression of CD25 seen in HIV-1 infection is due to $CD4^+$ and $CD4^+CD45RO^+$ lymphocyte depletion, however, even within these subsets there is a selective loss of $CD25^+$ cells. Although, increases in IL-2Rβ were detected in the $CD8^+$ and $CD45RO^+$ subsets from HIV-1^+ patients, the $CD8^+CD45RO^+$ subset expressed levels comparable with the control group. This indicates that HIV-1 infection does not cause a selective increase in the receptor but rather induces a shift towards a $CD8^+CD45RO^+$IL-2Rβ^+ phenotype.

The activation induced upregulation of CD25

In the next part of the study, the expression of CD25 after stimulation with anti-CD3 was investigated. Lymphocytes were stimulated in bulk cultures supplemented with rIL-2 and the expression of CD25 on $CD4^+$ and $CD8^+$ lymphocytes measured daily by direct IF using CD25-FITC and CD4/CD8-PE conjugates. During data acquisition a wide gate incorporating both live and dead cells was used and 5,000 events collected. When the data were subsequently analysed, only the viable PE positive cells were gated. The number of viable cells and developing lymphoblasts was determined using a combination of dye exclusion and cell size. The mean values are shown in Fig. 5.7 and representative individuals are illustrated in Fig. 5.8.

In HIV-1^- individuals the upregulation of CD25 was already apparent in

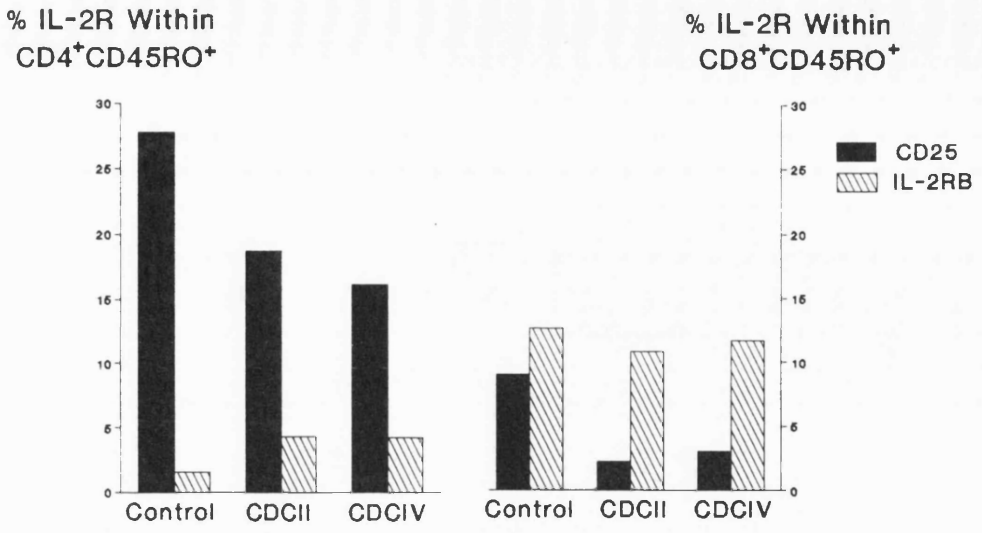


Figure 5.6 The presence of CD25 and IL-2Rβ on CD45RO$^+$ T cell subsets

The presence of CD25 and IL-2Rβ in CD4$^+$CD45RO$^+$ and CD8$^+$CD45RO$^+$ T cell subsets was determined in 5 HIV-1$^-$ controls and 9 HIV-1$^+$ individuals. Purified CD45RO$^+$ T cells were investigated by two colour, indirect IF using the combinations; anti-CD25 or anti-IL-2Rβ plus GAM IgG-PE and anti-CD4 or anti-CD8 plus GAM IgG2a/IgM-FITC.

Table 5.3 The expression of CD25 and IL-2Rβ on CD45RO+ T cell subsets[a]

		$CD4^+CD45RO^{+b}$		$CD8^+CD45RO^+$	
	n	CD25	IL-2Rβ	CD25	IL-2Rβ
$HIV\text{-}1^-$ Control	5	27(23-36)[c]	2(1-3)	9(4-17)	13(7-21)
$HIV\text{-}1^+$ CDCII	5	19(11-25)	4(3-6)	2(0-3)	11(5-16)
$HIV\text{-}1^+$ CDCIV	4	16(5-21)	4(3-6)	3(1-4)	12(4-21)

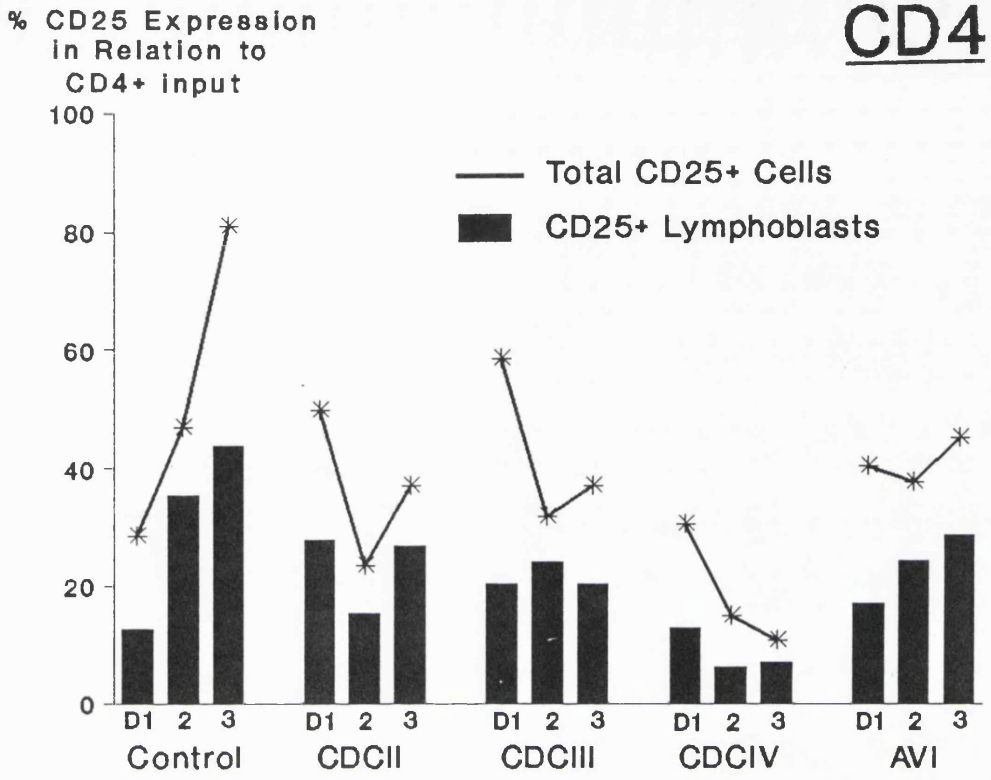
[a] The expression of CD25 and IL-2Rβ was measured on a CD45RO enriched population prepared from E^+NK^- T cells by removal of CD45RA+ lymphocytes. The $CD4^+$ and $CD8^+$ subsets were identified using FITC conjugated reagents.

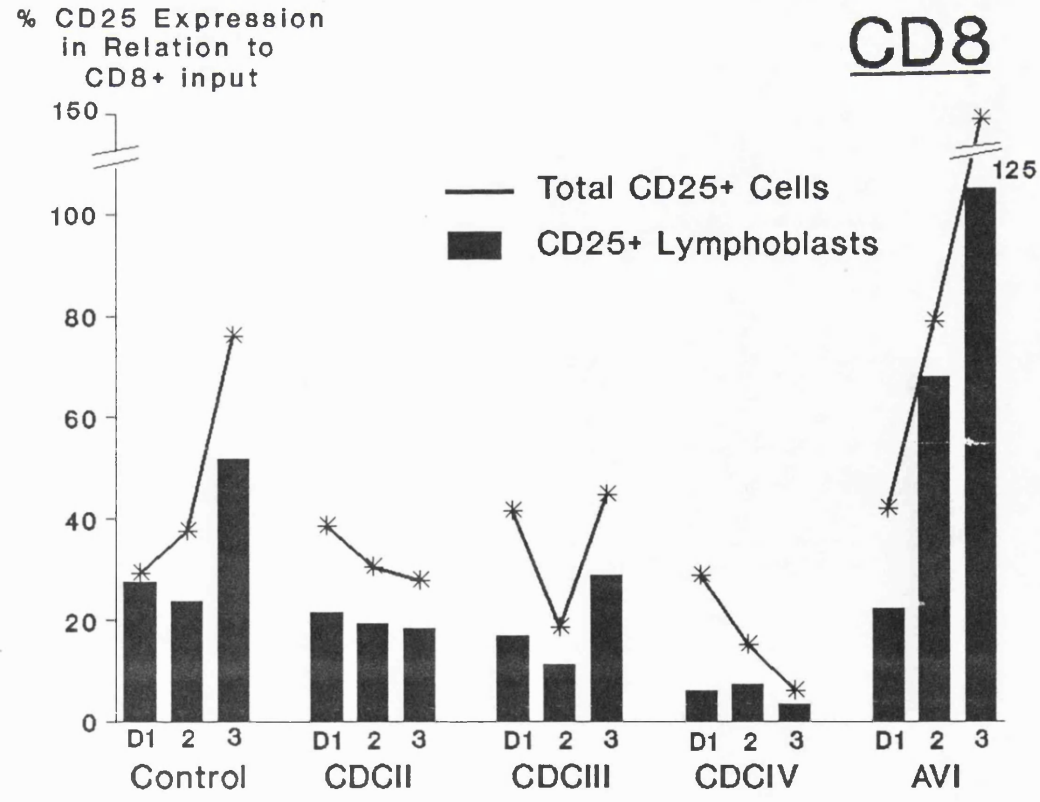
[b] IL-2R expressionon $CD4^+$ and $CD8^+$ cells was measured by two colour IF. During data acquisition the FITC positive cells were gated and 5,000 events collected.

[c] The data shown is the percentage of PE positive cells within the gated population; mean (range).

Figure 5.7 Time course of CD25 expression in $CD4^+$ and $CD8^+$ T cell subsets

PBMC cultured in the presence of anti-CD3 and rIL-2 were investigated daily by direct IF using CD25-FITC and CD4/CD8-PE conjugates. The total number of $CD25^+$ cells and lymphoblasts present in the cultures was determined by microscopic and flow cytometric analysis. A total of 9 HIV-1^-, 20 HIV-1^+ and 5 AVI individuals were investigated. The data is shown as the mean percentage of total $CD25^+$ cells and lymphoblasts present at the time points indicated in relation to the initial input of $CD4^+$ or $CD8^+$ lymphocytes on day 0.


% CD25 Expression
in Relation to
CD4+ input
CD4
100
80
60
40
20
0
Total CD25+ Cells
CD25+ Lymphoblasts
D1 2 3
Control
CDCII
CDCIII
CDCIV
AVI


% CD25 Expression
in Relation to
CD8+ input
CD8
150
100
80
60
40
20
0
125
Total CD25+ Cells
CD25+ Lymphoblasts
D1 2 3
Control
CDCII
CDCIII
CDCIV
AVI

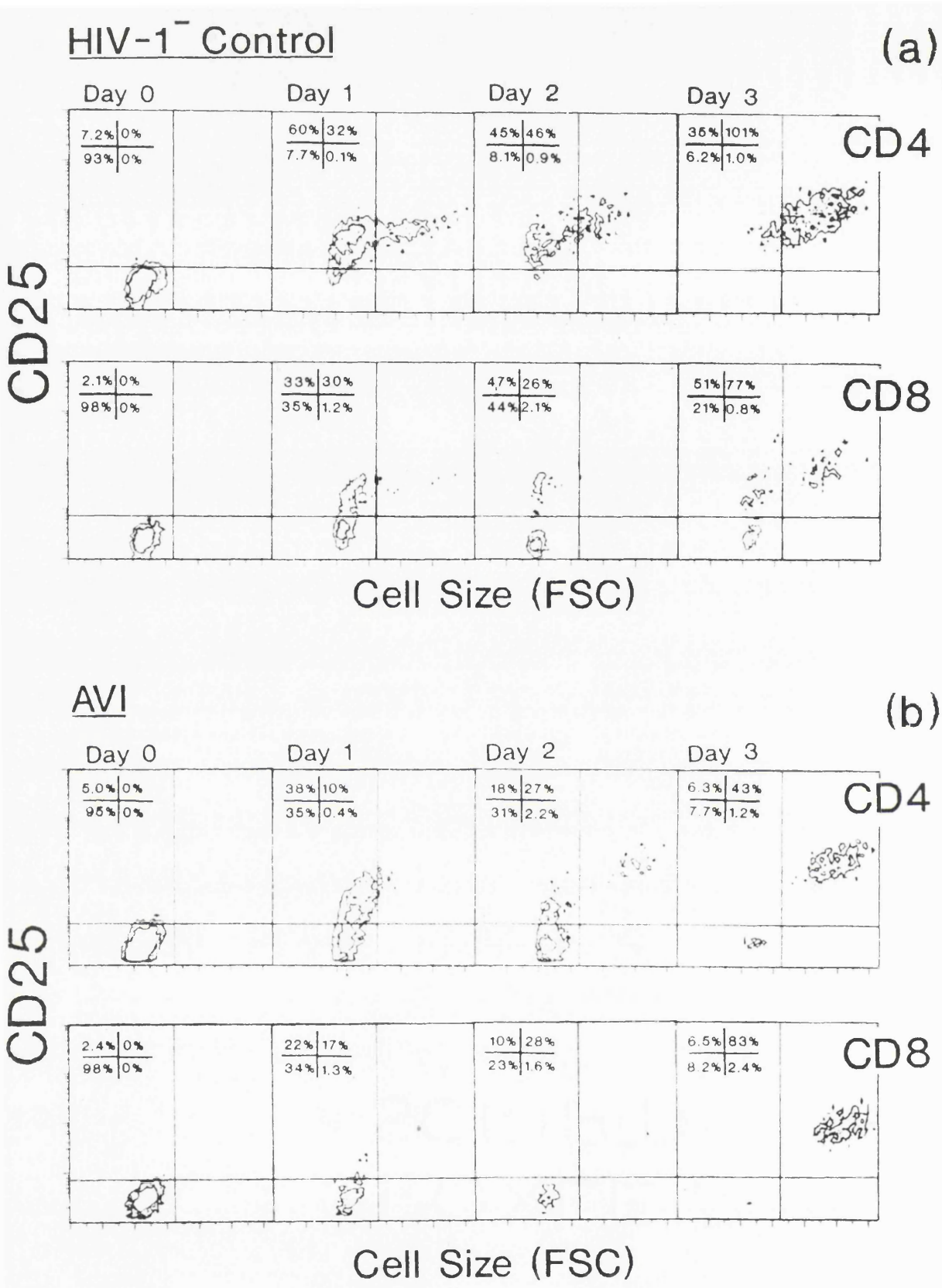
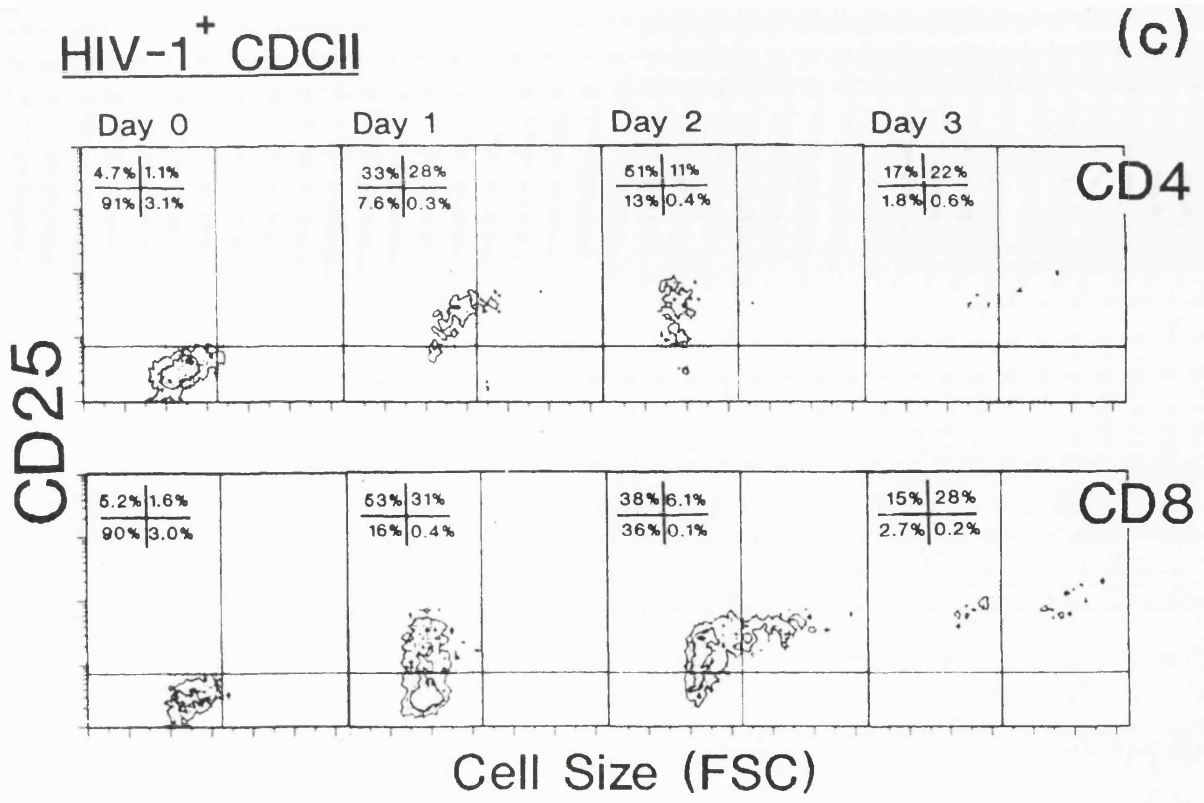
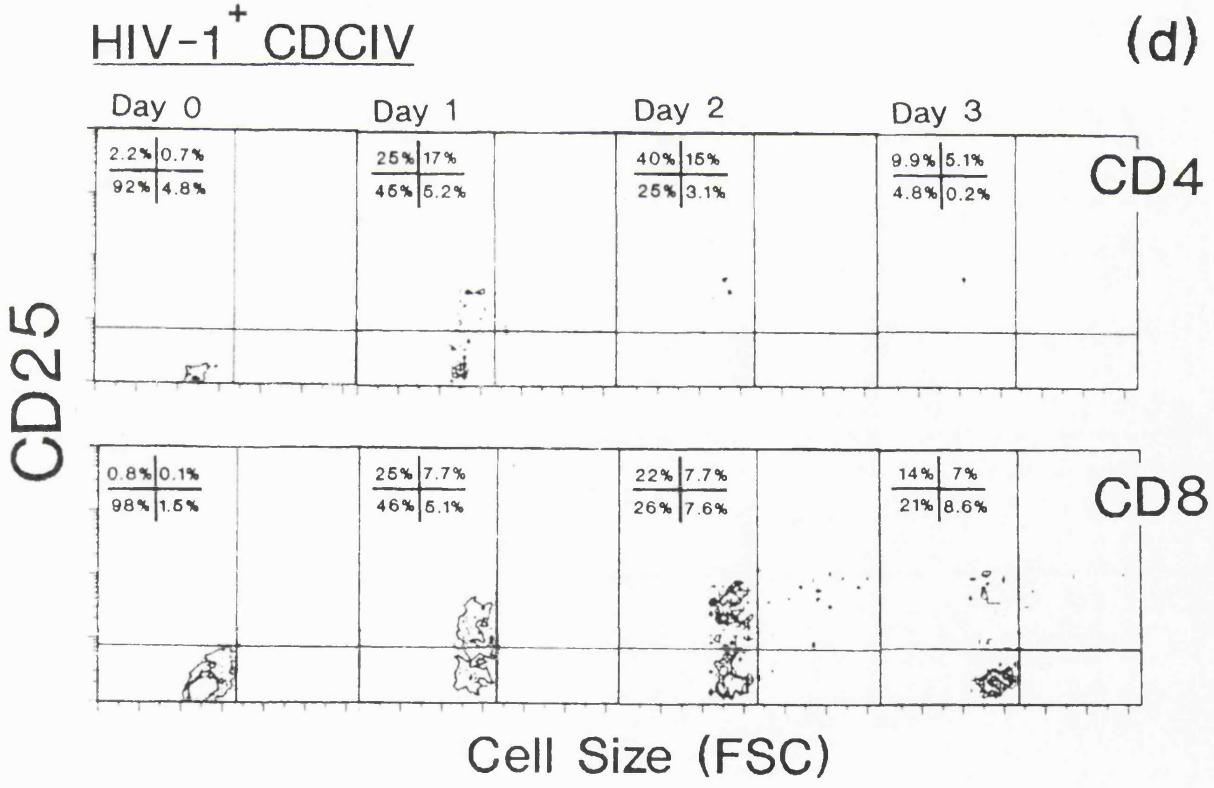


Figure 5.8 Time course of CD25 expression in anti-CD3 stimulated cultures

Representative example of an HIV-1$^-$ control (a), AVI (b), HIV-1$^+$ CDCII (c) and HIV-1$^+$ CDCIV (d) individual. The cells were investigated daily by direct IF using CD4/CD8-PE and CD25-FITC.





During flow cytometric analyses 5,000 ungated events were acquired and viable, $CD4^+$ or $CD8^+$ cells were subsequently gated. The cell size (FSC, x-axis) versus CD25 expression (y-axis) is shown. The numbers in the quadrants refer to the percentage of $CD4^+$ or $CD8^+$ lymphocytes in relation to the initial input on day 0.

both $CD4^+$ and $CD8^+$ subsets (25-30%) after 24 hours in culture, while the cells were still small or intermediate in size (Fig. 5.8a). Thereafter, the cells increased in size until by day 3 44% (range 18-73%) of $CD4^+$ and 52% (range 19-152%) of $CD8^+$ lymphocytes had transformed into $CD25^+$ lymphoblasts. A striking feature of cultures from HIV-1^+ patients was the failure of the cells to develop into lymphoblasts despite the initial increase in CD25 on day 1. In all HIV-1^+ individuals CD25 was upregulated in both $CD4^+$ and $CD8^+$ populations after 24 hours in culture and involved a similar proportion of lymphocytes as found in the seronegative (Fig. 5.7). Thereafter, the number of $CD25^+$ cells declined (Fig. 5.7) indicating that some lymphocytes die after they have acquired IL-2 receptor. Nevertheless, a proportion of cells transformed into lymphoblasts and it was in fact possible to expand the few responding cells and to generate IL-2 dependent cell lines that could be maintained for some weeks.

In the HIV-1^+ patients a small proportion of cells failed to acquire CD25 after 24 hours stimulation with anti-CD3. The size of this population declined with time but whether this was due to subsequent acquisition of CD25 or the death of the cells was not clear.

In the AVI patients investigated (Fig. 5.7; Fig. 5.8b) the number of $CD4^+$ or $CD8^+$ T cells that acquired CD25 after 24 hours was similar or increased when compared to the number of lymphoblasts that subsequently developed ($CD4^+$ day 1 40%, day 3 45%; $CD8^+$ day 1 42%, day 3 152%). This suggests that none of the cells that responded normally to the stimulus and expressed CD25 died and that AALD is not operating in this group. The cell death therefore occurred in the population that failed to upregulate CD25.

The intensity of CD25 after activation

Using the simply cellular method for fluorescence quantitation, no difference was noted between levels of CD25 expression in the control and patient groups. Maximum MFI's were achieved by all groups after 3 days in culture and no significant difference was found in either $CD4^+$ or $CD8^+$ lymphocyte subsets (range 54-102 x 10^3 MPC). Thus AALD occurs after the upregulation of CD25 and does not result from deficient CD25 expression.

Discussion

It has been proposed that defective IL-2 receptor expression could account for the poor proliferative responses of lymphocytes from HIV-1^+ patients (Prince & John 1986, Greene, Bohnlein & Ballard 1989). However, previous reports have given conflicting information concerning the expression of IL-2R in HIV-1 infection.

In this study it was found that $CD4^+CD45RO^+$ T cells constitutively express low levels of CD25. However, when the equivalent subset in HIV-1^+ patients was investigated, these were found to have a reduced CD25 expression indicating a preferential loss of the $CD4^+CD45RO^+CD25^+$ subset in HIV-1 infection. This confirms the findings of Zola et al (1991), who found a reduced expression of CD25 in HIV-1 infected patients. Although this was more pronounced in AIDS patients, it was independent of $CD4^+$ lymphocyte count, probably due to variations in the $CD4^+CD45RO^+$ population.

Lymphocytes which constitutively express CD25 are thought to represent primed cells which have recently encountered antigen (Taga et al 1991). The loss of this population in HIV-1 infection might therefore occur as a direct result of HIV-1 cytopathicity. The virus is known to infect $CD4^+$ lymphocytes (Klatzman et al 1984a,b, Dalgleish et al 1984) and as replication is triggered by lymphocyte activation (Zagury et al 1986, Rabson et al 1988), the decrease in $CD25^+$,$CD4^+$,$CD45RO^+$ cells may result from the deletion of infected cells after stimulation *in vivo*. In support of this it has recently been demonstrated that the removal of $CD25^+$ T cells prior to infection with HIV-1 dramatically reduces the levels of p24 and that although both $CD25^+$ and $CD25^-$ cells can be infected, only $CD25^+$ cells release viral proteins (Ramilo et al 1993). Alternatively, it has been documented that cell lines and peripheral T cells infected *in vitro* with HIV-1 or preincubated with gp120, down regulated CD25 expression after activation (Hofmann et al 1990, Oyaizu et al 1990, Sahraoui et al 1992). Also, plasma from HIV-1^+ individuals has been shown to suppress the proliferative response of normal PBMC to PHA through a decrease in IL-2 production and CD25 expression (Farmer et al 1986, Donnelly et al 1986) suggesting that an indirect mechanism might also contribute to the deficient constitutive expression of CD25.

In other chronic viral infections eg Hepatitis B virus (HBV), although there is

an increase in T cells expressing the activation marker HLA-DR, no increase in CD25 is found (Nouri-Aria et al 1988). Similarly, no increase in CD25 was found in the acute stages of CMV, EBV or VZV infection (Mercante et al 1991, Ebihara et al 1990, Yoshiike et al 1991) again despite the increased expression of class II. Interestingly, in acute EBV or CMV induced mononucleosis, an increase in soluble CD25 (sCD25) in serum was reported (Tomkinson et al 1987, Marcante et al 1991). Such increases have also been reported in HIV-1 infection (Honda et al 1989, Scott-Algara et al 1991). Therefore, it may be possible that CD25 upregulated during immune stimulation *in vivo* is cleaved from the surface of T cells as part of a feedback control mechanism. The loss of CD25 on $CD4^+$ cells might therefore not be HIV-1 specific and could be a measure of immune activation in these individuals.

The IL-2Rβ is of particular importance as it contains the signal transducing part of the IL-2R complex (Smith et al 1988, Hatakeyama et al 1989). After removal of NK cells which express high levels of IL-2Rβ (Tsudo et al 1987), the $CD8^+CD45RO^+$ subset from normals was found almost exclusively to express IL-2Rβ. When this subset was compared in normal and HIV-1 infected patients no change was seen in the percentage of cells expressing the receptor. Although both increased and decreased levels of IL-2Rβ have been reported in HIV-1 infection (Sahraoui et al 1992; Zambello et al 1992, Vanham et al 1993) no study has previously investigated equivalent subsets in normal and $HIV\text{-}1^+$ patients. The discrepancies between this and previous studies could therefore result from proportional differences in $CD8^+CD45RO^+$ lymphocytes.

The expression of CD25 is upregulated after normal lymphocyte stimulation. This is an early event occurring during the first 12 hours and is maintained throughout the cell cycle (Uchiyama, Broder & Waldman 1981). By clearly defining and enumerating viable lymphocytes and lymphoblasts, we have shown that this early upregulation of CD25 occurs normally in $HIV\text{-}1^+$ patients. However, the number of lymphoblasts which subsequently develop is reduced. In general, discrepancies in the reported levels of CD25 after stimulation *in vitro* can be accounted for by differences in experimental procedures. Those studies which investigated viable cells after stimulation reported no alterations in CD25 expression compared to normals (Lane et al 1985, Creemers et al 1986, Hofmann et al 1989,

Allouche et al 1990). In this study the number of CD25 molecules per cell after stimulation for 72 hours was estimated by quantitative IF at 54-102 x 10^3/cell in HIV-1^-, HIV-1^+ and AVI patients. This is in agreement with the quantitative study by Lane et al 1985 who showed CD25 levels of 58 x 10^3 and 45 x 10^3 molecules/cell in normal and HIV-1^+ patients respectively. The amount of CD25 also relates to the number of high and low affinity receptors for IL-2. Based upon IL-2 binding studies the number of IL-2 receptors, both high and low, was estimated at 44 x 10^3/cell (Foxwell et al 1990) which is also in agreement with the results for CD25 obtained using quantitative immunofluorescence.

In conclusion, the lack of CD25 or IL-2Rβ are not responsible for the proliferative defects previously described (chapter 3). However, we cannot rule out a possible defect in the expression of the IL-2Rγ chain particularly in view of the recent association between mutations in the IL-2Rγ gene and X-linked severe combined immunodeficiency (Noguchi et al 1993).

CHAPTER 6

THE SIGNIFICANCE IF LOW BCL-2 EXPRESSION IN HIV-1 INFECTION AND THE ROLE OF APOPTOSIS IN SPONTANEOUS AND ACTIVATION ASSOCIATED CELL DEATH

Introduction

During acute viral infections there is a greatly expanded circulating $CD8^+$ T cell population which arises in response to, and is directed against, the invading pathogen (Enssle & Fleisher 1990, Ebihara et al 1990). Many of these $CD8^+$ T cells have a blastic appearance and express high levels of HLA-class II and CD45RO, indicating immune stimulation *in vivo* (Miyawaki et al 1991). Indeed, isolated $CD8^+$ T cells are highly cytotoxic (Enssle & Fleisher 1990, van Binnendijk et al 1990) supporting their role as an "effector" population. This CD8 lymphocytosis is transient, the absolute number of circulating T cells quickly returning to normal upon resolution of the disease (Tomkinson et al 1987, Cauda et al 1987, Cheeseman 1988, Miyawaki et al 1991). Thereafter, individuals retain a circulating pool of memory T cells specific for the pathogen which can easily be detected in CTL or proliferation assays. Therefore, a mechanism must exist *in vivo* for the safe removal of effector cells while at the same time generating a memory population.

It has recently been demonstrated that T cells from patients with acute EBV induced infectious mononucleosis die by apoptosis after short-term culture *in vitro* (Uhehara et al 1992, Moss et al 1985) suggesting that death in this population is pre-programmed within the cell. Similarly, spontaneous and activation induced death in HIV-1 infection are reported to occur by apoptosis (Meyaard et al 1992, Groux et al 1992) indicating an analogous pre-programming. Thus, under certain circumstances cells may be induced to die as part of a normal mechanism for the safe removal of effector cells and the maintenance of lymphocyte homeostasis. If this mechanism is somehow permanently or abnormally "switched on" in HIV-1 infection then apoptosis might account for CD4 depletion and immune dysfunction.

The *bcl-2* gene has been shown to play a fundamental role in the control of cell death in a variety of systems. Therefore, the presence of Bcl-2 in T cells from $HIV\text{-}1^-$, $HIV\text{-}1^+$ and AVI patients was investigated using flow cytometric and microscopic methods. As this protein is found in the cytoplasm, a number of

permeabilising and staining protocols were compared to determine the level of Bcl-2 expression in these individuals. In addition the presence of cells in apoptosis was investigated using a variety of techniques to determine if cell death in HIV-1 infection could be identified as apoptosis.

Methods

Standardisation of cytoplasmic staining procedures for FACScan analysis

It has previously been reported that germinal centre B-blasts do not express Bcl-2 while resting B and T cells within the lymphoid tissue express levels detectable by IF immunofluorescence (Hockenbery et al 1991). An unconjugated anti-Bcl-2 reagent (clone 124, IgG1, Dako Ltd, High Wycombe, UK) was titrated out on tonsil sections using an indirect staining procedure with GAM IgG-FITC (SBA). The antibody concentration which gave the clearest distinction between the negative germinal centre and positive B-cell corona (10μl culture supernatant in 50μl PBS per section) was then used to study Bcl-2 staining on cytospin preparations of PBMC from normal and patient groups. These studies showed that Bcl-2 was expressed in the cytoplasm of the majority of PBL in normal individuals but that a proportion (10-20%) of cells did not express the protein. This negative population was increased in AVI patients. To more closely investigate Bcl-2 expression in T cell subsets a method was developed to allow the combined analysis of cell surface and intracellular antigens by flow cytometry. This method involved the permeabilisation of the cell membrane without destroying membrane antigen expression or altering lymphocyte morphology.

Three permeabilising reagents were compared using the following technique; *Procedure*: PBMC were adjusted to 2 x 10^6/ml in PBSA and 100μl added to a 5ml test tube. Cell surface antigens were stained as described in chapter 2. After the final wash the cells were fixed with 4% paraformaldehyde in PBS for 2 minutes at room temperature and than washed once with PBSA. The pellet was resuspended in 50μl of FCS and the cells cooled on ice for 5 minutes. Cells were then permeabilised with either (i) 150μl 1% saponin (ii) 1.0ml 0.3% tween (Schmid Uittenbogaart & Giorgi 1991) or (iii) 500μl 1:1 acetone/methanol solution. The cells were incubated for 15 minutes on ice and then washed twice with ice-cold PBSA.

Because the cells were fragile after the permeabilising procedure all washes were carried out at 1000rpm for 10 minutes. Indirect staining procedures were then carried out as normal. To check if the permeabilising procedure had been successful, propidium iodide (20μg/ml; 1.0ml) was added to the cells. This dye fluoresces when bound to DNA but can only gain access to the nucleus if the membrane has been permeabilised. As a negative control purified mouse IgG at 10μg/ml was used in place of anti-Bcl-2.

All three reagents caused some change in cell scatter (Fig. 6.1) on the FACcan but this was most pronounced when cells were permeabilised with saponin (Fig 6.1a). None of the procedures were found to cause any change in membrane expression of the markers used in the study including; CD3, CD4, CD8, CD45RA, CD45RO. Although both GAM-FITC and -PE second layers gave similar percentage results for bcl-2 expression, staining with FITC was very weak and tended to merge with the negative control while PE staining was generally brighter (Fig. 6.2). Using saponin all cells stained brightly for Bcl-2 (Fig. 6.1b). However, the negative population seen in cytospin preparations was not apparent. Using tween, no Bcl-2 staining was detected (Fig. 6.1d) despite positive propidium iodide uptake, suggesting the detergent may interfere with the Bcl-2 protein or that the pore sizes induced by tween were too small to allow entry of the antibodies. The most discriminating of the techniques which also compared well with the results obtained from cytospins was the acetone/methanol permeabilisation (Fig. 6.1f). This method did not significantly alter cell scatter profiles on the FACScan and was therefore the preferred technique for the measurement of Bcl-2 expression in cell suspension by flow cytometry.

Triple colour immunofluorescence studies of lymphoid tissue by confocal microscopy

The expression of Bcl-2 was also investigated on cryostat sections of lymphoid tissue from control and HIV-1$^+$ individuals. In particular it was of interest to determine if HIV-1 infection induced a change in Bcl-2 expression on T cell subsets. As three colour studies would provide more information concerning any change within T cell subsets, conventional epifluorescence techniques were combined

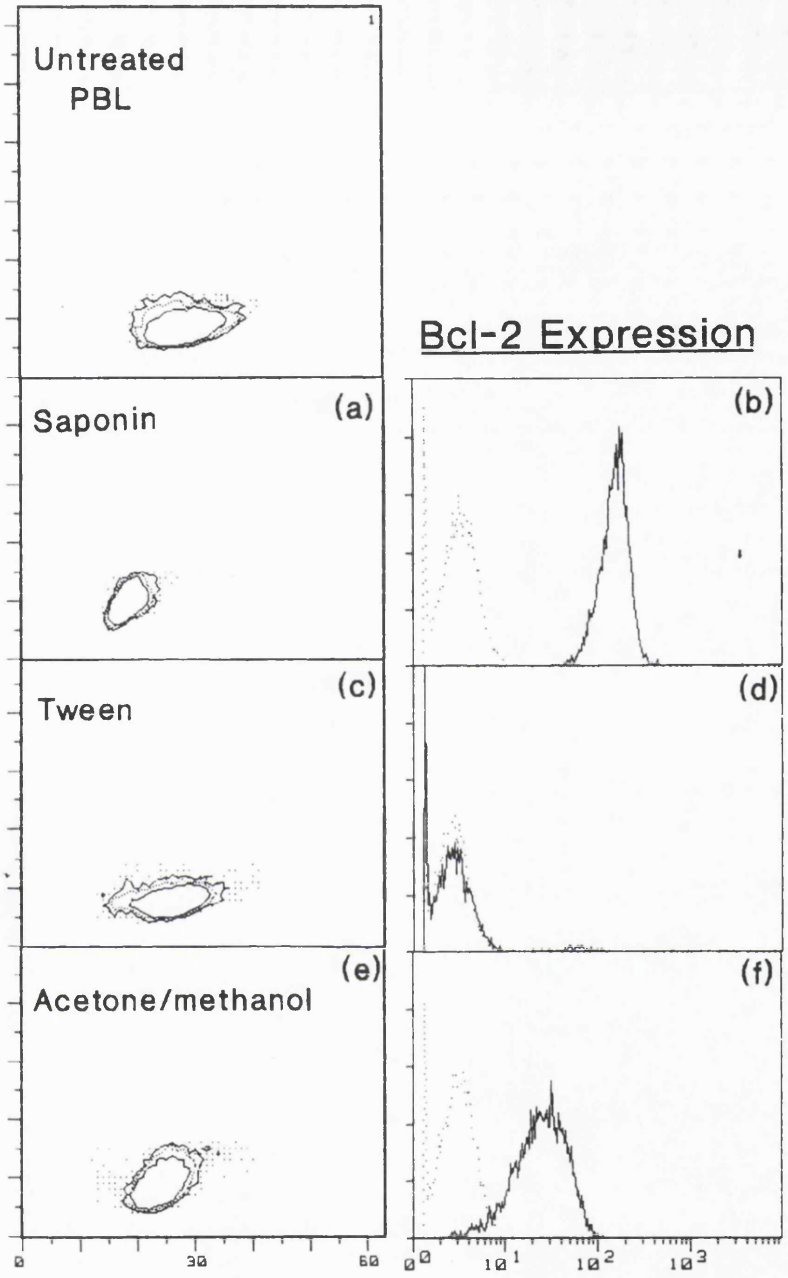


Figure 6.1 Comparison of three permeabilising reagents for the measurement of Bcl-2 in cell suspension

Three reagents saponin (a,b), tween (c,d) and acetone/methanol (e,f) were compared for their effects on cell scatter profiles (FSC x-axis, SSC y-axis; a,c,e) and the intensity of Bcl-2 expression using an indirect immunofluorescence technique with GAM-IgG1-PE (b,d,f). The control for the immunofluorescence studies is shown in dotted lines, right hand panel

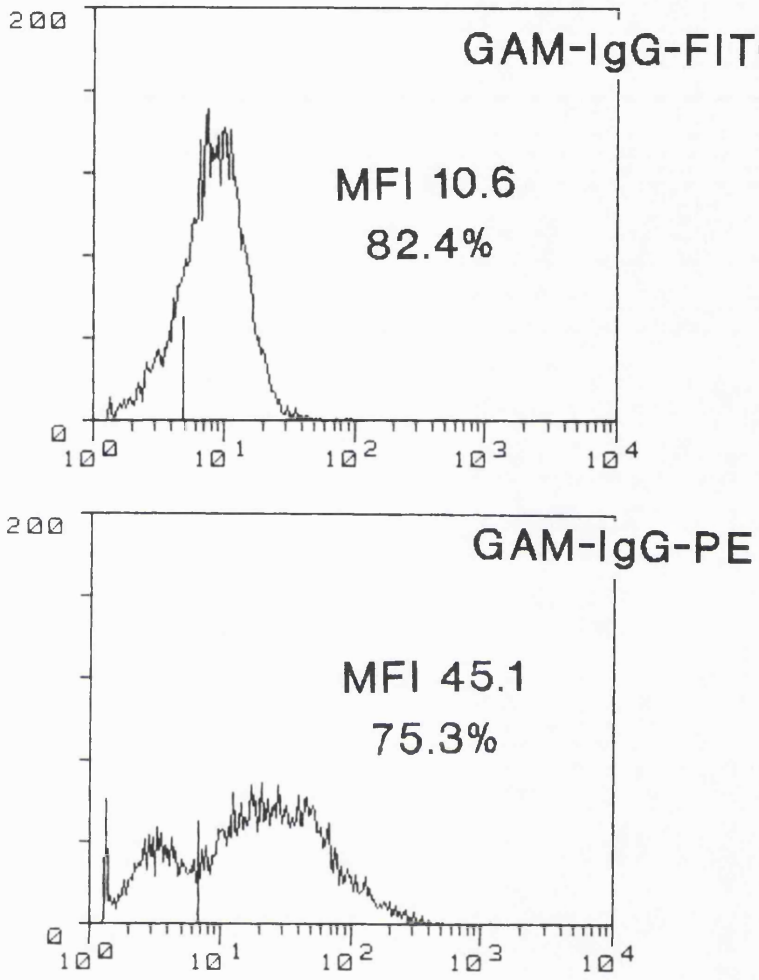


Figure 6.2 Comparison of FITC and PE second layers in cytoplasmic staining of Bcl-2

PBMC from an AVI patient with acute VZV were investigated for Bcl-2 expression by indirect IF using either GAM-IgG-FITC or -PE. Cytospin preparations of the same cell suspension were studied in parallel and results were in agreement with the FACS*can* data (77%).

with three colour IF using confocal microscopy.

In confocal microscopy the specimen is scanned by a spot of laser light and only in focus fluorescence emissions are collected. This has the effect of reducing the out-of-focus blur associated with epifluorescence. In addition, the Krypton/Argon mixed gas laser has three laser lines at 488nm, 568nm and 647nm, allowing three fluorochromes to be used simultaneously.

Procedure: Three fluorochromes with peak excitation wavelengths in the region of the laser lines were chosen. (i) FITC; excites at 488nm and emits at 520nm; (ii)Lissamine rhodamine (LR; Jackson Immunoresearch Laboratories Inc, West Grove, PA, USA); excites at 557nm and emits at 595; (iii) Cy5 (Jackson Immunoresearch Laboratories Inc); excites at 650nm and emits at 660nm. These reagents were used together with the appropriate filter block sets to prevent the fluorochromes bleeding into other channels. Indirect staining procedures were used throughout. The second layers were GAM-IgG2a-FITC, GAM-IgM-LR and streptavidin-Cy5, used in a three layer staining procedure with GAM-IgG1-biotin. Sections were stained as for epifluorescence procedures. Controls included mouse Ig and Ig-biotin together with the second layers. Also single and double combinations of each fluorochrome were tested in the three laser lines to ensure the filter blocks were correct and to check background, non-specific levels of fluorescence.

Quantitation of Bcl-2 expression

The quantitation of Bcl-2 in tissue sections was achieved by measuring the optical or pixel density of the fluorescence staining using confocal microscopy. For this procedure it is essential that all staining protocols are standard and that machine settings, laser voltage and acquisition times are always uniform. The method involves the capture of different fields on the section without prior exposure to either UV or laser light. This field was then scanned for $CD8^+$ cells which were marked and the Bcl-2 intensity measured. The information obtained using this technique is similar to the MFI value from a flow cytometer. Towards the end of this study, a directly FITC-conjugated anti-Bcl-2 reagent became available (Dakopatts Ltd) and this was used together with Quantum beads to confirm levels of Bcl-2 staining.

Measurement of cells in apoptosis

Because the precise steps leading to apoptosis are unknown, there is no single characteristic that unequivocally defines the process. There are however a number of morphological features that are shared by the majority of cells undergoing apoptosis. One very early event is the condensation and fragmentation of the nucleus. Other changes include rounding of the cell, blebbing of the plasma membrane, cytoplasmic condensation and the breakdown of cells into membrane bound apoptotic bodies. These morphological changes are very easily identified in cytocentrifuge preparations after May-Grunewald-Giemsa staining.

Procedure: Cytospins were first air dried then fixed by immersing in a jar of methanol for 10-20 minutes. They were then transferred to a staining jar containing May-Grunwald's stain (Merck Ltd) freshly diluted with an equal volume of buffered water. The slides were stained for 5 minutes and then transferred without washing to a jar containing Giemsa's stain (Merck Ltd) freshly diluted with nine volumes of buffered water. After staining for 10-15 minutes the slides were washed in three or four changes of water and left to stand in water for 2-5 minutes to allow differentiation to take place. The slides were air dried and mounted in D.P.X. (Merck Ltd)

This procedure had the advantage that it was simple to perform and involved very little manipulation of the samples which might damage already fragile cells. It was also quantitative in that the proportion of cells in apoptosis was determined by simple counting. In addition, neutrophils undergoing apoptosis were easily distinguished from apoptotic lymphoid cells. This is particularly important in HIV-1 infection as PBMC prepared by centrifugation over ficoll are frequently contaminated by granulocytes. This method is however time consuming and can be subjective in cases where apoptosis is not immediately obvious.

A key biochemical event of apoptosis is the activation of an endonuclease which cleaves double stranded DNA into oligosomal fragments visible by gel electrophoresis. This method was used to confirm the presence of apoptosis in the cultures

Procedure: DNA was extracted from 2 x 10^6 lymphocytes. The cell suspension was pelleted and the cells lysed with 500ml lysis buffer (10mM Tris pH7.5; 1mM

EDTA; 0.2% TritonX 100) for 2 minutes at room temperature. The DNA was precipitated by the addition of 100μl 5M NaCl and 600μl ice-cold propanol after incubation at -20°C overnight. The following day the tubes were centrifuged at 10,000rpm for 15 minutes, the supernatant discarded and the DNA pellet washed with 70% ethanol. The pellet was air dried and then dissolved in 20μl Tris/EDTA buffer. The contaminating RNA was removed by the addition of 2μl RN-ase (0.5mg/ml, DN-ase Free RN-ase, Boehringer Mannheim, Lewes East Sussex), incubated at 37°C for 30 minutes. The samples were then heated to 65°C for 10 minutes and 5μl of loading buffer added (46% glycerol, 50mM EDTA, 0.2% bromophenol blue) prior to loading. The DNA was run on a 1.5% agarose gel (Bethesda Research Laboratories, Gaitherburg, USA) in TBE running buffer (10mM Tris; 1mM EDTA; 5mM Boric acid) for 2 hours at 80V. The DNA was visualised by washing the gel in excess Ethidium bromide (5mg/ml; 10μl/100ml TBE buffer) for 30 minutes and viewed under fluorescence.

Although this method allows the visualisation of fragmented DNA it is poorly adapted to quantitation. However, using the above procedure in which a very limited number of purification steps are used, allows a crude comparison of cells provided the same starting numbers are compared.

Results

Cytoplasmic staining for Bcl-2 in cell suspension for FACSscan analysis

For flow cytometric studies on Bcl-2 expression, cell suspensions were permeabilised with acetone/methanol and stained using an indirect technique with GAM-IgG1-PE conjugate second layer. In all studies, staining was confirmed on cytospin preparations of cell suspensions. Using this procedure a suspension of normal tonsil was investigated for Bcl-2 expression as this tissue contains both positive (B corona, paracortical T cells) and negative (germinal centre B cells) subsets (Fig. 6.3). The $CD38^{bright}$ population represent the B lymphoblasts within the germinal centre and were universally $Bcl\text{-}1^{-}$ (Fig. 6.3a&b). The IgD^{+} B cells contained both a $Bcl\text{-}2^{+}$ and $Bcl\text{-}2^{-}$ fraction but were all lymphocytic.

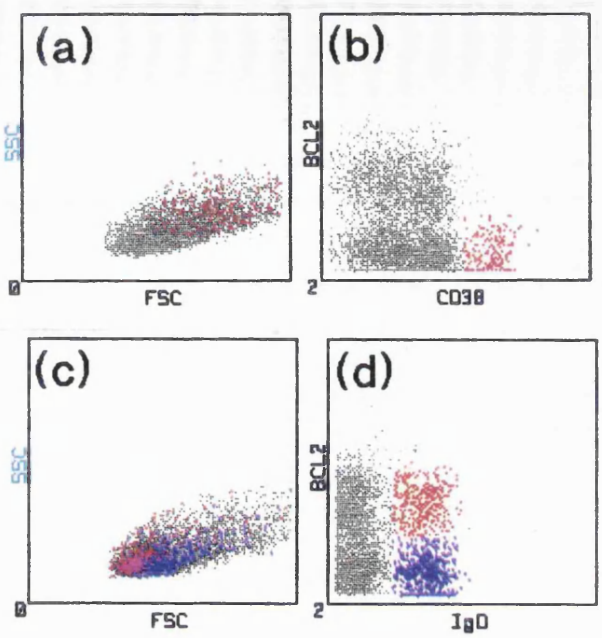


Figure 6.3 Bcl-2 expression in normal tonsil suspension

The presence of Bcl-2 in the cytoplasm was detected in cell suspension by FACS*can* analysis using an acetone/methanol permeabilisation procedure. Normal tonsil was double stained with CD38-FITC and Bcl-2 plus GAM-IgG1-PE (a,b) or IgD-FITC (c,d). The figure shows the presence of $CD38^{bright}$ blasts in red (a) which lack Bcl-2 while a proportion of small IgD^+ cells (c) are $Bcl\text{-}2^+$ (d)

The expression of Bcl-2 by T cell subsets

PBMC were isolated and examined for Bcl-2 expression using the cytoplasmic staining procedure for FACScan analysis. This was a dual colour method which allowed the analysis of Bcl-2 within T cell subsets identified by membrane markers. The majority (80%) of $CD3^+$ T cells from normal individuals (n=10) expressed Bcl-2 (Table 6.1; Fig. 6.4). A minor $Bcl\text{-}2^-$ population was however consistently found in each individual tested mainly among the $CD45RO^+$ subset (37%). When $CD4^+$ and $CD8^+$ subsets were compared both were found to contain a $Bcl\text{-}2^-$ component (CD4 18%; CD8 17%). Interestingly, an inverse relationship between CD45RO and Bcl-2 was often seen with those cells strongly expressing CD45RO lacking Bcl-2 and vice versa (Fig. 6.4). The reciprocal $CD45RA^+$ ($CD45RO^-$) population also contained some $Bcl\text{-}2^-$ cells (11%) but significantly fewer than the $CD45RO^+$ lymphocytes ($p<0.005$). As the $CD45RO^-$ population contained a B cell and NK cell component, the expression of Bcl-2 on $CD45RO^-$ T cells could not clearly be determined using whole PBMC. Therefore, purified subsets were prepared from E^+, NK depleted T cells using negative depletion procedures (Chapter 2). The Bcl-2 negative T cells were invariably found within the $CD45RO^+$ population in both $CD4^+$ (23%) and $CD8^+$ (20%) subsets (Fig. 6.5c,d) and few (<5%) were found in $CD45RA^+$ cells (Fig. 6.5a,b). In addition, the intensity of Bcl-2 was consistently lower in the $CD45RO^+$ cells compared to the $CD45RA^+$.

When the Bcl-2 expression of T cells from normal and $HIV\text{-}1^+$ individuals were compared, the $Bcl\text{-}2^-$ subset within the $CD3^+$ T cell population was expanded particularly among the CDCIV patients ($p<0.005$; Table 6.1). This was due to changes within the $CD8^+$ and $CD45RO^+$ subsets which contained significantly more $Bcl\text{-}2^-$ cells than the normal group ($p<0.01$) while Bcl-2 levels within $CD4^+$ lymphocytes were unchanged. The AVI patient group showed the greatest decline in Bcl-2 expression within the $CD3^+$ T cells (54%; $p<0.001$). All T lymphocyte subsets investigated showed lower than normal Bcl-2 expression but a similar pattern was seen, with $CD8^+$ and $CD45RO^+$ cells showing the most significant expansion of the $Bcl\text{-}2^-$ subset ($p<0.001$).

The low Bcl-2 expression in $HIV\text{-}1^+$ and AVI patients was confirmed on

Table 6.1 The expression of Bcl-2 by T cell subsets from HIV-1$^-$ control, HIV-1$^+$ and AVI patients

Groups	n	CD3	CD4	CD8	CD45RO$^+$	CD45RO$^-$
HIV-1$^-$ Control	10	80(73-84)[a]	82(63-95)	83(68-93)	63(53-73)	89(78-96)
HIV-1$^+$ CDCII	7	67(44-85)*[b]	70(60-80)^{NS}	53(37-75)****	52(37-75)*	77(56-89)*
CDCIV	5	58(45-81)***	79(70-93)^{NS}	66(43-83)**	46(31-68)**	60(40-84)****
AVI	14	54(33-67)****	70(48-82)*	56(17-89)****	44(16-70)**	66(46-89)****

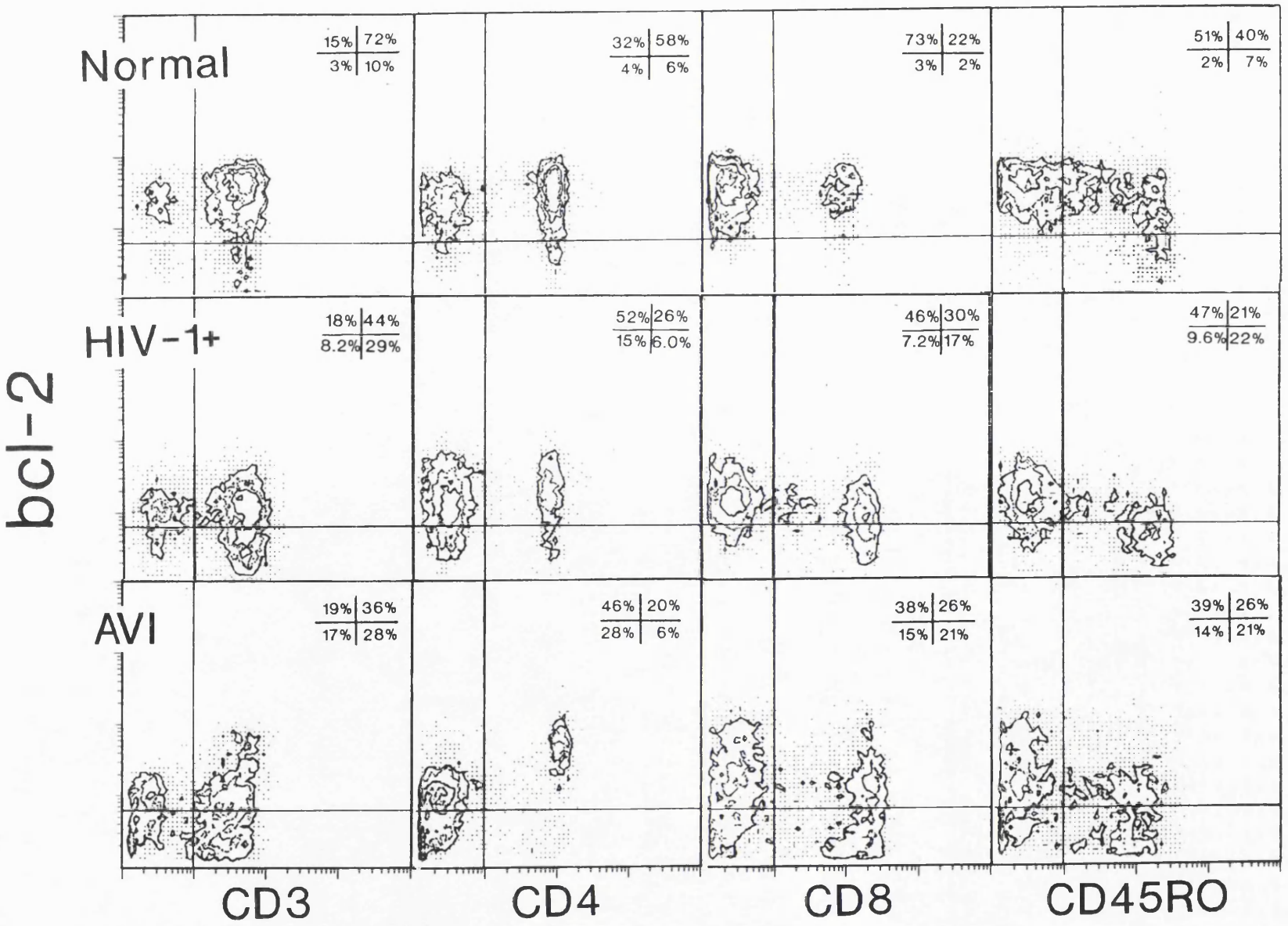
[a] The proportion of bcl-2$^+$ cells within PBMC T cell subsets analysed by two-colour immunofluorescence. The results represent the mean percentage (and range) of bcl-2$^+$ cells in the different subsets as shown.

[b] The mean values for each group were compared to the normal using the Student's t test; * $p<0.05$, ** $p<0.01$, *** $p<0.005$, **** $p<0.001$.

NS Not significantly different from normal

Figure 6.4 Bcl-2 expression in T cell subsets

The expression of Bcl-2 by T cell subpopulations from a normal individual, an HIV-1^+, asymptomatic and a patient with acute EBV infection. PBMC were analysed for co-expression of Bcl-2 (y-axis) with other T cell markers (x-axis) by double-colour immunofluorescence. During acquisition, lymphoid cells were gated and 5,000 cells collected. The percentages shown represent the proportion of cells in each quadrant of the fluorescence gates, set using negative control antibodies.


Normal
HIV-1+
AVI
bcl-2
CD3
CD4
CD8
CD45RO
15% 72%
3% 10%
32% 58%
4% 6%
73% 22%
3% 2%
51% 40%
2% 7%
18% 44%
8.2% 29%
52% 26%
15% 6.0%
46% 30%
7.2% 17%
47% 21%
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15% 21%
39% 26%
14% 21%

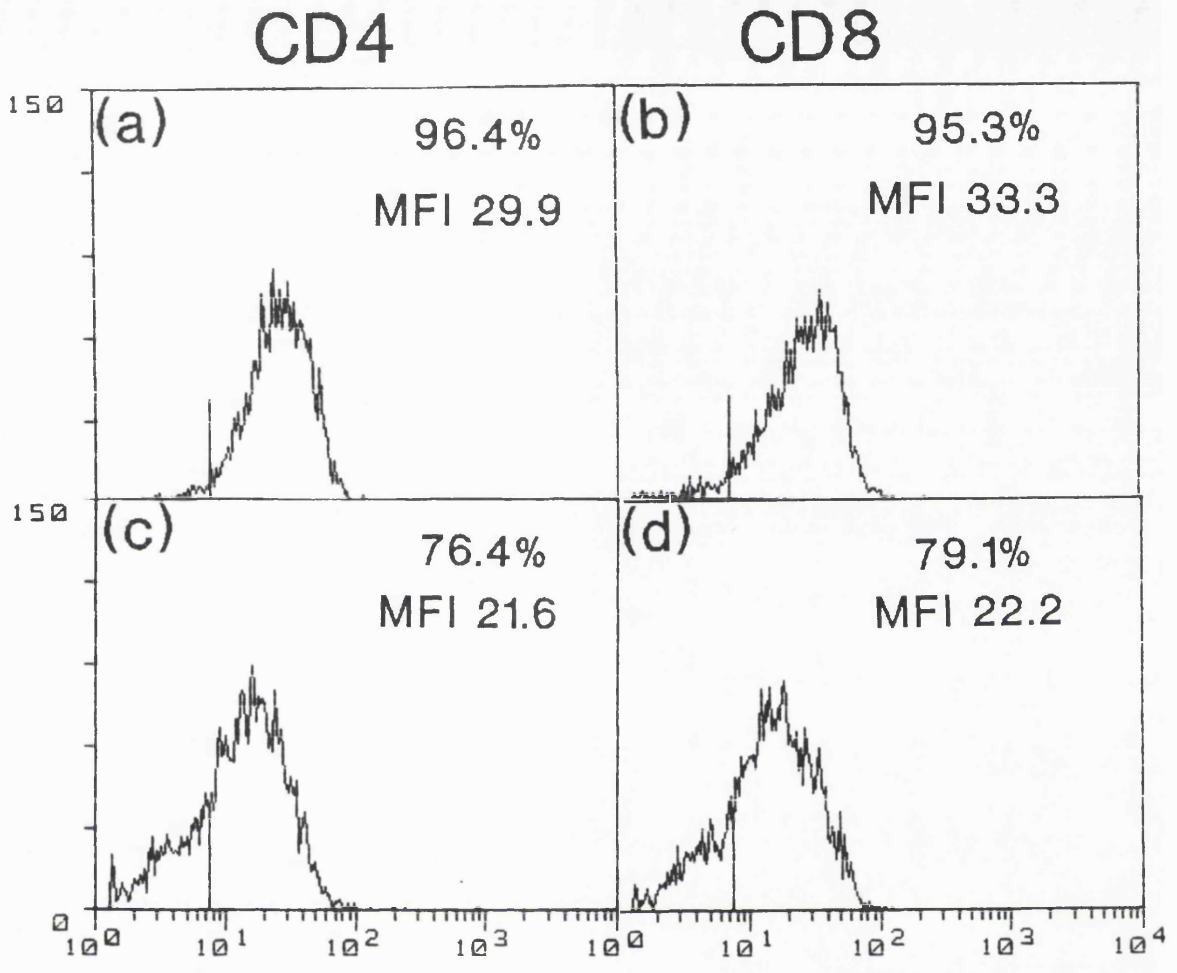


Figure 6.5 The expression of Bcl-2 in purified CD45RA$^+$ and CD45RO$^+$ T cells

Purified CD45RA$^+$ (a,b) and CD45RO$^+$ (c,d) T cell subsets were prepared from one normal individual and the expression of Bcl-2 on the CD4$^+$ (a,c) and CD8$^+$ (b,d) subsets determined by two colour immunofluorescence. During FACS*can* analysis the CD4$^+$ or CD8$^+$ cells were gated (5,000 cells) and the levels of Bcl-2 within the gate determined. The percentages shown are the proportion of Bcl-2$^+$ cells for each subset set using negative control antibodies. MFI mean fluorescence intensity

cytospins of PBMC double stained with CD8 and Bcl-2 (Fig. 6.6). Note that in the control individual the majority of the $CD8^+$ lymphocytes express Bcl-2 (Fig. 6.6a&b) while in the AVI patient shown all of the $CD8^+$ cells are $Bcl\text{-}2^-$.

The expression of Bcl-2 by normal T cells after activation in vitro

The differences in Bcl-2 expression between the $CD45RO^+$ and $CD45RA^+$ T cell subsets shown previously suggested that changes in the expression of Bcl-2 might be linked to T cell differentiation as a result of recent priming. To investigate this possibility further, purified $CD4^+CD45RA^+$ and $CD8^+CD45RA^+$ populations were stimulated with PHA and the levels of Bcl-2 determined during the transition from CD45RA to CD45RO (Fig. 6.7). Before stimulation both of the purified populations were >95% $CD45RA^+$ and $Bcl\text{-}2^+$. After 3 days of stimulation with PHA 79% of the $CD4^+$ and 78% of $CD8^+$ T cells had started to express CD45RO but the majority (92%) of both $CD4^+$ and $CD8^+$ T cells remained $Bcl\text{-}2^+$. After 5 days 93% of $CD4^+$ and 92% of $CD8^+$ T cells expressed CD45RO and a bimodal distribution of Bcl-2 had developed in both $CD4^+$ and $CD8^+$ subsets (34% and 26% $Bcl\text{-}1^-$ respectively). This $Bcl\text{-}2^-$ population was still observed after 7 days of activation. Therefore, the appearance of $Bcl\text{-}2^-$ cells is associated with lymphocyte activation and differentiation towards a $CD45RO^+$ phenotype.

Low Bcl-2 expression by $CD8^+$ lymphocytes in lymph nodes from HIV-1^+ individuals

The low expression of Bcl-2 in peripheral blood T cells from HIV-1^+ patients was even more apparent when lymphoid tissue was investigated. The expression of Bcl-2 on lymphocytes subsets determined using both epifluorescence and confocal microscopy, revealed an expansion of $CD8^+$ lymphocytes which lacked Bcl-2 in the HIV-1 infected lymph node (Table 6.2). This occurred in both the paracortex (pc) and germinal centre (gc) areas where as few as 5% of $CD8^+$ cells retained a $Bcl\text{-}2^+$ phenotype. The loss of expression of Bcl-2 on $CD8^+$ lymphocytes in lymphoid tissue was confirmed in cell suspension using a semi-quantitative technique on the confocal microscope. Cytospin preparations of tonsil and lymph node suspensions were examined using the dual excitation filters on the confocal

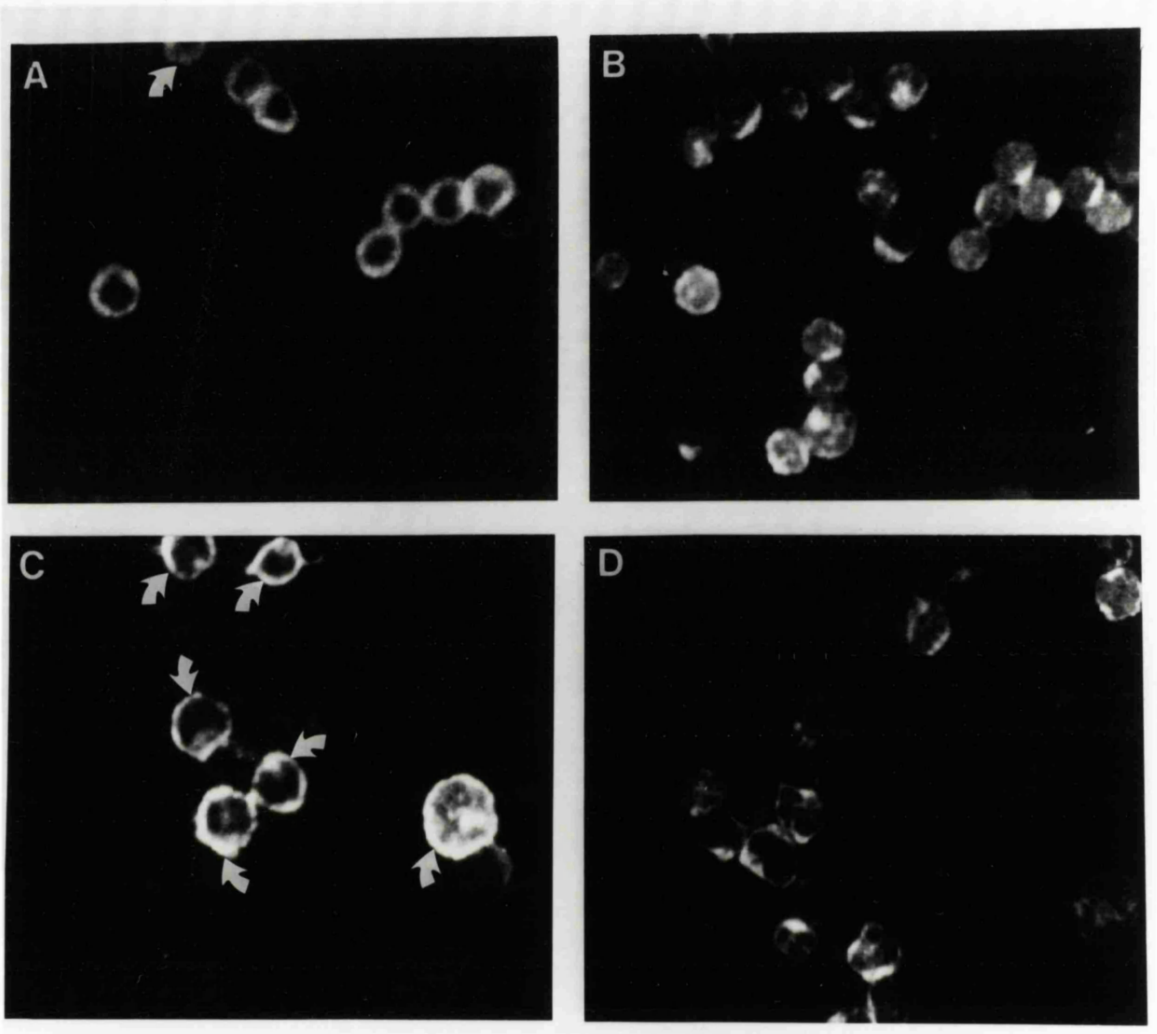


Figure 6.6 The expression of Bcl-2 on cytospins

The absence of Bcl-2 on $CD8^+$ T cells from AVI patients was confirmed in cytospin preparations investigated by conventional immunofluorescence microscopy. PBMC from one normal (A,B) and one patient with acute EBV infection (C,D) were double stained with CD8μ plus GAM-IgM-TRITC (A,C) and Bcl-2 plus GAM-IgG-FITC (B,D). The arrows indicate the presence of $CD8^+Bcl\text{-}2^-$ lymphocytes.

Figure 6.7 The changes in Bcl-2 expression after PHA activation

Purified $CD4^+CD45RA^+$ (A-D) and $CD8^+CD45RA^+$ (E-H) T cell populations were investigated for Bcl-2 expression in comparison with the appearance of CD45RO reactivity. The numbers shown are the percentages of cells in each quadrant of the fluorescence gates, set using negative control antibodies.

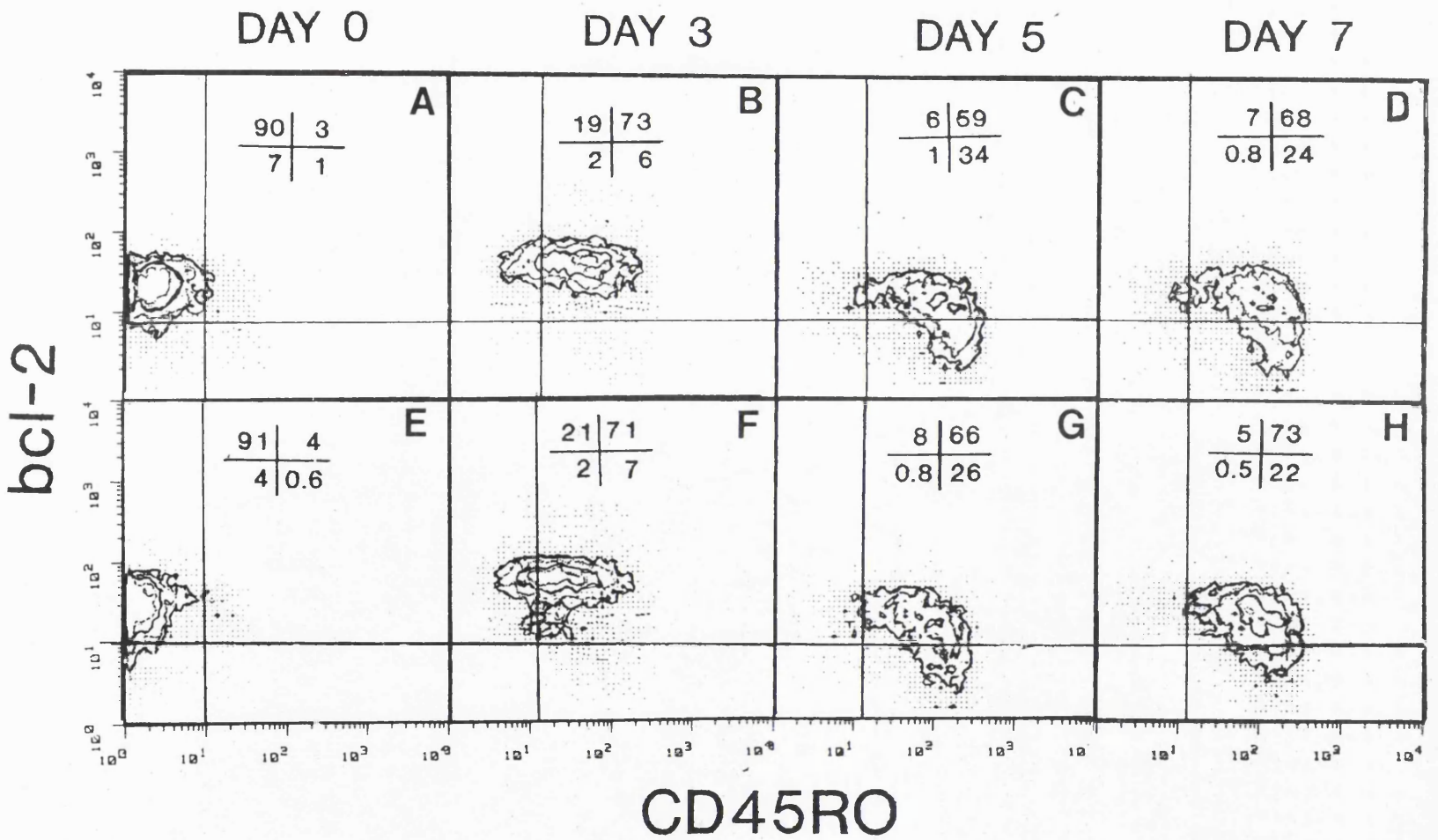

DAY 0
DAY 3
DAY 5
DAY 7
A
90 | 3
7 | 1
B
19 | 73
2 | 6
C
6 | 59
1 | 34
D
7 | 68
0.8 | 24
E
91 | 4
4 | 0.6
F
21 | 71
2 | 7
G
8 | 66
0.8 | 26
H
5 | 73
0.5 | 22
bcl-2
CD45RO

Table 6.2 The expression of Bcl-2 on $CD8^+$ T cell subsets in the lymph node[a]

	Normal Tonsil	HIV-1^+ Lymph Node	
	PC[b]	GC	PC
$CD8^+Bcl\text{-}2^+$	80(62-88)[c]	13(5-50)	33(18-79)
$CD8^+CD45RO^+$	24(14-31)	>95	78(52-95)
$CD8^+CD45RO^+$ $Bcl\text{-}2^-$	40(0-66)	79(70-90)	75(20-96)

[a] Tissue sections from 8 normal tonsils and 9 HIV-1+ lymph nodes were investigated by two and three colour immunofluorescence for expression of CD8,CD45RO and Bcl-2. Two colour studies were performed using conventional epifluorescence microscopy. Three colour studies were performed on the confocal microscope (see text)

[b] CD8+ lymphocytes are not found within the germinal centre.

[c] The numbers shown are the median and range

PC Paracortex

GC Germinal centre

microscope. The $CD8^+$ lymphocytes stained with LR were identified in one channel and the intensity of the Bcl-2/FITC staining for each cell determined in channel two. As shown in Fig. 6.8a, the mean intensity of Bcl-2 on $CD8^+$ lymphocytes was far lower in the HIV-1^+ individual, (<50% of the normal tonsil) due to the presence of Bcl-2^- and Bcl-2^{low} cells (Fig. 6.8c).

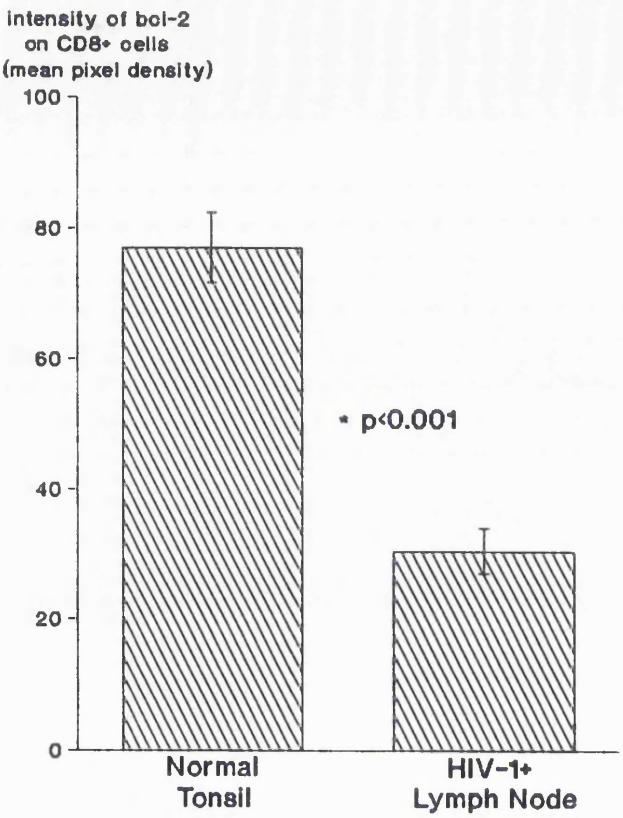
As many of the $CD8^+$ lymphocytes infiltrating the HIV-1^+ lymph node co-express CD45RO, studies were performed using triple colour IF to determine if the low expression of Bcl-2 was simply due to this population shift or to changes within the $CD45RO^+$ population. Using confocal microscopy tissue sections from normal tonsil and HIV-1^+ lymph nodes were triple stained using the combination CD45ROγ2a plus GAM-IgG2a-FITC, CD8μ plus GAM-IgM-LR and Bcl-2 plus GAM-IgG1-biotin together with streptavidin-Cy5. A representative example is illustrated in Fig. 6.9 which shows a paracortical area from one seronegative and one HIV-1 infected individual. In Fig. 6.9a&c $CD8^+$ cells in green are shown together with $CD45RO^+$ cells in blue, the double positive cells appearing cyan. Note that the HIV-1^+ patient has many more $CD8^+$ lymphocytes and that these are mainly $CD45RO^+$. The second doublet (Fig. 6.9b&d) shows CD8 in green together with Bcl-2 in red which combine to form yellow ($CD8^+$,Bcl-2^+). In the normal tonsil the majority of $CD8^+$ cells express Bcl-2 (yellow) including in this individual all of the $CD45RO^+CD8^+$ cells. Overall the HIV-1^- tissue shows a red/yellow staining pattern indicating that most cells express Bcl-2. In contrast, green predominates in the HIV-1^+ individual due to the absence of Bcl-2.

Triple staining using CD8, CD45RO and Bcl-2 on sections from 8 separate HIV-1^- and 9 HIV-1^+ individuals revealed a similar loss of Bcl-2 on $CD8^+$ cells in HIV-1 infection (Table 6.2). The majority, >70% of $CD8^+$ cells in both the paracortical areas and germinal centres of the HIV-1 infected tissue had a primed, $CD45RO^+$ phenotype. Of these, >75% lacked expression of Bcl-2 compared to only 40% in the normal tonsil. Taken together this data suggests that the loss of Bcl-2 on $CD8^+$ cells in lymph nodes is not simply due to an accumulation of $CD8^+$,$CD45RO^+$ lymphocytes but results from changes within this subset.

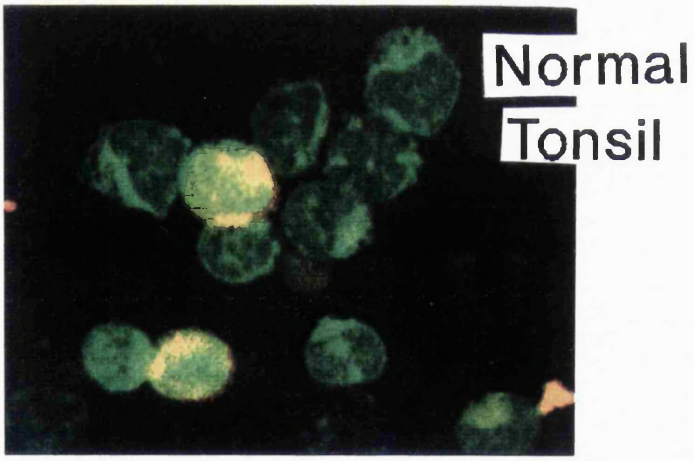
Figure 6.8 The intensity of bcl-2 staining in tonsil and HIV-1$^+$ lymph node

Cell suspensions of normal tonsil (b) and HIV-1$^+$ lymph node (c) were investigated for Bcl-2 expression using the confocal microscope. Cytospin preparations were double stained with CD8μ plus GAM-IgM-LR (red) and Bcl-2 plus GAM-IgG-FITC (green). The intensity of Bcl-2 on the CD8$^+$ cells was determined for each cell by measuring the pixel density of the FITC fluorescence (a). The results are shown as the mean pixel density of 100 CD8$^+$ cells $\pm$ sem.

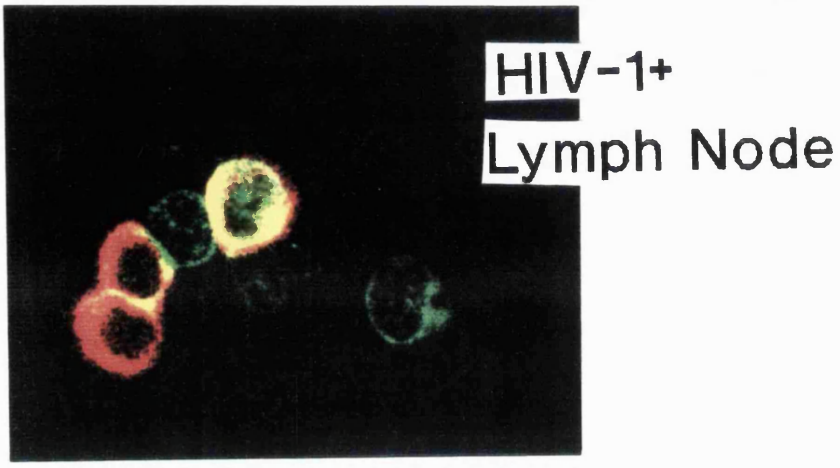
(a)


intensity of bcl-2
on CD8+ cells
(mean pixel density)
100
80
60
40
20
0
* p<0.001
Normal
Tonsil
HIV-1+
Lymph Node

(b)


Normal
Tonsil

(c)


HIV-1+
Lymph Node

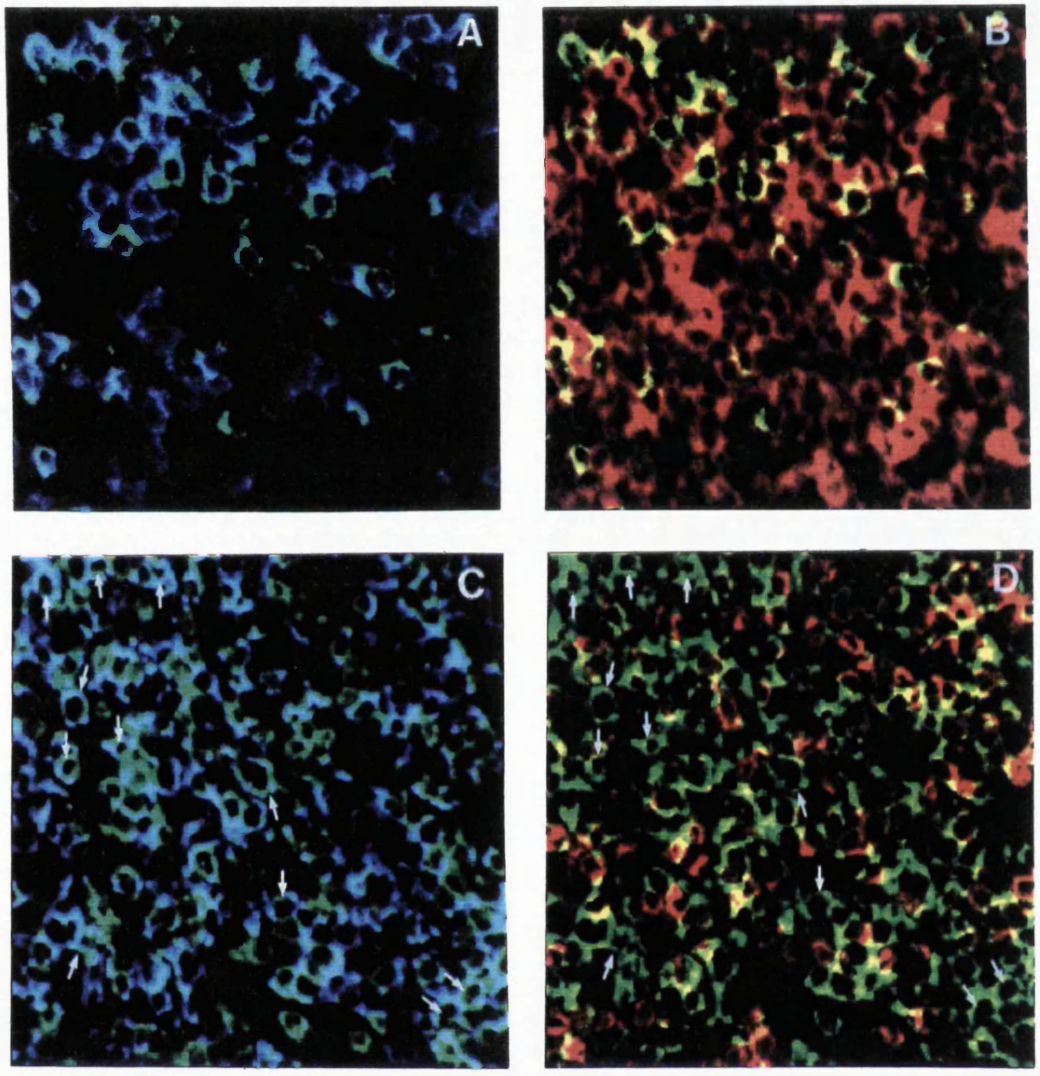


Figure 6.9 Triple colour study of CD8,CD45RO and Bcl-2 in tissue section using confocal microscopy

Sections of normal tonsil (A,B) and HIV-1^+ lymph node (C,D) were triple stained using the combination CD8-lissamine rhodamine, CD45RO-FITC and Bcl-2-biotin plus streptavidin-Cy5. The data are shown as two sets of doubles. In the first CD8 in green combines with CD45RO in blue to form doubles in cyan (A,C). In the second CD8 in green combines with Bcl-2 in red to form doubles in yellow (B,D). The arrows show the presence in the HIV-1^+ individual of $CD8^+CD45RO^+$ cells that lack Bcl-2. Two paracortical areas are shown.

The association between low Bcl-2 and apoptosis

To investigate if the low expression of Bcl-2 by T cells from HIV-1 and AVI patients reflected their susceptibility to death by apoptosis, PBMC were isolated and apoptosis measured after culture. Apoptosis was determined morphologically in cytospin preparations stained with May Grunwald Giemsa. These were clearly distinguishable from live cells by the decrease in cell volume and the presence of fragmented nuclei (Fig. 6.10a). Apoptosis by these criteria was confirmed by DNA electrophoresis where oligosomal DNA fragmentation was observed in AVI and HIV-1 patients but was absent in seronegatives (Fig. 6.10b). The expression of Bcl-2 on freshly isolated PBMC from HIV-1^-, HIV-1^+ and AVI individuals was determined by flow cytometry. The cells were then cultured in the presence or absence of anti-CD3 plus rIL-2 and analysed daily in cytocentrifuge smears for the morphological signs of apoptosis. After only 24 hours in culture in the absence of any stimuli significantly more apoptotic cells were found in the AVI patients compared to both the seronegative and HIV-1^+ individuals (HIV-1^- 2.4%; HIV-1^+ 8.2%; AVI 20.0%; Table 6.3). Furthermore, there was a strong negative correlation between the proportion of Bcl-2^+ cells in the fresh samples and the presence of apoptotic cells ($r=-0.89$; $p<0.001$; Fig. 6.11). This was also true when AVI and HIV-1^+ individuals were analysed separately (AVI $r=-0.94$ $p<0.001$; HIV-1 $r=-0.85$ $p<0.001$) but was less clear in the seronegative group where few cells in apoptosis were observed.

When lymphocytes activated with anti-CD3 were similarly investigated, no correlation was seen between Bcl-2 expression on freshly isolated cells and the degree of apoptosis after 3 days in culture ($r=0.34$; not significant). In fact, after activation very few classically apoptotic cells could be identified. This is illustrated in Fig. 6.12 which shows the appearance of lymphocytes from HIV-1^- and HIV-1^+ individuals after activation for 3 days with anti-CD3 plus rIL-2. After mitogenic stimulation, many of the lymphocytes from the seronegative individual transformed into large lymphoblasts (*; Fig. 6.12a) while in the HIV-1^+ individual shown (Fig. 6.12b) there are very few lymphoblasts but instead large amounts of nuclear material and cell debris. Although some small lymphocytes and cells with condensed nuclei can be seen, there is no evidence of extensive, activation induced apoptosis. This

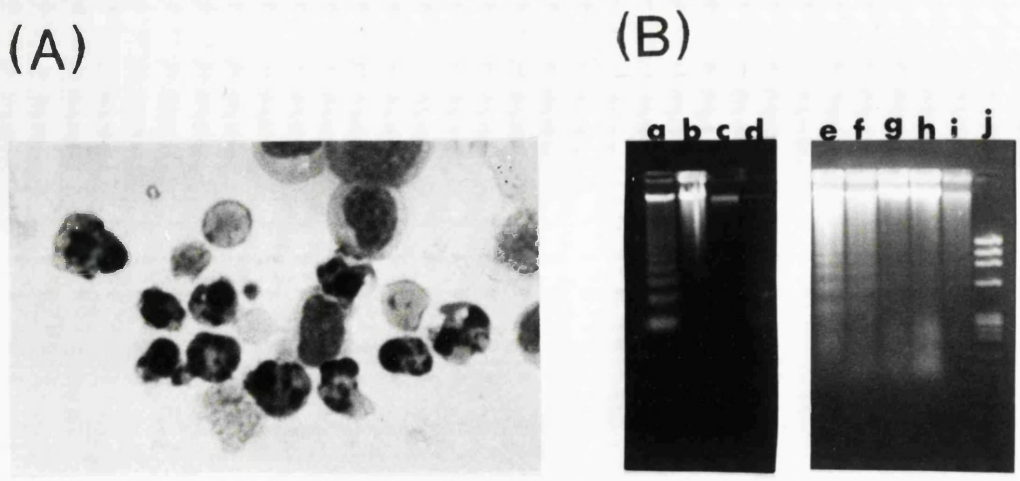


Figure 6.10 Detection of cells in apoptosis

Cytospins of PBMC cultured overnight were stained with MGG and examined for the presence of cells with the morphological features of apoptosis. These are seen as small shrunken cells with condensed and fragmented nuclei (A). Apoptosis was confirmed by DNA electrophoresis (B). The results shown are; dexamethasone treated rat thymocytes (a) and PBL from an individual with VZV (b), a normal (c) and an HIV-1^+ individual (d) after 24 hours in culture without stimulation. In the second part of the figure DNA was extracted from lymphocytes after three days in culture with or without stimulation with anti-CD3; HIV-1^+ unstimulated (e), HIV-1^+ stimulated (f), normal unstimulated (g), normal stimulated (h), control day0 (i), DNA markers EcoRIII-HIND (j)

Table 6.3 The association between low Bcl-2 and apoptosis

	n	Bcl-2[a]	Apoptosis[b]
HIV-1$^-$	10	81.3±3.2[c]	2.4±0.6
HIV-1$^+$	11	73.5±2.8^{NS}	8.2±1.8*
AVI	22	54.9±5.5**	20.0±3.5***

[a] The percentage of Bcl-2$^+$ lymphocytes was determined on PBMC prior to culture using the permeabilisation procedure outlined. The data are shown as mean ± sem

[b] The number of cells in apoptosis was determined after 24 hours in culture by morphological criteria.

[c] The mean values were compared to normal using the Students t test; * $p<0.01$, ** $p<0.005$, *** $p<0.002$

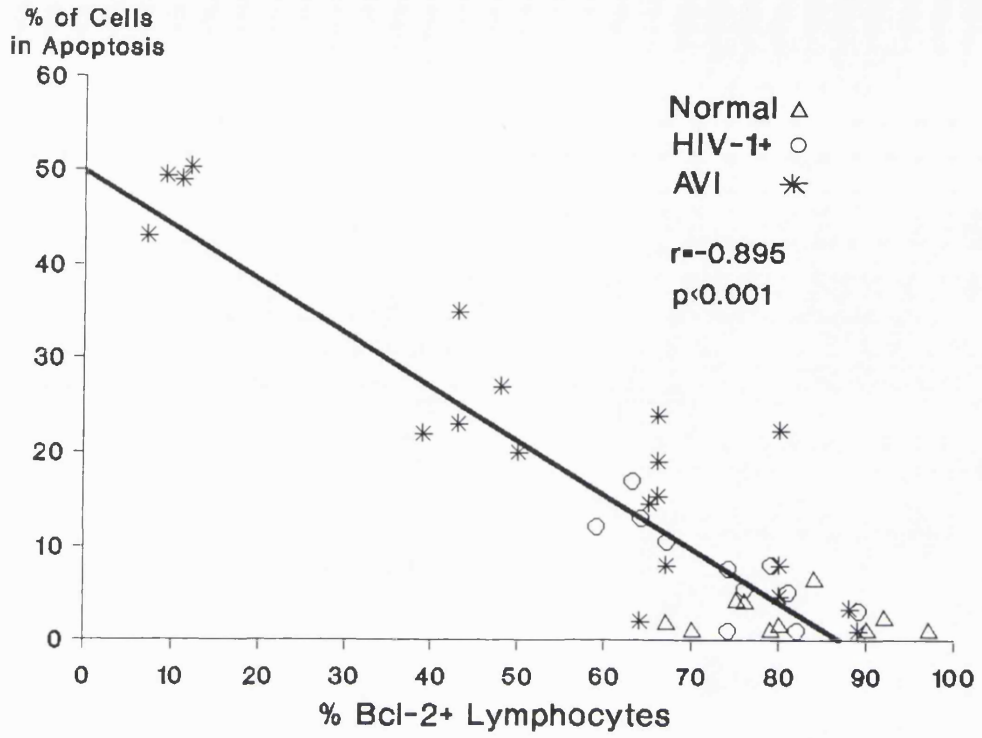


Figure 6.11 The association between low Bcl-2 expression and apoptosis in AVI and HIV-1$^+$ patients

PBMC were stained for Bcl-2 and analysed by flow cytometry (x-axis). Samples of the same cells were also cultured for 24 hours without stimulation and the number of cells in apoptosis determined by morphology after MGG staining (y-axis). Samples from normal blood (△) and from individuals with acute EBV or VZV (✳) and HIV-1$^+$ patients (○) are shown.

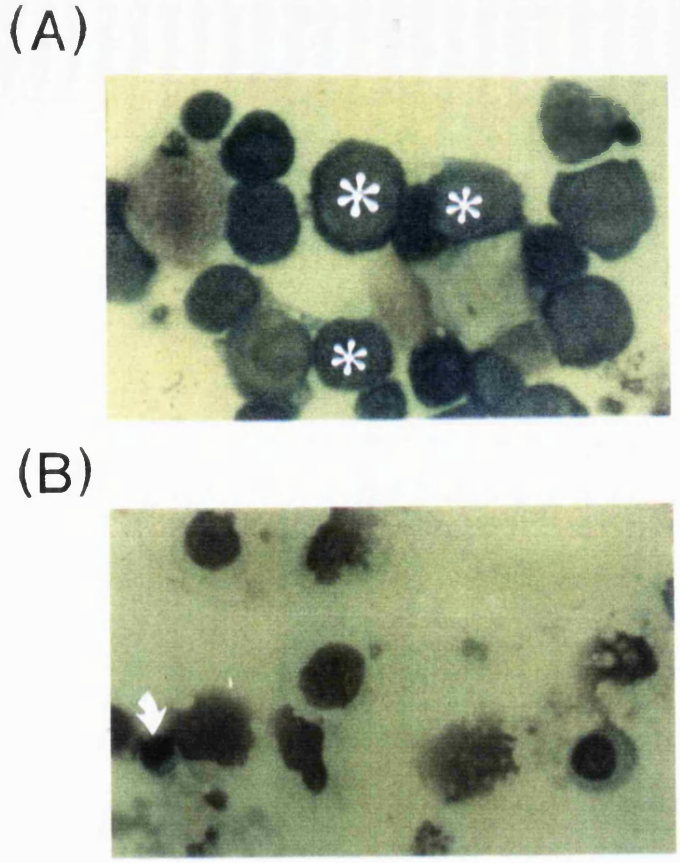


Figure 6.12 The appearance of lymphocytes after activation

PBMC from HIV-1$^-$ (A) and HIV-1$^+$ (B) individuals were investigated for the presence of apoptotic cells after stimulation with anti-CD3 for 3 days. The appearance of the cells after MGG staining is shown. * lymphoblast

was investigated further using DNA electrophoresis in which DNA was extracted from cells cultured for 48 hours in the presence or absence of anti-CD3 plus rIl-2. The number of cells at the start of the culture was the same (2 x 10^6) and the entire well was harvested for DNA extraction. Therefore, assuming equal efficiency during the extraction procedure, the amount of DNA recovered from each well and loaded onto the gel should be equivalent. However, in no individual tested (n=10) was any increase in the degree of DNA fragmentation noted after activation (Fig. 6.10B). Although this method of investigating apoptosis does not lend itself easily to quantitation, in combination with the morphological data it does suggest that AALD is not occurring via classical apoptosis.

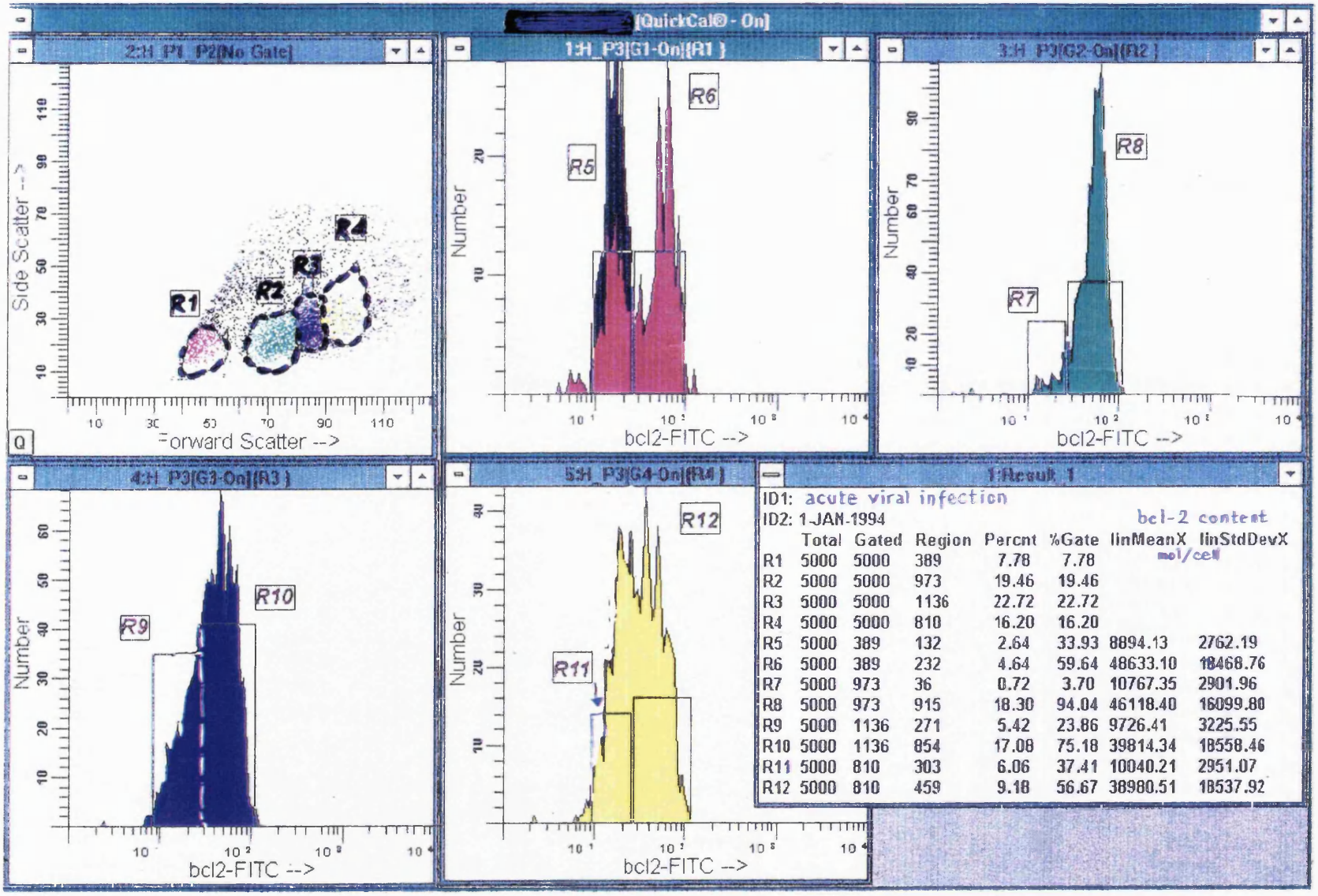
Quantitation of Bcl-2 expression using Quantum Simply Cellular beads

The results of a quantitative investigation of Bcl-2 expression are illustrated in Fig. 6.13 which shows PBMC from an AVI patient stained with Bcl-2-FITC. Various lymphocyte populations were colour coded according to size; red dead cells and debris; green blue and yellow are small, intermediate and large lymphocytes respectively. The background Bcl-2 staining using this reagent was set at 10,000 mol/cell (regions 5,7,9 & 11). From this figure it is clear that as cells increase in size the intensity of Bcl-2 decreases, confirming that the blast-like cells in the peripheral blood of individuals with acute viral infections are low in Bcl-2.

Discussion

Previous studies have shown that the expression of Bcl-2 is closely involved in the regulation of lymphocyte survival associated with selection or deletion of germinal centre B lymphoblasts (Liu et al 1989, Liu et al 1991, Nunez et al 1991) and cortical thymocytes (Hockenberry et al 1991, Sentman et al 1991, Siegel et al 1992). In these tissues the loss of expression of Bcl-2 is linked with cell death while upregulation of the protein in transgenic mice increases the lifespan of the cells and can prevent selection (Strasser et al 1991, Siegel et al 1992).

As shown in chapter 4, in both HIV-1^+ and AVI patients, activated T cells expressing CD45RO develop *in vivo* (Froebel et al 1991, Prince & Jensen 1991, Miyawaki et al 1991). These *in vivo* activated peripheral blood populations were



	Total	Gated	Region	Percnt	%Gate	linMeanX	linStdDevX
R1	5000	5000	389	7.78	7.78		
R2	5000	5000	973	19.46	19.46		
R3	5000	5000	1136	22.72	22.72		
R4	5000	5000	810	16.20	16.20		
R5	5000	389	132	2.64	33.93	8894.13	2762.19
R6	5000	389	232	4.64	59.64	48633.10	18468.76
R7	5000	973	36	0.72	3.70	10767.35	2901.96
R8	5000	973	915	18.30	94.04	46118.40	16099.80
R9	5000	1136	271	5.42	23.86	9726.41	3225.55
R10	5000	1136	854	17.08	75.18	39814.34	18558.46
R11	5000	810	303	6.06	37.41	10040.21	2951.07
R12	5000	810	459	9.18	56.67	38980.51	18537.92

found to contain a Bcl-2$^-$ component that was expanded compared to normal individuals. As naive, CD45RA$^+$ T cells develop a primed CD45RO phenotype as a consequence of stimulation (Sanders, Makgoba & Shaw 1988, Beverley 1990, Akbar et al 1988, Clement 1992) this suggested that Bcl-2 might be down regulated during lymphocyte activation. This possibility was confirmed in isolated CD45RA$^+$ lymphocytes which were found to lose Bcl-2 expression during the transition from CD45RA to CD45RO *in vitro*. Therefore, both acute and chronic *in vivo* stimulation as well as mitogen induced activation *in vitro* lead to the development of a CD45RO$^+$ population which lacks Bcl-2.

The CD8$^+$ lymphocytosis seen in acute viral infection is transient (Tomkinson et al 1987, Cauda et al 1987, Cheeseman et al 1988, Miyawaki et al 1991) suggesting a rapid clearance of the cells *in vivo*. Indeed, it has been demonstrated that these cells die rapidly by apoptosis after culture (Moss et al 1985, Uehara et al 1992). Thus a decrease in Bcl-2 expression renders cells prone to apoptosis and may provide mechanism for the control of T cell numbers after virus induced polyclonal expansion. The correlation between Bcl-2 and apoptosis also holds true for the spontaneous cell death seen in HIV-1 infection. Interestingly, the addition of IL-2 to the activated CD45RO$^+$ T cells increased Bcl-2 expression and rescued the cells from apoptosis (Akbar et al 1993). Therefore, the lack of sufficient local levels of IL-2 might lead to a decreased Bcl-2 expression and predispose apoptosis *in vivo*. Such a scenario might be envisaged in HIV-1 infection where levels of IL-2 are decreased due to CD4$^+$ T cell depletion.

In contrast, no association between Bcl-2 expression and AALD was found in this study indicating that Bcl-2 is not involved in this form of cell death. Additionally, based on morphology and DNA fragmentation studies, AALD could not identified as a classically apoptotic phenomenon. In a recent review on programmed cell death (PCD) Schwartz & Osborne (1993) noted that PCD and apoptosis are not synonymous. Not all PCD occurs by apoptosis, for instance during insect metamorphosis dying cells do not display the classical signs of apoptosis, including chromatin condensation and DNA fragmentation. Similarly, apoptosis can occur in the absence of new gene expression, for instance the DNA fragmentation induced in target cells by cytotoxic lymphocytes (Russell & Dobbs 1980, Duke

Chervenak & Cohen 1983). Therefore, the AALD described here may be another example of a PCD phenomenon which does not occur by apoptosis. Interestingly, one group who noted apoptosis in HIV-1 infected individuals after stimulation with anti-CD3 were unable to show any correlation or increase with disease progression (Meyaard et al 1994). However, levels of apoptotic cell death were far lower than the AALD described here, further suggesting that AALD is not classical apoptosis. Alternatively, AALD could be an example of necrotic cell death induced perhaps by some viral protein that interferes with normal cell division. Indeed there is some evidence that dividing cells are affected by HIV-1 infection. For example, many of the gut and skin complaints of patients could reflect problems of epithelial or epidermal cell regeneration. In addition, patients also suffer hair loss that might also be related to poor cell division. In this respect, viral proteins such as Tat and Nef are known that are known to interfere with normal lymphocyte function (Viscidi et al 1989, Purvis et al 1992) could be implicated.

It still remains a possibility that AALD is also an apoptotic phenomenon but that due to the timing of the experiment the classical signs were missed. There is only a narrow window during which it is possible to measure DNA fragmentation and if activated cells apoptose at approximately similar times early after stimulation, this might account for the results. Although a daily analysis of cells after stimulation did not reveal extensive apoptosis, a detailed time course involving a more quantitative assessment of apoptosis, such as the detection of DNA breaks by *in situ* nick translation (Gorczyca et al 1993), might prove useful to validate the findings.

Factors other than Bcl-2 protein expression may be involved in cell death after activation. Recently, two *bcl-2* related genes *bax* and *bcl-X* have been reported (Boise et al 1993, Oltval et al 1993). Bcl-X exists in two forms, Bcl-XS and Bcl-XL the latter of which functions similarly to Bcl-2 and protects cells from apoptosis. The Bax protein forms heterodimers with Bcl-2 and modifies its function (Yin et al 1994). Thus, *bcl-X* or *bax* may be involved in AALD. In addition, activated $CD45RO^+$ T cells from individuals with viral infections show increased expression of Fas/APO-1, indicating that they may be primed for Fas-mediated cell death.

Interleukin-2 is likely to be one of the key molecules involved in keeping activated T cells cycling. However, such continuous stimulation is probably not the

only mechanism whereby mature T cells are maintained. For instance, the co-culture of apoptosis prone lymphocytes with tissue stroma-derived fibroblasts can extend viability and slow down apoptosis. Studies have shown that the viability of activated T cells can be extended without significant proliferation after co-culture on fibroblasts and that they lose their blast-like morphology (Scott, Pandolfi & Kurnick 1990). These studies suggest that stromal factors may support the survival of T cells in a quiescent state and that different microenvironments may influence the fate of activated T cells.

The greatly expanded $CD8^+$,$Bcl\text{-}2^-$ population seen in the lymph nodes of HIV-1 infected individuals may reflect the influence of stromal factors on T cell survival *in vivo* as such Bcl-2 negative cells would be expected to die rapidly in the absence of a positive signal. The $CD8^+$,$CD45RO^+$ lymphocytes in the lymph node show an even more marked loss of Bcl-2 expression than the equivalent population in peripheral blood. Recent data suggests that the lymph node microenvironment is not sufficient to promote the survival of all of these cells as, using propidium iodide staining, there is clear evidence of apoptosis among the $CD8^+Bcl\text{-}2^-$ population (Bofill et al submitted). Similarly, the huge expansion of $CD8^+$ cells in the lymph nodes during acute infectious mononucleosis is associated with areas of necrosis within the tissue. This might be expected if large numbers of cells are in apoptosis and the macrophages in the paracortex are unable to phagocytose the overwhelming number of dying cells.

This study suggests that the loss of Bcl-2 and subsequent death by apoptosis may be a normal physiological response to pathogen induced proliferation, designed to maintain lymphocyte homeostasis. However, the reasons why cells die after activation are less clear. Studies on cell lines and PBL *in vitro* point to a role for HIV-1 virus and viral gp120 in cell death by apoptosis (Terai et al 1991, Martin Matear & Vyakarnam 1994). The cross-linking of gp120 on the CD4 molecule, followed by stimulation of the cell through the TCR causes an activation induced apoptosis of $CD4^+$ lymphocytes (Banda et al 1992). This may be particularly relevant in vivo in view of the discovery of gp120 in the serum of $HIV\text{-}1^+$ patients (Oh et al 1992). Therefore, the loss of $CD4^+$ lymphocytes by apoptosis may in part be explained either by infection with HIV-1 or by the presence on the cell surface of

gp120 bound to the CD4 molecule. This however does not explain the cell death observed in the $CD8^+$ population.

Interestingly, it was reported by Groux et al (1992) that co-stimulation with anti-CD28 can decrease apoptosis in HIV-1 infection after activation with the superantigen SEB, suggesting that co-stimulation could prevent AALD.

CHAPTER 7

THE LOSS OF CD28 EXPRESSION IN HIV-1 INFECTION: ASSOCIATION WITH AALD

Introduction

There is increasing evidence that cross-linking of the TCR alone is insufficient for T cell activation and that a second signal derived from receptor ligand interactions between the T cell and APC are required for optimal stimulation (Meuller, Jenkins & Schwartz 1989). One such second signal is provided through the interaction of the CD28 antigen on T cells with either anti-CD28 (Pierres et al 1988, Ledbetter et al 1985, Ledbetter et al 1990) or with its specific ligand B7/BB1 (Linsley et al 1991) which is expressed on a variety of APC (Freedman et al 1991, Young et al 1992). Such co-stimulation greatly enhances T cell activation via the TCR or the CD2 molecule, providing an alternative pathway to TCR engagement which is insensitive to Cyclosporin A (June et al 1989). Currently, the engagement of CD28 is the strongest known second signal in human T lymphocyte activation (reviewed in Lui & Linsley 1992).

In HIV-1 infection, co-stimulation with anti-CD28 has been reported to return the proliferative responses of anti-CD3 or anti-CD2 stimulated cells to normal levels (Gruters et al 1990; Van-Noesel et al 1990) and to prevent superantigen or PWM induced apoptosis of $CD4^+$ lymphocytes (Groux et al 1992). However, other studies have shown that a sub-population of $CD8^+$ T cells which express the activation associated antigen HLA-DR have a decreased response to co-stimulation through anti-CD28 (Pantaleo et al 1990b). Also, infection of $CD4^+$ clones with HIV-1 leads to an inhibition of stimulation through CD28 (Laurence et al 1989).

The CD28 antigen is expressed on >95% of $CD4^+$ and 50% of $CD8^+$ lymphocytes (Hansen, Martin & Nowinski 1980, McMichael & Gotch 1987), but is absent from CD3-negative, $CD16^+$ NK cells (Nagler et al 1989). Nevertheless, despite the importance of CD28 as a co-stimulatory molecule in lymphocyte activation, its expression in HIV-1 infected individuals has not been fully documented. In particular, the expression of CD28 has not been correlated with the phenotypic shifts in lymphocyte subsets which occur in these individuals nor related to the functional defects characteristic of this infection. Two studies on small groups

of HIV-1 infected individuals showed decreased CD28 expression on a subset of $CD8^+$ cells (Gruters et al 1991, Saukkonen Kornfeld & Berman 1993) but these were not confirmed as *bona fide* $CD3^+$ T cells. In another study of $HIV\text{-}1^+$ haemophiliacs $CD4^+$ cells showed lower than normal proportions of $CD28^+$ cells (Dianzani et al 1988). There is no data on the expression of CD28 in AVI patients or its potential role in preventing spontaneous apoptosis in this group.

One possible explanation for the activation induced cell death in HIV-1 infection could be the loss of CD28 antigen on T lymphocytes leading to the absence of an obligatory second signal during activation. Therefore the aims of this chapter were;

(1) To investigate the expression of CD28 in various lymphocyte subsets in normal, $HIV\text{-}1^+$ and AVI individuals.

(2) To compare this with the blast transformation and proliferation of the same cell populations.

(3) To determine if co-stimulation via CD28 could prevent AALD

Methods

Analyses of CD28 expression on T lymphocyte subsets

The percentages of cells expressing CD28 were determined by two and three colour IF studies using the whole blood technique (Chapter 2). The absolute numbers were calculated from the lymphocyte counts for each patients obtained from routine haematology. In some cases, absolute numbers were calculated directly using the Cytoron*Absolute*.

Two and three colour IF combinations were used to measure CD28 expression in whole blood from patient groups. For two colour studies CD28 plus GAM-IgG1-PE was combined with one of either; CD3 or CD4 plus GAM-IgG2a-FITC or CD8 plus GAM-IgM-FITC. Controls for these double combinations were Mouse Ig at 5μg/ml plus the GAM-Ig second layers. To more closely investigate the $CD3^+$,$CD28^-$ population, the following triple combinations were employed; CD28-PE, CD3-biotin plus strep-tricolor and one of the following; (a) CD8-FITC (b) CD57 plus GAM-IgM-FITC (c) CD38 plus GAM-IgM-FITC (d) CD45RO-FITC (e) HLA-DR-FITC

DNA synthesis measured using bromodeoxyuridine (BrdU)

Lymphocyte cultures were stimulated with PHA as previously described (Chapter 2). Four hours prior to harvesting, BrdU (Amersham International plc, Amersham, UK) at 10μM was added to the cultures. At the end of the culture period the cells were harvested and membrane stained in suspension with CD57 and CD28. Cell smears were then prepared and stored as described (Chapter 2). To visualize the membrane staining cytospins were incubated with GAM-IgM-LR (for CD57) and GAM-IgG1-biotin plus strep-Cy5 (for CD28). The lymphocytes that had incorporated BrdU were identified using an anti-BrdU antibody (Amersham plc) together with GAM-IgG2a-FITC. This triple colour combination was viewed using confocal microscopy

Results

The expression of CD28 on T cells

Initial studies to investigate the normal distribution of CD28 on lymphocyte subsets revealed that 98$\pm$2.4% (n=10) of $CD28^+$ cells were $CD3^+$ T cells. This constituted about 90% of the total $CD3^+$ population while Ig^+ B-cells, $CD4^+$ monocytes and $CD3^-$ natural killer (NK) cells, defined by the expression of CD16, CD56 or CD57, were negative.

A comparison between CD28 expression in HIV-1^+ and AVI patients revealed that the vast majority of $CD28^+$ lymphocytes, as with the HIV-1^- control group, were seen within the $CD3^+$ population. Nevertheless, the percentage of $CD3^+$ T cells which expressed CD28 was reduced in both AVI patients and HIV-1^+ individuals at every stage of the disease ($p<0.001$; Table 7.1). This was also reflected in a significant decline in absolute numbers of $CD3^+$,$CD28^+$ cells in HIV-1^+ patients compared to the normal control ($p<0.01$; $p<0.001$; Table 7.1) but not in the AVI patients where the lymphocytosis masked any percentage shifts. These changes in CD28 expression were due to an expansion of the $CD3^+$,$CD28^-$ population which was significantly increased in every patient group compared to the control group (control 0.14$\pm$0.03; HIV-1^+ CDCII 0.68$\pm$0.13, CDCIII 1.04$\pm$0.1, CDCIV 0.47$\pm$0.14; AVI 0.46$\pm$0.08; $p<0.001$; Fig. 7.1)

The majority of $CD4^+$ T cells in AVI patients and in the HIV-1^+, CDCII and

Table 7.1
The expression of CD28 on $CD3^+$ T-Cells

			$CD3^+CD28^+$	
		n	Percentage	Absolute Number[a]
HIV-1^-	Control	10	89.8 ± 0.7	1.27 ± 0.116
HIV-1^+	CDCII	22	$49.8 \pm 6.3^{***b}$	$0.70 \pm 0.141^{**}$
	CDCIII	8	$27.6 \pm 8.3^{***}$	$0.40 \pm 0.131^{***}$
	CDCIV	13	$42.9 \pm 4.3^{***}$	$0.36 \pm 0.131^{***}$
AVI		16	$65.9 \pm 5.6^{***}$	1.55 ± 0.132^{NS}

[a] The percentage and absolute number of CD28+ lymphocytes within the CD3+ T cell population was determined by two colour immunofluorescence in HIV-1^-, AVI and HIV-1 infected individuals. The absolute number of lymphocytes x 10^9/L in peripheral blood was an estimate based on the percentages obtained in combination with the lymphocyte counts for each individual. These observations were confirmed using lysed whole blood method for counting $CD3^+$,$CD28^-$ cells with the Cytoron*absolute* (Ortho)
[b]The data were compared to the normal control group using the Students T-test; $^{***}p < 0.001$; $^{**}p < 0.01$

Figure 7.1 The contribution of $CD28^+$ and $CD28^-$ lymphocytes within the $CD3^+$, $CD4^+$ and $CD8^+$ subsets from normal controls and HIV-1 infected individuals

Two colour IF was used to determine the percentage of $CD28^+$ and $CD28^-$ subsets within T lymphocyte populations. The total number of $CD3^+$, $CD4^+$ or $CD8^+$ lymphocytes is shown as the total height of the bar while the shaded areas represent the mean percentage of $CD28^+$ or $CD28^-$ cells within each group.

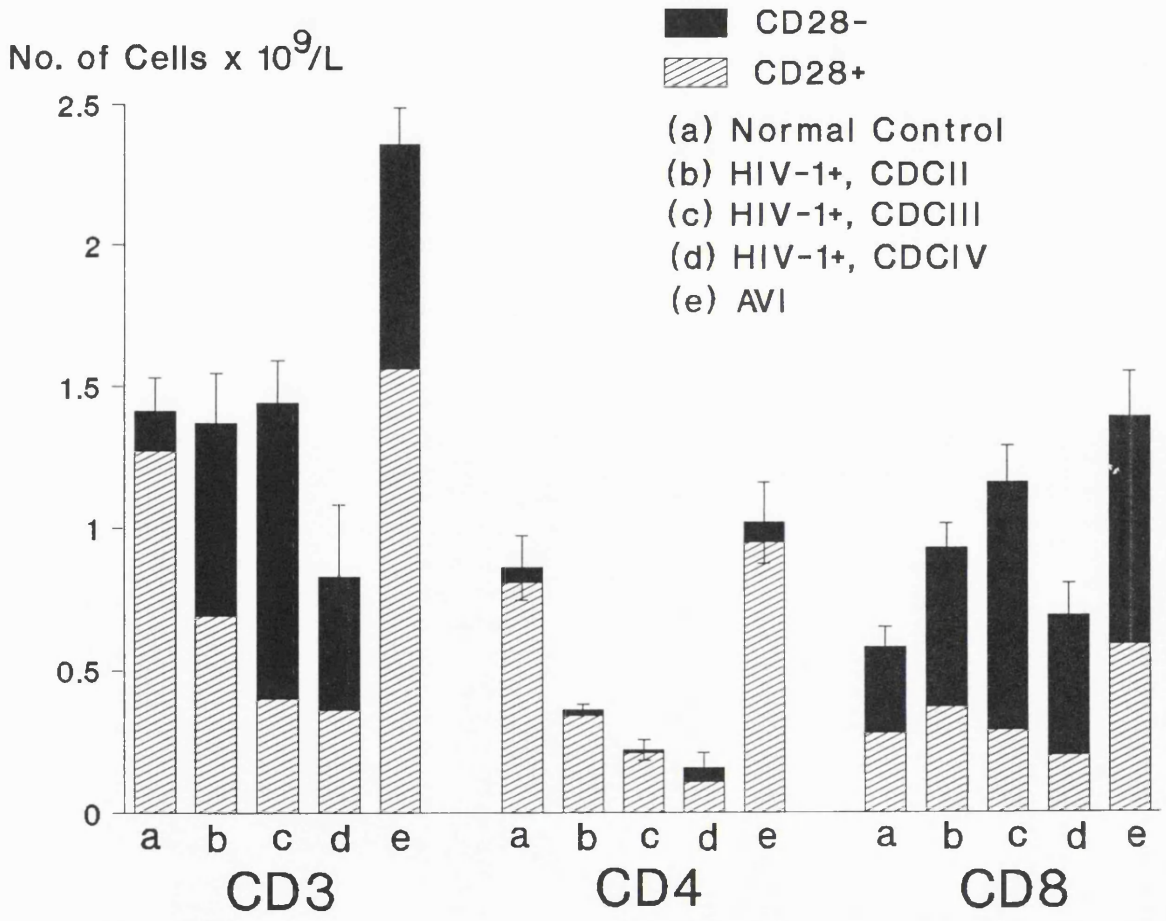

No. of Cells x 10^9/L
CD28-
CD28+
(a) Normal Control
(b) HIV-1+, CDCII
(c) HIV-1+, CDCIII
(d) HIV-1+, CDCIV
(e) AVI
2.5
2
1.5
1
0.5
0
a b c d e
CD3
a b c d e
CD4
a b c d e
CD8

Table 7.2 The proportion of $CD28^+$ and $CD28^-$ lymphocytes within $CD3^+$, $CD4^+$ and $CD8^+$ lymphocyte subsets

		n	CD3	$CD28^-$ *$CD28^+$*	CD4	$CD28^-$ *$CD28^+$*	CD8	$CD28^-$ *$CD28^+$*
$HIV\text{-}1^-$	Control	10	1.41±0.12[a]	9.2±1.4[b] *90.8±1.4*	0.86±0.11	5.7±1.2 *94.3±1.2*	0.58±0.07	52.0±6.7 *47.7±6.7*
$HIV\text{-}1^+$	CDCII	22	1.38±0.17	50.2±6.3 *49.8±6.3*	0.36±0.02	14.7±5.9 *85.3±1.2*	0.93±0.08	59.9±6.7 *47.9±6.7*
	CDCIII	8	1.43±0.16	72.4±8.3 *27.6±8.3*	0.22±0.04	4.6±5.8 *95.4±5.8*	1.16±0.13	73.3±6.5 *26.7±6.5*
	CDCIV	13	0.82±0.26	57.2±4.3 *42.8±4.3*	0.16±0.05	46.0±12.7 *55.0±12.7*	0.68±0.12	69.7±4.9 *30.3±4.9*
AVI		16	1.36±0.13	34.1±5.6 *65.9±5.6*	1.02±0.14	7.1±2.8 *92.9±2.8*	1.39±0.16	57.3±5.8 *42.7±5.8*

[a] The absolute number of $CD3^+$, $CD4^+$ and $CD8^+$ lymphocytes was based on the percentages obtained in combination with the lymphocyte counts for each individual

[b] The percentage of $CD28^+$ and $CD28^-$ lymphocytes within the $CD3^+$,$CD4^+$ or $CD8^+$ lymphocyte populations was determined by two colour immunofluorescence

This data is shown graphically in Fig. 7.1

CDCIII groups still expressed CD28 (CD28% in CD4; control 94.3±1.2, CDCII 85.3±5.6, CDCIII 96.4±6.7, AVI 92.9±2.8). Only in HIV-1$^+$, CDCIV individuals could one detect a substantial drop in the proportion of CD28$^+$,CD4$^+$ cells (CDCIV 54.9±13.9) but due to the decrease of the CD4$^+$ lymphocytes the overall absolute numbers of CD4$^+$,CD28$^-$ T cells remained the same during the whole course of HIV-1 infection (control 0.05±0.01, CDCII 0.02±0.001, CDCIII 0.01±0.002 and CDCIV 0.05±0.027 x 10^9/l; Fig. 7.1).

It follows from these results that the increased numbers of CD28$^-$ T cells in both AVI and HIV-1 disease were present in the CD8$^+$ population. In both patient groups the CD8 lymphocytosis was mainly due to a selective increase of CD8$^+$,CD28$^-$ lymphocytes (control 0.30±0.026, CDCII 0.56±0.07, CDCIII 0.87±0.15, CDCIV 0.49±0.09, AVI 0.80±0.08 x10^9/L; $p<0.003$; Fig.7.1). The CD8$^+$,CD28$^+$ population remained relatively constant throughout the course of HIV-1 infection (control 0.28±0.07; CDCII 0.37±0.04; CDCIII 0.29±0.06; CDCIV 0.20±0.045) but in AVI patients, both CD28$^+$ and CD28$^-$ lymphocytes were increased (CD8$^+$,CD28$^+$ 0.59±0.08; CD8$^+$,CD28$^-$ 0.8±0.08 x 10^9/L). Nevertheless, in both acute and chronic viral infections there is a dominance of CD28$^-$ lymphocytes within the CD8$^+$ population.

Phenotypic features of CD28$^-$ T cells

As both AVI and HIV-1 infections are associated with phenotypic changes in peripheral blood lymphocytes, studies were performed using triple colour IF to more clearly define the rare CD3$^+$,CD28$^-$ lymphocyte subset in control and the dominant CD3$^+$,CD28$^-$ cells in the patient groups. Triple combinations using CD3-biotin, CD28-PE plus a third FITC-conjugated marker were therefore standardised. The third markers chosen were those shown in this study (chapter 4) and by other investigators to be increased in HIV-1 infection particularly within the CD8$^+$ lymphocyte subset and included CD45RO, HLA-DR, CD38 and CD57.

CD8: Using the triple combination CD3, CD28 and CD8, it was found that in all individuals the majority of CD3$^+$,CD28$^-$ cells were CD8$^+$ (control 55±12.9%, HIV-1$^+$ 89.8±2.1%, AVI 85.5±5.8%; Table 7.3). Additionally, CD3$^-$,CD8$^+$ NK cells were entirely CD28 negative and this population was not increased in either

Table 7.3 The compostion of the $CD3^+CD28^-$ population[a]

	Within $CD3^+$ T Cells					
	$CD8^+$	$CD45RO^+$	$HLA\text{-}DR^+$	$CD38^+$	$CD57^+$	$CD57^-CD28^-$
Control[b]	17.3±3.4	38.8±3.8	<1%	21.1±3.9	6.9±1.5	3.1±0.7
HIV-1^+	57.9±5.5	47.8±3.1	34.2±5.7	44.5±6.2	26.7±5.0	18.4±2.9
AVI	57.8±8.1	62.9±6.2	41.8±8.4	75.9±10.6	9.5±2.7	56.4±4.7
	Within $CD3^+CD28^-$ T Cells					
	$CD8^+$	$CD45RO^+$	$HLA\text{-}DR^+$	$CD38^+$	$CD57^+$	$CD57^-CD28^-$
Control	55.0±12.9	52.6±10.8	<1%	<1%	65.4±5.2	34.6±5.2
HIV-1^+	89.8±2.1	57.7±6.0	33.9±9.9	45.7±2.0	54.2±7.1	44.7±8.0
AVI	85.5±5.8	71,9±11.5	65.6±9.5	87.4±6.7	29.4±7.4	70.4±7.4

[a] The contribution of various lymphocyte subsets to the $CD3^+CD28^-$ population was investigated by triple colour immunofluorescence studies. During data analysis the $CD3^+$ or $CD3^+CD28^-$ populations were gated and the percentage of the test antibody determined within this gate.

[b] 10 normal, 10 HIV-1^+ and 9 AVI individuals were investigated

patient group. However, there was an increase in the percentage of $CD3^+$,$CD8^+$,$CD28^-$ T cells compared to the control group (control 25.6±6.6, HIV-1 64.5±4.9, AVI 60.7±5.4).

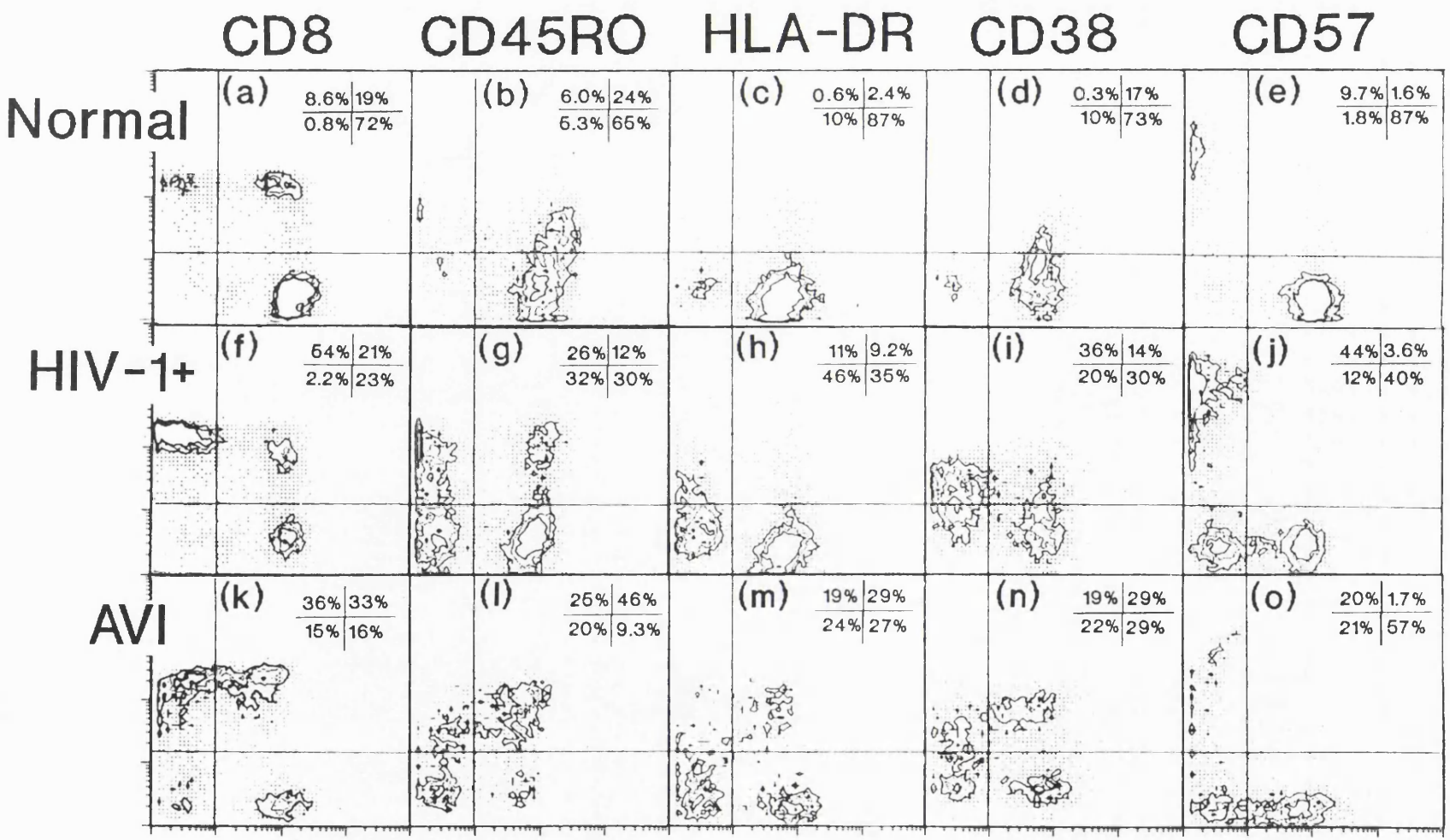
CD45RO: As demonstrated in chapter 4, in both HIV-1^+ and AVI patients there is a switch within the $CD8^+$ lymphocyte population towards a $CD45RO^+$, primed phenotype. In HIV-1^- individuals, among the few $CD3^+$,$CD28^-$ cells 52.6±10.8% were $CD45RO^+$ (Fig. 7.2b, Table 7.3). However, this represented less than 10% of the total number of $CD3^+$,$CD45RO^+$ cells. In HIV-1 infection, a larger proportion of $CD8^+$ lymphocytes expressed CD45RO, and the absolute numbers of $CD3^+$,$CD28^-$,$CD45RO^+$ cells were greatly increased (control 0.05±0.01, HIV-1^+ 0.30±0.02; $p<0.001$). Within the increased $CD3^+$,$CD28^-$ subset 57.7±6.0% were $CD45RO^+$; the $CD28^-$ component comprising up to 60% of the entire $CD3^+CD45RO^+$ population (Fig. 7.2g). The greatly expanded $CD45RO^+$ population in AVI individuals also contained a large (61.1±9.4%) $CD28^-$ component which made up the majority (71.9±11.5%) of the $CD3^+$,$CD28^-$ population. However in these acute patients there was also an increased number of $CD28^+$,$CD45RO^+$ lymphocytes (Fig. 7.2l).

HLA-DR: In both HIV-1^+ and AVI individuals, the number of $CD3^+$ lymphocytes expressing the activation marker HLA-DR are increased (Table 7.3). In the HIV-1^- control group $CD3^+$ lymphocytes expressing HLA-DR were seldom found (Fig. 7.2c). This activation marker was expressed on 34.2±5.7 and 41.8±8.4% of $CD3^+$ T cells in HIV-1^+ and AVI individuals respectively. While in HIV-1 infection the increase in HLA-DR expression occurred almost exclusively in the $CD28^-$ population (Fig. 7.2h), in AVI patients both $CD3^+$,$CD28^+$ and $CD3^+$,$CD28^-$ lymphocytes showed increased expression (Fig. 7.2m.)

CD38: The antigen CD38 is also increased in HIV-1 infection, although whether this is a function of activation or immaturity has been the subject of some debate (Giorgi & Detels 1989, Salazar-Gonzalez et al 1885, Janossy et al 1992). In seronegative donors, low levels of CD38 ($<5 \times 10^3$ molecules/cell) were detected on a proportion (21.1±3.9%) of $CD3^+$ T cells; these were predominantly $CD28^+$ (>80%; Fig. 7.2d). The increase in $CD3^+$,$CD38^+$ lymphocytes in HIV-1 infection was, however, due to an expansion of the $CD3^+$,$CD38^+$,$CD28^-$ population. These

Figure 7.2 FACScan profile of CD28 in $CD3^+$ lymphocytes from control and HIV-1^+ individuals

Triple colour combinations of CD3 and CD28 plus one of CD8, CD57, CD38, HLA-DR or CD45RO were used to illustrate the distribution of $CD28^-$ in $CD3^+$ T cells. During data acquisition a gate was set up around the $CD3^+$ cells. The figure shows CD28 (x-axis) and the third marker as illustrated (y-axis). Data from one representative control (a-e), one HIV-1^+ CDCII individual (f-j) and one acute HIV-1 infected patient (k-o) are shown. The numbers represent the percentage of cells in each of the quadrants.


CD8
CD45RO
HLA-DR
CD38
CD57
Normal
HIV-1+
AVI
(a) 8.6% 19% 0.8% 72%
(b) 6.0% 24% 5.3% 65%
(c) 0.6% 2.4% 10% 87%
(d) 0.3% 17% 10% 73%
(e) 9.7% 1.6% 1.8% 87%
(f) 54% 21% 2.2% 23%
(g) 26% 12% 32% 30%
(h) 11% 9.2% 46% 35%
(i) 36% 14% 20% 30%
(j) 44% 3.6% 12% 40%
(k) 36% 33% 15% 16%
(l) 25% 46% 20% 9.3%
(m) 19% 29% 24% 27%
(n) 19% 29% 22% 29%
(o) 20% 1.7% 21% 57%

$CD38^+$ cells constituted 45.7±2.0% of the $CD3^+$,$CD28^-$ lymphocytes. Cells bearing this marker were greatly increased in AVI patients (75.9±10.6% of $CD3^+$ T cells) indicating that in these individuals CD38 represents an activation marker. Again cells expressing CD38 made up a large proportion (87.4±6.7%) of the $CD3^+$,$CD28^-$ population but expression of this marker was also increased on $CD3^+$,$CD28^+$ cells (Fig. 7.2n).

CD57: A fourth marker of particular interest in HIV-1 infection is CD57. Lymphocytes expressing CD57 are increased in peripheral blood and alveolar lavages from HIV-1^+ individuals and have been shown to have suppressor cell activity in these patients (Sadat-Sowti et al 1991). When $CD57^+$ lymphocytes were investigated, CD57 and CD28 were found on separate lymphocyte populations in all individuals tested and few (<5%) double positive cells were seen (Fig. 7.2e,j,o). The percentage of $CD3^+$,$CD57^+$ cells was increased in HIV-1 infection (control 6.9±1.5; HIV-1 26.7±5.0; $p<0.001$) but was not significantly altered in AVI individuals (9.5±2.7%). Most of the $CD57^+$ cells were $CD3^+$,$CD28^-$ and represented a significant proportion of the $CD3^+$,$CD28^-$ population in control and HIV-1^+ individuals (control 63.6±4.9; HIV-1 54.2±7.1). Importantly, in HIV-1 infection there remained a particularly large proportion of $CD3^+$,$CD57^-$, $CD28^-$ cells (0.35±0.04, 0.42±0.087 and 0.37±0.53 $x10^9$/L in the CDCII, III and IV groups, respectively). These cells were rare in the control blood (<0.05 $x10^9$/L) but represented the majority of $CD3^+$,$CD28^-$ cells in AVI patients (70.4±7.4%).

Summary: Although both acute and chronic viral infections are characterised by an increase in the proportion of T cells expressing the activation markers CD45RO, HLA-DR and CD38 these subsets differ in each patient group with respect to CD28 expression. The more chronic condition (HIV-1) leads primarily to a loss of CD28 expression and the other markers form overlapping populations within this group of cells. In acute infections where cells have experienced short-term stimulation *in vivo*, although many $CD28^-$ cells are seen there are also large numbers of activated cells that retain a $CD28^+$ phenotype. This data suggests that the loss of CD28 does not occur after short-term activation but may require long-term or chronic stimulation. Similarly, the expansion of $CD57^+$ T lymphocytes may be an important indicator of chronic stimulation.

CD28 expression in lymphoid tissue

Cell suspensionsof HIV-1$^+$ lymph node and tonsil from seronegative controls wereinvestigated for CD28 expression by FACS analysis. The huge expansion of CD3$^+$,CD8$^+$, CD28$^-$ T cells seen in the peripheral blood of HIV-1 infected individuals (HIV-1$^-$ 25.6±6.1%, HIV-1$^+$ 64.5±4.9%) was not found to such a great extent in lymphoid tissue. A comparison of blood versus tonsil from seronegatives showed that CD28 expression was higher in the CD8$^+$ in the tissue (HIV-1$^-$ blood 75.4%, tonsil 87.0%). This was even more marked in HIV-1 infection (HIV-1$^+$ blood 35.5%, lymph node 68%). One reason for this may be the absence of CD57$^+$,CD8$^+$ T cells in lymphoid tissue from all individuals investigated (<5%). This suggests that CD28$^-$ T cells do not home to lymphoid tissue and are primarily a blood associated population. Interestingly, the CD4$^+$ T cells including the CD57$^+$ subset found within the germinal centres, retain a CD28$^+$ phenotype.

The proliferative defects of CD28$^-$ lymphocytes in HIV-1 infection

In the next part of the study the defective activation response of lymphocytes from HIV-1$^+$ individuals were related to the presence of CD3$^+$,CD28$^-$ cells. Lymphocytes from HIV-1$^+$ individuals and normal controls were stimulated with PHA plus rIL-2 and the numbers of lymphoblasts recovered on day 3 related to the initial input of CD28$^+$ cells. In 56 HIV-1$^+$ individuals investigated there was a highly significant positive correlation between the number of CD28$^+$ cells present at the start of the culture and the number of lymphoblasts recovered after 3 days activation ($p<0.001$; Fig. 7.3). This correlation was further tested on CD28$^-$ cells following the removal of CD28$^+$ T lymphocytes. Less than 10% of this population from HIV-1$^+$ individuals were able to transform into lymphoblasts after activation with PHA despite the addition of rIL-2 (Fig. 7.4). Conversely, the removal of CD57$^+$ (CD28$^-$) lymphocytes proportionally increased the number of lymphoblasts recovered (Fig. 7.4), indicating that in this assay the residual CD28$^+$ cells from HIV-1$^+$ donors are not handicapped and can respond to mitogens.

To further demonstrate that CD28$^-$ T cells, but not CD28$^+$, have a defective proliferative response, we investigated the phenotypic features of cells surviving

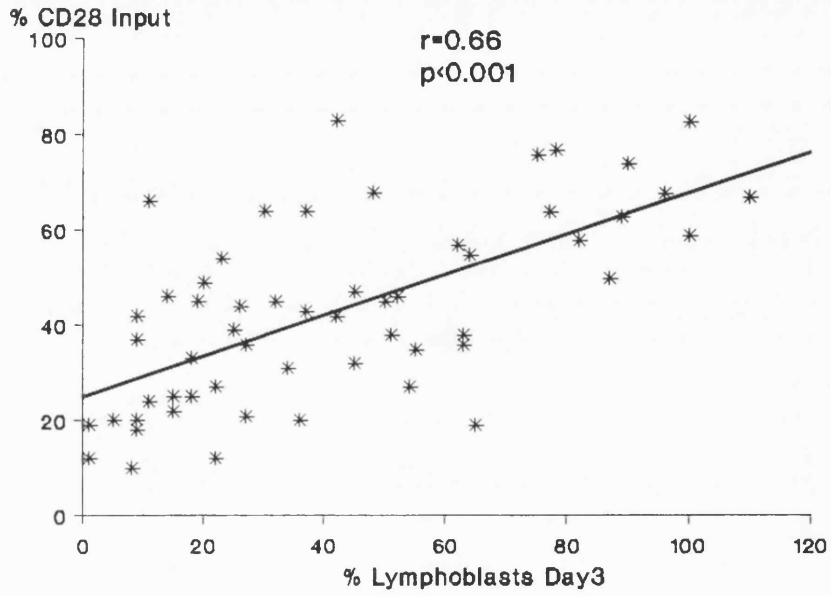


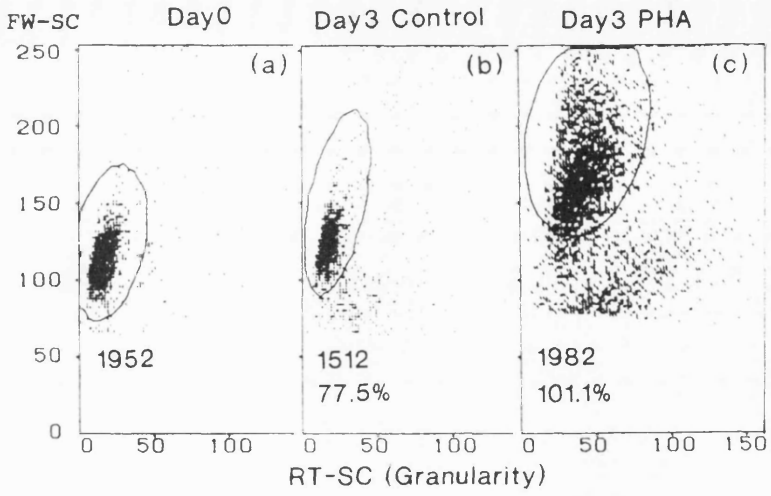
Figure 7.3 The correlation between CD28 expression and lymphocyte activation responses

PBMC from 56 HIV-1$^+$ individuals were assayed for their expression of CD28 before activation with PHA. The percentage of lymphoblasts developing in the cultures compared to the total cell input (x-axis), is plotted against the percentage of CD28$^+$ cells present in the culture prior to activation (y-axis). Regression analysis confirmed the highly significant relationship between these two variables.

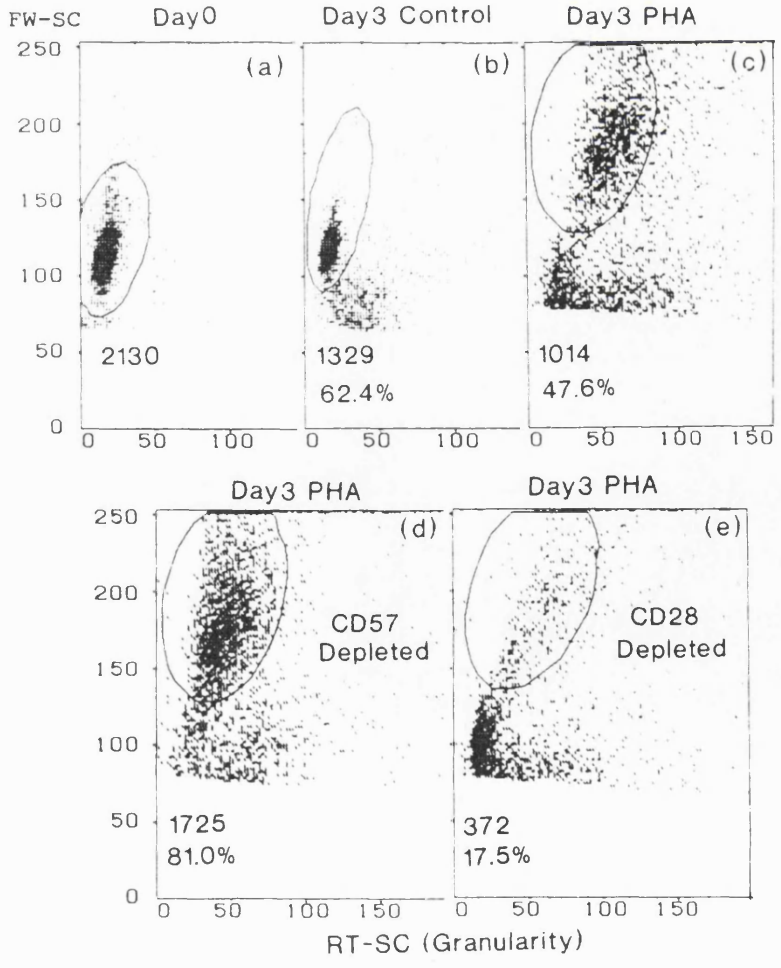
Figure 7.4 Cytoron*Absolute* profiles of PBMC before and after culture *in vitro*

Profiles of PBMC from an HIV-1^{-} (Part I)and HIV-1^{+}, CDCII individual (Part II). The figure shows cells on isolation (a) and after culture for three days with or without PHA (b & c). Part II shows the effect of removal of either $CD57^{+}$ or $CD28^{+}$ cells on the blastogenic response. The numbers shown are the lymphocyte counts within the gated areas. The percentages are the proportion of gated lymphocytes in relation to the input at the start of the culture.

Part I



Part II



after 3 days activation (Fig. 7.5). The majority (95±2.0%) of lymphoblasts recovered from the HIV-1$^-$ control cultures were CD28$^+$. Among the fewer remaining blasts detected in the cultures from HIV-1$^+$ donors, the CD28 positivity was high (89±3.70%) and the absolute blast count at day 3 corresponded to the original CD28$^+$ input at day 0. Thus the CD28$^+$ cells selectively transformed while the CD28$^-$ cells died. In addition, very few CD57$^+$ blasts were seen in both the control and HIV-1$^+$ samples (control 2.6±0.36; HIV-1$^+$ 5.5±0.63) and the rare surviving CD57$^+$ blasts co-expressed low levels of both CD57 and CD28, indicating that the expression of CD28 might have contributed to their survival. Some other CD57$^-$,CD28$^-$ lymphoblasts constituted $<10\%$ of the total blast population but these were B cells.

The expression of CD25 on CD28$^-$ lymphocytes after stimulation

When investigated for CD25 (IL-2Rα chain) expression after 2 days in culture, the majority of lymphocytes from normal or HIV-1$^+$ donors expressed the IL-2R (Fig. 7.6a). This CD25$^+$ population included both CD57$^+$ and CD28$^-$,CD57$^-$ lymphocytes. However, in contrast to CD28$^+$ cells, the CD28$^-$ population were small lymphoid cells which failed to transform into lymphoblasts as measured by their cell size. By day 3 of culture, these CD25$^+$, CD28$^-$,CD57$^+$ cells had perished.

The inability of CD57$^+$ and CD28$^-$ T cells to transform into lymphoblasts was confirmed in studies using BrdU uptake to measure cells in G2/M phase of the cell cycle. Three colour studies with CD28 and CD57 in combination with anti-BrdU were performed on cytospins using confocal microscopy. These showed that after 48 hours stimulation with PHA, CD57$^+$ cells were present as small, BrdU$^-$ lymphoid cells and that only CD28$^+$ cells had taken up BrdU and were in G2/M of cycle (Fig. 7.6b).

Bcl-2 expression on CD28$^-$ lymphocytes

When spontaneous cell death in AVI and HIV-1$^+$ patients was investigated, no statistically significant correlation could be found between this phenomenon and the lack of the CD28 antigen. It was therefore perhaps not surprising that in triple

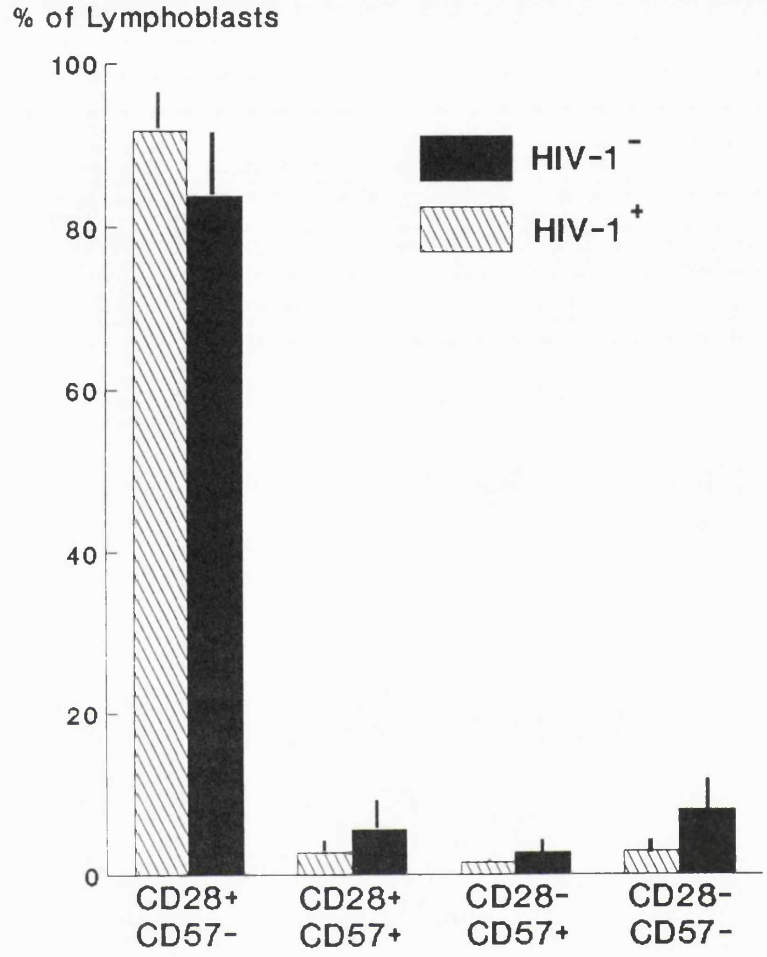


Figure 7.5 Phenotypic study of the lymphoblasts developing in PHA stimulated cultures after 3 days

The phenotype of the lymphoblasts recovered after activation with PHA was determined with respect to CD57 and CD28. During data acquisition a gate was set up around the large lymphoblasts based on their forward and side scatter profiles. The results are shown as the percentage of total lymphoblasts from 5 HIV-1^{-}, normal controls and 10 HIV-1^{+} individuals.

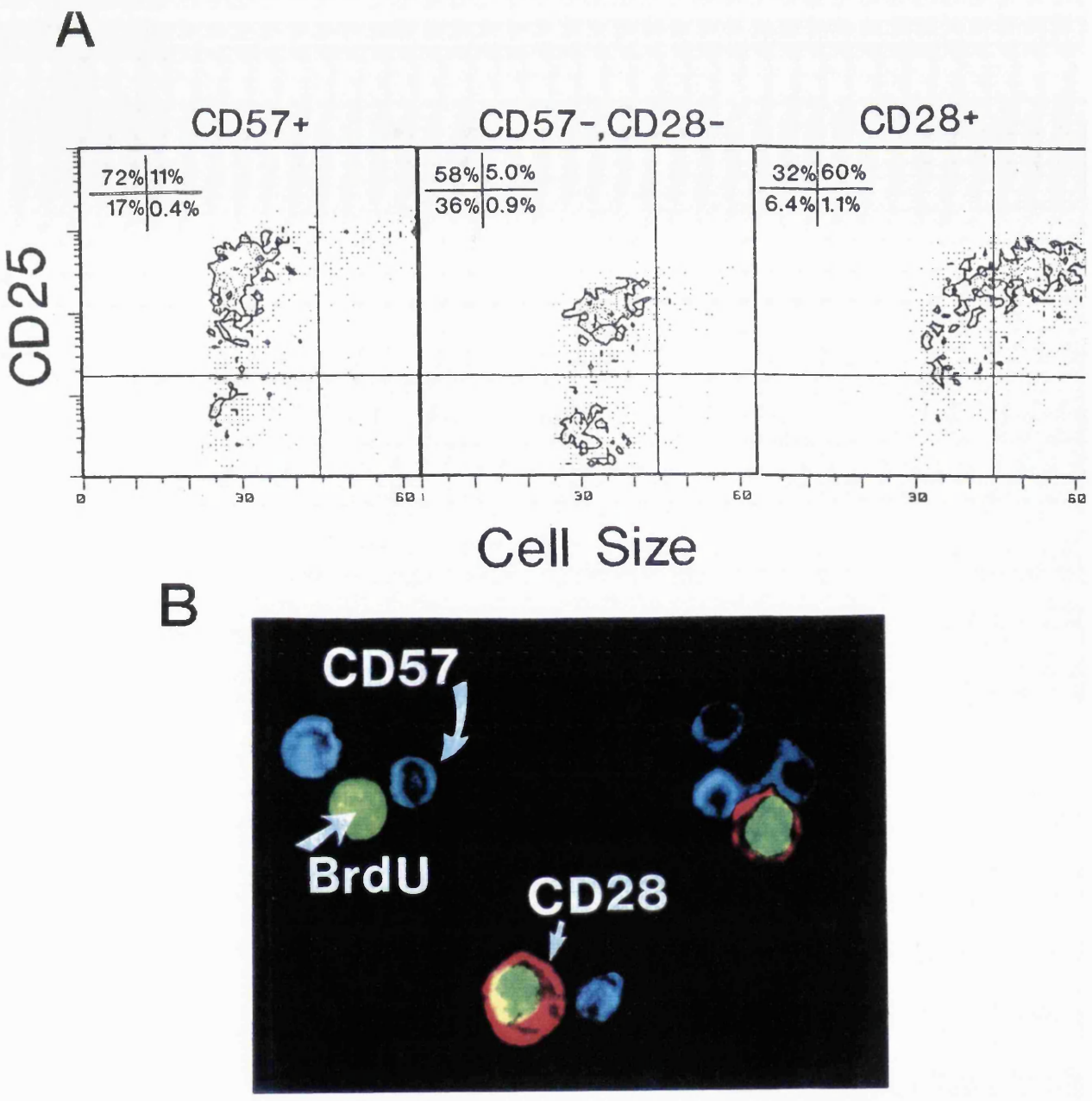


Figure 7.6 The defective activation response of CD28$^-$ lymphocytes

The early upregulation of CD25 was investigated on CD57$^+$, CD57$^-$,CD28$^-$ and CD28$^+$ lymphocytes after 2 days in culture with PHA (a). During data acquisition the cells were gated according to their CD57 and CD28 phenotypes and the cell size (x-axis) in relation to CD25 expression (y-axis) plotted. The result from the representative patients shown indicate that although CD28$^-$ lymphocytes can upregulate CD25 they fail to transform into lymphoblasts. This was confirmed in cells incubated with BrdU (b). Lymphocytes stimulated with PHA for 2 days were pulsed with BrdU and the phenotype of the BrdU$^+$ blast cells investigated by three colour immunofluorescence using confocal microscopy. The BrdU$^+$ nuclei are shown in green, the CD28$^+$ membrane stain in red and the CD57$^+$ membrane stain in blue. The BrdU and CD28 combine to form the yellow staining pattern. Note that the CD57$^+$ cells are small and BrdU negative (blue).

marking studies using the combination CD3-biotin, CD28-PE and cytoplasmic Bcl-2-FITC, both $CD28^+$ and $CD28^-$ T cells were found to contain a $Bcl\text{-}2^-$ component (Fig. 7.7a). This was investigated more closely in purified $CD28^-$ T cells prepared by negative depletion procedures from E^+ T cells (chapter 2). Cytospin preparations were double stained with CD57-TRITC and Bcl-2-FITC and the intensity of Bcl-2 expression was determined using a semi-quantitative technique on the confocal microscope. Using this procedure the $CD28^-$ population were found to contain cells with a range of Bcl-2 intensities (Fig. 7.7b). Those which lacked expression of Bcl-2 were mainly $CD57^-$,$CD28^-$ while the majority (>85%) of $CD57^+$ lymphocytes retained Bcl-2 expression (Fig. 7.7c). This occurred in both HIV-1^- and HIV-1^+ individuals.

The effect of co-stimulation via CD28 on activation responses

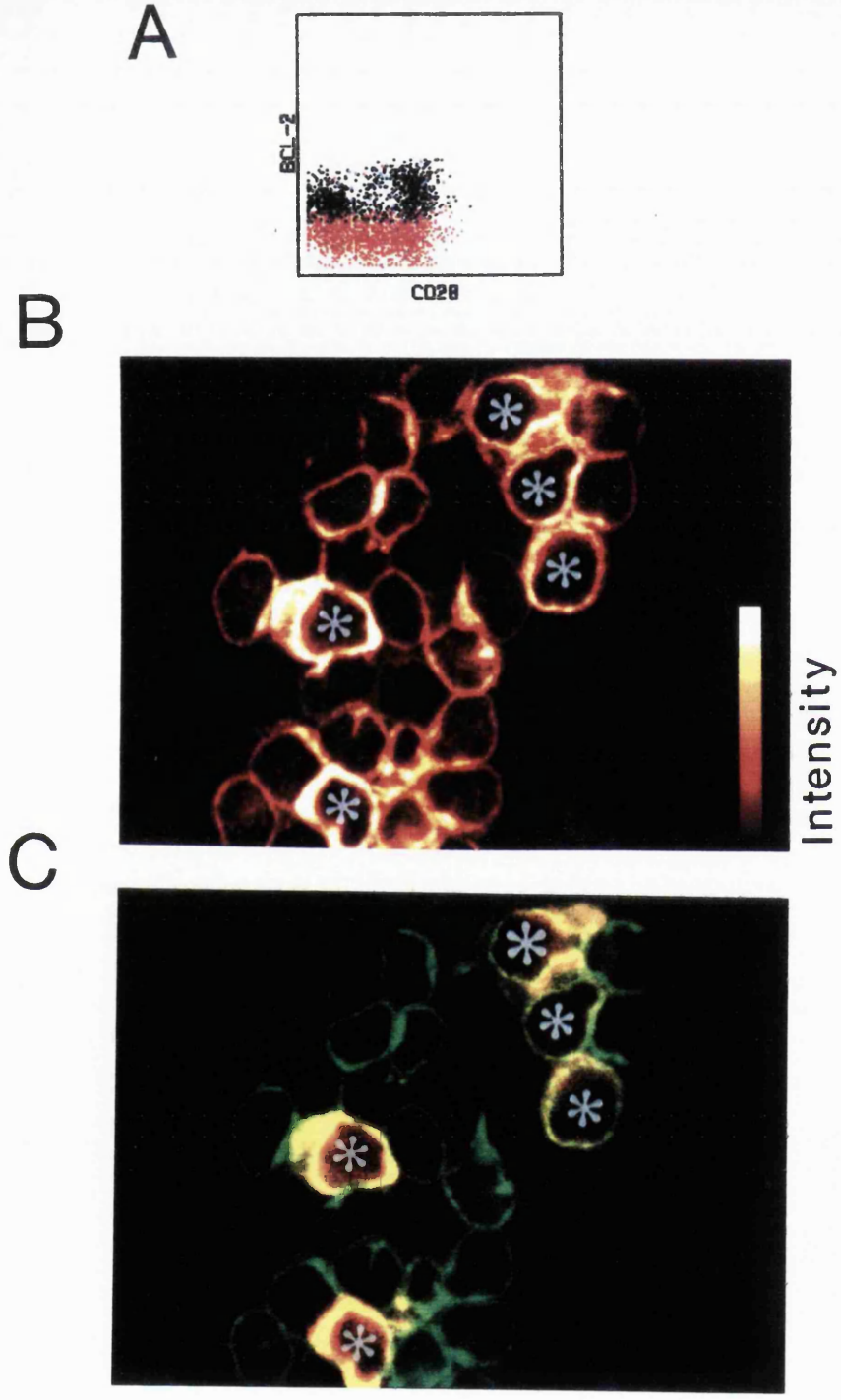
It has been reported that co-stimulation via CD28 restores the defective proliferative response of lymphocytes from HIV-1 infected individuals (Gruters et al 1990, Van Noesel et al 1990) and prevents activation induced apoptosis (Groux et al 1992). To evaluate this in relation to CD28 expression, cells were stimulated with combinations of anti-CD2 antibodies. These were used in suboptimal concentrations as this mitogenic activity is significantly boosted by adding anti-CD28. PHA stimulated cells were the positive controls. Lymphocyte activation was measured by both a bulk assay of ^{3}H-thymidine uptake (cpm; Fig. 7.8a,b) and by determining lymphoblast counts at day 3 in relation to the original T cell input (Fig. 7.8c,d). In all cases the magnitude of response closely correlated with the expression of CD28 (cpm: $r=0.797$, $p=0.006$ and % lymphoblasts: $r=0.834$ $p=0.003$).

As also shown in Fig. 7.8, individuals responded to each of the mitogens tested. In addition, when anti-CD28 was added as a co-stimulus to CD2 there was a significant increase in thymidine uptake. In both the cultures of lymphocytes from normal donors and HIV-1^+ individuals the enhancement was of a similar degree (220-240%), indicating that this pathway of lymphocyte activation is still intact in $CD28^+$ cells. On the other hand, the addition of anti-CD28 to cultures of lymphocytes from HIV-1^+ individuals did not reconstitute the number of lymphoblasts observed at day 3 to the much higher levels seen in cultures from

Figure 7.7 Bcl-2 is expressed within the $CD28^-$ T lymphocyte population

Part A: PBMC were triple stained with Bcl-2-FITC, CD28-PE and CD3-biotin plus streptavidin tricolour. Lymphocytes were gated on CD3 positivity and the expression of Bcl-2 and CD28 determined within this population. Note that $Bcl\text{-}2^+$ cells are distributed equally within the $CD28^+$ and $CD28^-$ T lymphocytes. A representative example from one $HIV\text{-}1^+$, CDCII individual is shown. Part B: E^+ T lymphocytes were depleted of $CD28^+$ lymphocytes and cytospin preparations stained for Bcl-2 and CD57. The figure shows a confocal image of the intensity of Bcl-2 within the $CD28^-$ T lymphocytes. Note that not all $CD28^-$ cells are $Bcl\text{-}2^-$. Part C: The same confocal image showing CD57 positive cells (*). Note that these same $CD57^+$ cells are $Bcl\text{-}2^+$ (* part A).

Fig 4.7



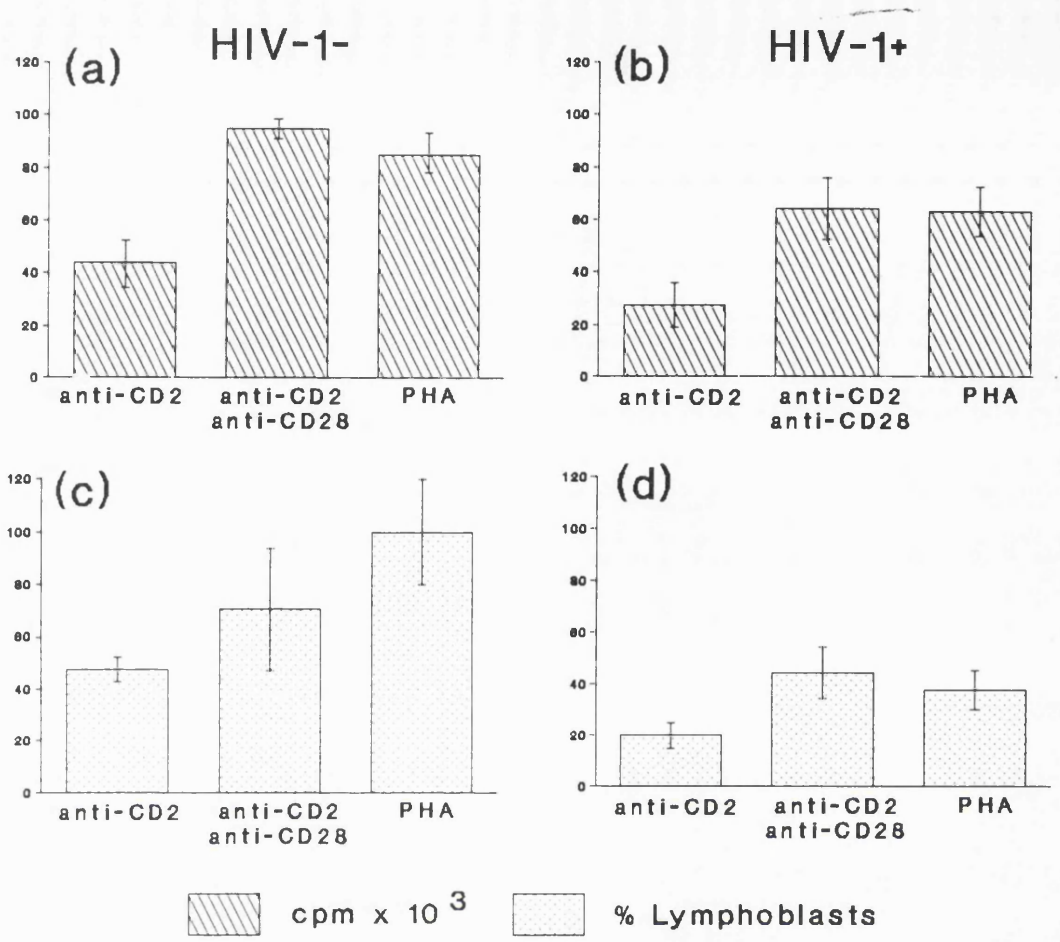


Figure 7.8 The effect of co-stimulation via anti-CD28 on the activation responses of lymphocytes from HIV-1$^-$ and HIV-1$^+$ individuals

PBMC from 5 HIV-1$^-$ controls (a,c) and 9 HIV-1$^+$ individuals (b,d) were activated with three different stimuli; combinations of anti-CD2, anti-CD2 plus anti-CD28 or PHA. The activation response was measured by ^{3}HTdR incorporation (a,b) and lymphoblast recovery (c,d).

HIV—1$^-$ healthy donors. Neither the thymidine uptake nor the recoverable blast cell counts were fully normalized (Fig. 7.8b,d). These results show that, as expected, the defective activation of CD28$^-$ T cells in HIV-1$^+$ individuals cannot be corrected by co-stimulation via anti-CD28.

Discussion

We have demonstrated that in asymptomatic individuals with HIV-1 infection CD3$^+$ T cells accumulate which fail to express CD28. Such cells form only a minor population in HIV-1$^-$ individuals where virtually all CD28$^-$ cells are members of the NK cell family and do not express CD3. This enriched CD3$^+$,CD28$^-$ population in HIV-1 infection is essentially composed of CD8$^+$ T cells and is responsible for the CD8 lymphocytosis seen throughout the whole course of disease. The data on CD28$^-$,CD8$^+$ cells extends the findings of Gruters et al. 1991 who in a study of five HIV-1$^+$ individuals demonstrated a shift within the CD8$^+$ population to a CD28$^-$ phenotype without documenting these cells' CD3 positivity. Although CD4$^+$,CD28$^-$ cells have been described previously (Dianzani et al 1988) our study shows that such cells are rare. Similarly, during acute viral infections there is an accumulation of CD28$^-$ T cells, again primarily within the CD8$^+$ subset.

In HIV-1 infection there is an increase in the number of cells expressing the activation associated markers CD45RO, HLA-DR and CD38 (Froebel et al 1991, Prince & Jensen 1991b, Giorgi & Detels 1989, Salazar-Gonzalez et al 1985, Stites et al 1986). In this study lymphocytes with these phenotypes were found to form overlapping populations within the CD3$^+$CD28$^-$ cells but were not increased in the CD3$^+$,CD28$^+$ population. However, in AVI patients although an increase in CD28$^-$ cells was noted, many of the activated (CD45RO$^+$, HLA-DR$^+$ or CD38$^+$) lymphocytes retained CD28 expression. This suggests that the loss of CD28 might only occur *in vivo* after long-term stimulation and is therefore a marker of chronic infection. Similarly, an increase in CD57$^+$ lymphocytes has been reported in many chronic conditions including the joints of patients with rheumatoid arthritis (RA; Burns, Tsai & Zvaifler 1992, Dupuy d'Angeac et al 1993 and bone marrow transplant recipients (Leroy et al 1986, Valardi et al 1988) and are also increased in HIV-1 infection (Lewis et al 1985, Giorgi et al 1987, Joly et al 1989). These cells

also lack expression of CD28 and are a major component of the $CD3^+$,$CD28^-$ population in HIV-1 infection. Patients with AVI did not show any increase in $CD57^+$ cells, further implicating CD57 as a marker of chronic immune stimulation. The absence of large numbers of $CD28^-$ or $CD57^+$ cells in either normal tonsil or HIV-1^+ lymph node suggests that this blood borne population does not normally home to lymphoid tissue. In contrast, broncho-alveolar lavage (BAL) from both seronegative and HIV-1^+ individuals contain a large proportion of $CD8^+$, $CD28^-$ and $CD8^+$,$CD57^+$ lymphocyte subsets (Saukkonen Kornfeld & Berman 1993) indicating that $CD8^+$,$CD57^+$,$CD28^-$ T cells are the normal $CD8^+$ T cell subset in the lung and that these cells also predominate in HIV-1 infection.

The CD28 antigen is the most important co-stimulatory molecule so far described on normal T cells as it transmits a second signal obligatory for T-cell activation (Jenkins et al 1991). We now confirm that the expression of CD28 is linked to the ability of T lymphocytes to undergo blastogenesis in response to PHA, anti-CD3 and -CD2 *in vitro* and also extend the relevance of these findings to HIV-1 disease. The $CD3^+$,$CD28^-$ population in the blood of individuals with HIV-1 infection are deficient in responding to the strongest mitogens including PHA, anti-CD3, combinations of anti-CD2 and anti-CD28 stimuli even in the presence of rIL-2. Both the $CD3^+$,$CD28^+$ and $CD3^+$,$CD28^-$ population transiently express CD25 but then perish in culture despite the addition of rIL-2. It has previously been reported that $CD57^+$, presumably $CD28^-$, lymphocytes from normal individuals do not respond to mitogenic stimuli as measured by BrdU uptake. A proportion of these cells remained lymphocytic by appearance after short term activation with PHA (Abo et al 1983, Campana, Coustan-Smith & Janossy 1988, Ruthlein James & Strober 1988) but could not be maintained *in vitro* for more than 48 hours (Prince & Jensen 1991), suggesting an inherent defect in these cells.

It has been demonstrated that activation of T-cells via ligation of TCR in the absence of a co-stimulatory signal induces a state of anergy (Meuller, Jenkins & Schwarz 1989). A powerful co-signal required for T cell activation is provided by the interaction between CD28 and its ligand B7/BB-1 (Linsley et al 1991) expressed on antigen presenting cells such as activated B cells (Yokochi, Holly & Clark 1981) and dendritic cells (Young et al 1992). While anergy is generally taken to be a

transient state of unresponsiveness it has recently been suggested that there are various levels of tolerance including anergy, and that anergic cells remain susceptible to further tolerogenic signals that eventually lead to deletion (Arnold, Schonrich & Hammerling 1993). For example, Liu & Janeway (1990) have shown in mice that activation of TH_1 clones in the absence of accessory cells causes cell death by an as yet undefined mechanism. The lack of expression of CD28 on T cells from HIV-1^+ individuals may therefore explain the activation induced cell death observed through the absence of a second signal. I t is perhaps important to note that a second ligand for B7 /BB1, CTLA4, has been described (Linsley et al 1992). This molecule is not present on resting cells but increases after activation and acts synergistically with anti-CD28 (Linsley et al 1992). The expression of this molecule, as well as other known to have co-stimulatory activity such as CD5, CD6, CD40 ligand and LFA-1 have not been fully investigated in HIV-1 infection.

Co-stimulation with anti-CD28 has been reported to return the proliferative responses to normal levels when measured by ^{3}H-TdR (Gruters et al 1990). These results have been interpreted to show that anti-CD28 is able to save some cells from cell death and/or apoptosis. Nevertheless, anti-CD28 accelerates the activation cycle of $CD28^+$ cells while it is unlikely to act upon the $CD28^-$ populations. Indeed, in our study we have confirmed that co-stimulation via anti-CD28 increased ^{3}H-TdR incorporation but this enhanced uptake was not due to the activation of $CD28^-$ cells which remained unresponsive. The quantitative results from the Cytoron*absolute* showed that the numbers of transforming lymphoblasts on day 3 were low despite adding anti-CD28, thus among mixed $CD28^+$ and $CD28^-$ T cell populations the co-stimulation via CD28 does not induce $CD28^-$ cells to regain their proliferative potential.

The functional commitments of $CD8^+$,$CD28^-$ lymphocytes are still ambiguous. Studies using purified $CD8^+$,$CD28^+$ and $CD8^+$,$CD28^-$ lymphocytes showed that only the $CD28^+$ fractions generated cytotoxic cells during culture with allogeneic cells (Damle et al 1983), while $CD8^+$,$CD28^-$ lymphocytes suppressed the MLR response. The $CD8^+$,$CD28^-$ population have also been shown to have suppressor activity in other systems (Lum et al 1982). In HIV-1^+ individuals $CD8^+$,$CD57^+$($CD28^-$) cells were found to secrete suppressor factors (Sadat-Sowti

et al 1991) although anti-viral activity resides within the $CD8^+$,$CD28^+$ subset (Landay, Mackewicz & Levy 1993). Other studies reveal that $CD3^+$,$CD8^+$,$CD28^-$ cells exhibit cytotoxic activity but only when freshly isolated from blood as these cells do not respond well to activation *in vitro* (Azuma, Phillips & Lanier 1993). The $CD57^+$ cells in the peripheral blood of individuals with HIV-1 infection also include some cells with CTL activity (Vanham et al 1990). These sets of data are not contradictory because $CD28^-$ T cells are likely to be a population of terminally differentiated effector cells which are cytotoxic only in short term cultures while secreting suppressor moieties.

The process which leads to the development of $CD28^-$,$CD8^+$ T cells during HIV-1^+ infection remains obscure. Studies on the cell line JA3 have shown that stimulation with anti-CD28 induces rapid modulation of CD28 (Moretta et al 1985). Similarly, stimulation of PHA activated lymphocytes with anti-CD28 results in a temporary decrease in both CD28 mRNA and surface expression of the protein (Linsley et al 1993). The interaction of CD28 with its ligand might therefore result in the loss of the antigen from the cell surface. To determine if CD28 had been modulated from the cell surface *in vivo* but was still detectable inside the cells, lymphocytes from HIV-1^+ individuals were investigated for the presence of cytoplasmic CD28; this was however undetectable. Furthermore, stimulation of PBL via anti-CD3 in short term cultures does not co-modulate CD28, indicating that these receptors are not physically linked (Moretta et al 1985). In fact, activation for 7 days in culture has been shown to increase CD28 expression (Lesslauer et al 1986). However, long-term cultures of T-cells in medium supplemented with rIL-2 results in a progressive loss of CD28 (Testi & Lanier 1989). It is therefore likely that the CD28 reduction may require some weeks of continuous activation to occur, in agreement with the phenotypic characteristics suggesting the differentiated features of $CD8^+$,$CD28^-$ cells.

As anticipated from the data in the previous chapter in which no statistically significant correlation was drawn between AALD and Bcl-2 expression, both $CD28^+$ and $CD28^-$ T cell subsets contained a Bcl-2^- component. Thus, $CD28^-$ T cells are not particularly susceptible to spontaneous cell death. Interestingly, $CD57^+$,$CD28^-$ T cells were Bcl-2^+, suggesting that this population are not short-

lived *in vivo* but will die if restimulated.

The appearance of $CD28^-$ T lymphocytes in the peripheral blood, as shown in this study, provides one mechanism for a severe functional immunodeficiency in asymptomatic HIV-1 infected individuals.

CHAPTER 8

THE CYTOLYTIC FUNCTION OF CD8$^+$ LYMPHOCYTES FROM HIV-1$^+$ INDIVIDUALS

Introduction

In HIV-1 infected individuals CD8$^+$ T cells characteristically exhibit a variety of phenotypic changes including, as shown in chapter 4, an increase in primed CD45RO$^+$,CD8$^+$ cells and also an increase in CD38 and HLA-DR expression (Salazar-Gonzalez et al 1985, Stites et al 1986, Giorgi & Detels 1989). In the previous chapter it was also shown that large numbers of CD8$^+$,CD28$^-$ and CD8$^+$,CD57$^+$ T cells appear. Cells of this phenotype are found both in the CD3$^+$ T cell and CD3$^-$ NK cell populations and it is the latter that is expanded in HIV-1 infection. The acquisition of CD57 on CD8$^+$ lymphocytes, and consequently the loss of CD28, are clinically associated with immunosuppression and occur after chronic stimulation *in vivo*. For example, CD8$^+$,CD57$^+$ cells appear in long-term bone marrow transplant survivors where they have been shown to have suppressor function (Leroy et al 1986) and in cardiac transplant recipients with CMV infections (Maher et al 1985). However, a number of studies have indicated that CD8$^+$,CD57$^+$ and CD8$^+$,CD28$^-$ T cells have a cytolytic function *in vitro* (Phillips & Lanier 1986, Azuma Phillips & Lanier 1993) and in fact represent *in vivo* primed CTL. As CD8$^+$ lymphocytes expressing CD45RO and HLA-DR have also been shown to be highly efficient CTL (Yamashita & Clement 1989, Akbar et al 1990, Vanham et al 1990) the expanded CD8$^+$ cells in HIV-1$^+$ patients might represent a cytotoxic effector population. The presence of large numbers of CTL in the peripheral blood of HIV-1$^+$ patients could play an important role in the pathogenesis of AIDS through the lysis of autologous lymphocytes by CTL (Lanzavecchia et al 1988, Siliciano et al 1988, Weinhold et al 1989, Zarling et al 1990, Grant Smail & Rosenthal 1994). Therefore, the aims of this chapter were to investigate the cytolytic activity of CD8$^+$ lymphocytes from HIV-1$^+$ patients and to determine the phenotype of the CD8$^+$ CTL. In addition, cytotoxicity was measured after stimulation *in vitro* to determine the role of AALD on the effector function of this subset.

It has been postulated that the breakdown in the follicular structure that occurs in lymph nodes from HIV-1$^+$ individuals might be due to the action of HIV-

1 specific CTL directed against FDC which are either infected with the virus or have immune complexes on their surface that trap virus (Racz et al 1985, Tenner-Racz et al 1986, Laman et al 1989). Indeed, studies on lymph nodes have demonstrated the presence of low levels of HLA class I restricted HIV-1 specific CTL activity both in freshly isolated cells and after restimulation of lymphocytes *in vitro* with autologous PHA blasts (Hoffenbach et al 1989, Hadida et al 1992). In addition, it was shown in chapter 6 and by other investigators (Racz et al 1986, Racz et al 1990) that large numbers of $CD8^+CD45RO^+$ cells infiltrate the germinal centres during HIV-1 infection. The next aim of this chapter was to investigate more closely CTL activity in HIV-1^+ lymph nodes and, using three colour immunofluorescence studies on the confocal microscope to determine if the $CD8^+$ cells infiltrating the germinal centres contained the cytotoxic granule proteins, TIA-1 and perforin, which are indicative of CTL function (Young et al 1989, Tian et al 1991).

Methods

Cytotoxicity assays

(i) Lectin dependant cytotoxicity (LDC) assay

The murine mastocytoma cell line P815 was used as the target cell in the LDC assay. The cell line was maintained in supplemented RPMI-1640 and was in the log phase of growth when used as a target.

Procedure: The P815 cells were harvested by centrifugation and the supernatant discarded. The pellet was resuspended and 3.5MBq of ^{51}Chromium, 50-500 Ci/g Cr, added in the form of Sodium chromate (ICN Biomedicals Ltd, High Wycombe, Bucks.) The target cells were incubated for 1 hour at 37°C to allow the Chromium to diffuse into the cells and then washed twice with PBS. The cells were finally adjusted to 5 x 10^4/ml in RPMI supplemented medium and 100μl aliquots dispensed into 96 well, round bottom microtitre plates (Gibco Brl).

Effector cells in RPMI-1640 supplemented medium were adjusted to give effector:target (E:T) ratios ranging from 50:1 to 1.5:1 and dispensed in triplicate into the wells together with the targets. Purified PHA (PHA-P, Welcome Diagnostics Ltd, Dartford) was then added to each well to give a final concentration of 1 ug/ml and the plates incubated at 37°C for 4 hours. The optimum concentration

of PHA-P and the time course of the assay were determined at the beginning of the study.

After the incubation period, 100ul aliquots of supernatant were removed from each well and transferred into LP2 tubes (Luckhams Ltd, Burgess Hill, Sussex). As the effector cells were obtained from HIV-1^+ patients, 20μl of 40% Formaldehyde was added to each tube which was then sealed with paraffin wax before removal for counting. The samples were counted on a Minaxi gamma counter (LKB Ltd, Croydon, Surrey) for 3 minutes and the percent cytotoxicity calculated using the formula:

$$\% \text{ Cytotoxicity} = \frac{\text{cpm experimental} - \text{cpm spontaneous}}{\text{cpm total} - \text{cpm experimental}}$$

where spontaneous release was that from target cells incubated in medium alone and total release was that from target cells incubated in medium containing 2.5% Triton X 100 (Sigma Ltd, Poole, Dorset). Any experiment where spontaneous release was >30% of the total release was discarded.

(ii) CD3 mediated redirected killing assay

The P815 cell line expresses on its surface a receptor for the Fc portion of mouse IgG and was therefore used as a target cell in the redirected killing assay .

Procedure: The same procedure was followed as for the LDC assay except that OKT3 (CD3, IgG2a) at a final concentration of 0.5μg/ml was added to each well instead of PHA.

(iii) HIV specific cytotoxicity assay

To measure HIV-1 specific cytotoxic cells, autologous B-cell lines were used as target cells.

Preparation of Epstein Barr Virus (EBV)

The cell line B95/8 is chronically infected with EBV and can be induced to release the virus if subjected to stress in culture. B95/8 was thawed and maintained in log phase in RPMI 1640 supplemented medium. To induce virus release the cells were adjusted to 2 x 10^5/ml in medium containing 2% FCS and cultured at 33°C for 2 weeks without changing the medium or splitting the culture. The cells were then

spun off and the supernatant passed through a 0.45 μm filter (Sartorius Ltd, Epsom, Surrey). The supernatant containing EBV was aliquoted and stored in liquid nitrogen.

B-cell transformation

PBMC were isolated from 20ml of blood as previously described. After the final wash, the cells were pelleted and 1ml of B95/8 supernatant containing EBV was added. The cells were incubated for 1 hour at 37°C, adjusted to 2 x 10^6/ml in RPMI-1640 supplemented medium and 1 ml aliquots dispensed into 24 well, flat bottom plates (Becton Dickinson, Cowley, Oxford). The plates were incubated at 37°C in 5% CO_2 and monitored for the appearance of clones. The EBV transformed cells became apparent after 14-21 days in culture but if no transformed cells were seen after 28 days the cultures were discarded. The B-cell lines were transferred to tissue culture flasks (Becton Dickinson, Cowley, Oxford.) and maintained in culture until stable. The B-cell lines were stored frozen in liquid nitrogen and thawed up 1 week prior to the cytotoxicity assay.

This transformation method resulted in a 70% success rate. Methods using PHA at the initiation of culture were less successful particularly when dealing with HIV-1^+ patients and were therefore abandoned. Certain patients were difficult to transform particularly HIV-1^+, CDCIV individuals. After repeated attempts, it was found that the addition of 0.1ug/ml cyclosporin A (Sandoz, Basel, Switzerland) into the cultures for the first 21 days could improve the transformation results from CDCIV patients.

HLA tissue typing

Polyclonal antibodies raised against HLA antigens were incubated with human PBMC and complement used to lyse any cell with the antibody bound to its surface. Cell lysis was visualised by light microscopy under phase contrast where dead cells gave a positive result compared to control wells containing human AB serum.

PBMC were prepared from 20ml of peripheral blood and adjusted to 1 x 10^6/ml in serum free RPMI-1640. Anti-HLA antibodies were dispensed into the wells of terasaki plates and the plates immersed in liquid paraffin (BDH Ltd, Dagenham,

Essex). One microlitre of cell suspension was added to each well and the plate tapped gently to aid mixing. The plates were incubated for 30 minutes at room temperature. Baby rabbit serum (Serological Reagents Ltd) was reconstituted with distilled water just prior to use and 5ul added to each well. After incubation at room temperature for 1 hour the cells were stained with 2μl of 5% Eosin in PBS and were finally fixed by the addition of 5μl 40% formaldehyde.

Infection of target cells with Vaccinia virus

EBV transformed B-cell lines were infected with Vaccinia virus constructs, obtained through the MRC AIDS Directed Program. These induced the expression of HIV*gag* determinants in the B-cell.

Procedure: Autologous and HLA A, B & C mismatched B-cell lines were grown up for each patient assayed. One million cells were pelleted and 1 plaque forming unit/cell of Vaccinia gag (VAC*gag*) or wild type Vaccinia (VAC*wr*) added. The cells plus virus were incubated for 1 hour at 37°C and resuspended in 5 ml of RPMI 1640 supplemented medium. The cells were left overnight at room temperature and used the next day as target cells in the cytotoxicity assay.

Assay Procedure: PBMC or lymph node suspension were isolated as described (Chapter 2), adjusted to 1 x 10^6/ml in RPMI 1640 supplemented medium and incubated at 37°C. One tenth of the cells were activated with 1μg/ml PHA-P overnight and, after washing x 3 with HBSS, returned to the other cells. The effector cells were left in culture for 7 days, harvested and, cell numbers allowing, used in cytotoxicity assays against the following targets:

(1) Autologous VAC*gag* infected B-cell lines
(2) Autologous VAC*wr* infected B-cell lines
(3) Allogeneic VAC*gag* infected B-cell lines
(4) Allogeneic uninfected B-cell line
(5) P815 plus PHA
(6) K562 (NK sensitive target)
(7) Kloek (LAK sensitive target)

PBMC and lymph node suspensions were also assayed directly against the above targets without preincubation with autologous PHA blasts.

Results

A comparison of CTL activity before and after re-stimulation In Vitro

(i) Cytotoxicity in Unstimulated PBMC

The total cytotoxic activity of freshly isolated PBMC was measured using the LDC assay in 27 normal HIV-1$^-$ donors, 48 HIV-1$^+$ donors (CDCII 17, CDCIII 16, CDCIV 15) and 11 AVI patients (Fig. 8.1). In HIV-1$^-$ individuals low levels of killing (8.5±1.6%; E:T 12:1) could be detected. This was mainly attributed to CD16$^+$ NK cells as removal of this population by complement lysis (Chapter 2) significantly reduced the levels of killing (1.7%; $p<0.007$; Table 8.1). In HIV-1$^+$ individuals there was a significant increase in the cytolytic activity of freshly isolated PBMC compared to the HIV-1$^-$ donors (CDCII 19.6±4.1%, CDCIII 16.9±2.9%, CDCIV 26.1±4.3%; E:T 12:1; $p<0.001$). Unlike HIV-1 specific activity which is reported to decline with disease progression (Weinhold et al 1988, Plata et al 1989, Joly et al 1989, Hoffenbach et al 1989), the ability of lymphocytes to kill P815 target cells in the LDC assay remained high throughout the course of the disease and in fact increased as patients progressed to CDCIV, AIDS. This increase in CTL activity in symptomatic AIDS patients was significant at E:T 6:1 and 12:1 compared to the CDCII and CDCIII individuals ($p<0.05$). Interestingly, in these CDCIV patients NK cells were found to contribute far less to the overall cytolytic activity (CDCII 63.4%, CDCIV 21%; Table 8.1). However, by far the greatest cytotoxicity was found in AVI patients who showed levels of killing up to 500% higher than the HIV-1$^-$ control group and 200% higher than the chronic HIV-1$^+$ donors (34.9±2.3%; E:T 12:1). Removal of NK cells from these patients reduced cytotoxicity by 60%, but residual cells still retained high levels of killing (13.2±0.2%; E:T 12:1; Table 8.1).

(ii) Cytotoxicity After Restimulation

Lymphocytes can acquire cytolytic function after mitogenic stimulation *in vitro* (Leeuwenberg et al 1985, Jung et al 1986). To test this ability in HIV-1$^+$ patients whole PBMC and CD16 depleted populations were stimulated *in vitro* with anti-CD3 and the cytotoxicity measured using the LDC assay. An initial time course study on PBMC from HIV-1$^-$ individuals showed that levels of killing increased

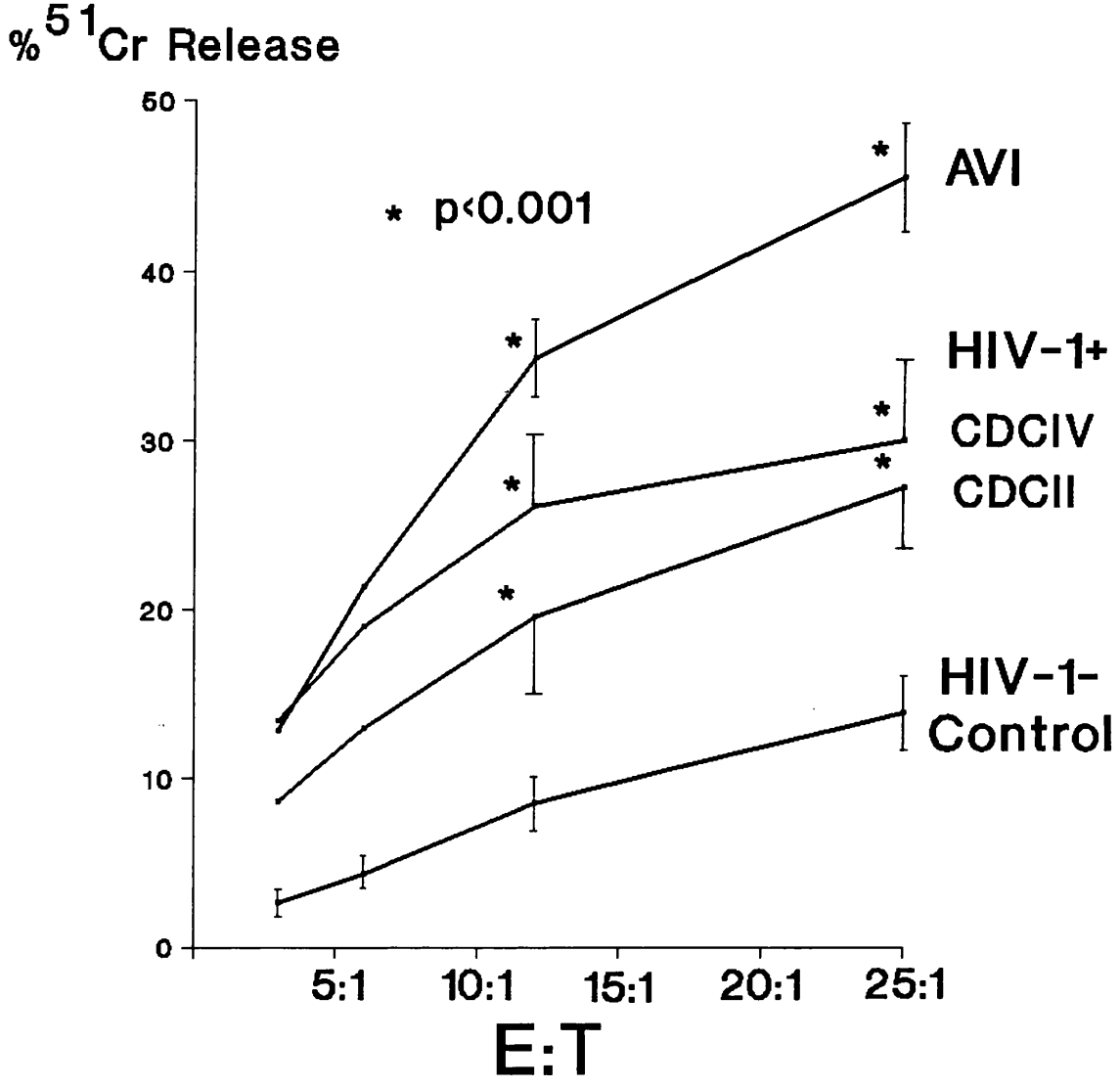


Figure 8.1 The cytolytic activity of PMBC measured using the LDC assay

The cytolytic activity of PBMC from 27 HIV-1^{-}, 17 HIV-1^{+} CDCII, 15 HIV-1^{+} CDCIV and 11 AVI individuals is shown. In this assay the target cell, P815 was incubated with the effectors at the ratios indicated in the presence of 1μg/ml PHA. Arithmetic means were compared to the HIV-1^{-} control using the students t-test; * $p < 0.001$

Table 8.1 The Effect of CD16+ Depletion on Cytolytic Activity[a]
Part I

	n	Without Activation PBMC	CD16 Depleted
HIV-1$^-$	10	8.3±1.6[b]	1.7±0.6**[c]
HIV-1$^+$ CDCII	6	20.2±5.5	7.4±2.3*
CDCIV	5	36.5±3.8	28.8±6.9
AVI	6	37.5±4.8	13.2±0.2

Part II

	n	After PHA Stimulation PBMC	CD16 Depleted
HIV-1$^-$	10	62.6±4.7	45.6±2.8*
HIV-1$^+$	9	39.7±4.5	21.8±5.5*

a The cytolytic activity of PBMC before and after removal of CD16$^+$ NK cells was compared in freshly isolated PBMC (Part I) and after 3 days activation with PHA plus rIL-2 (Part II).

b The percentage cytotoxicity at E:T 12:1 in the LDC assay is shown

c The data were compared using the paired Students t-test,
* $p<0.05$, ** $p<0.005$

rapidly after only 24 hours in culture (52%; E:T 12:1) reaching a peak of around 65% between days 2 and 3. Thereafter cytolytic activity slowly declined unless cultures were supplemented with IL-2 or restimulated. A comparison of HIV-1$^-$ and HIV-1$^+$ individuals revealed that the initial early increase in cytolytic activity during the first three days was absent in HIV-1$^+$ patients. This is illustrated in Fig. 8.2 which shows a representative example of one HIV-1$^-$ and one HIV-1$^+$ donor. Although the starting level of killing is higher in this HIV-1$^+$ individual compared to the HIV-1$^-$ control (Control 10.2%, HIV-1$^+$ 27.7%; E:T 12:1), the activation associated increase in cytotoxicity is delayed. In fact, activation results in an initial drop in activity over the first three days, and reaches those seen in the seronegative only by day 5.

Activation with anti-CD3 also induces CTL activity in CD3$^-$ T cells (Jung, Martin & Muller-Eberhard 1987) probably through the action of IL-2 on NK cells. As this could obscure any change in T cell mediated killing, the initial failure to increase cytotoxic killing was further investigated in a larger group of patients after removal of NK cells. When CD16$^+$ cells were removed prior to stimulation, the cytotoxicity that subsequently developed in both HIV-1$^-$ and HIV-1$^+$ cultures was reduced (Table 8.1) but was substantially higher than CTL activity in unstimulated cultures. A comparison of cytotoxicity in 10 HIV-1$^-$ and 10 HIV-1$^+$ individuals prior to culture and after 3 days activation with PHA plus IL-2 is shown in Fig. 8.3. From this it is clear that although initial levels of killing are higher in the HIV-1$^+$ group (HIV-1$^-$ 8.0$\pm$1.4%; HIV-1$^+$ 28.2$\pm$6.1; E:T 12:1; $p<0.001$), activation over this short time period does not significantly increase the amount of cytotoxicity in the HIV-1$^+$ group (34.7$\pm$5.1%). Lymphocytes from HIV-1$^-$ individuals on the other hand acquire high levels of killing after this short term stimulation (68.4$\pm$5.2%; $p<0.001$).

These results suggest that lymphocytes with CTL function are pre-activated in the blood of HIV-1$^+$ patients but cannot be efficiently activated by TcR ligation unlike the resting CD8$^+$ T cells present in HIV-1$^-$ individuals. The failure of lymphocytes from HIV-1$^+$ individuals to acquire cytolytic activity after activation *in vitro* was due to the rapid death of the responding cells. Activated cell cultures from HIV-1$^+$ donors adjusted for viable cells showed high levels of killing which were

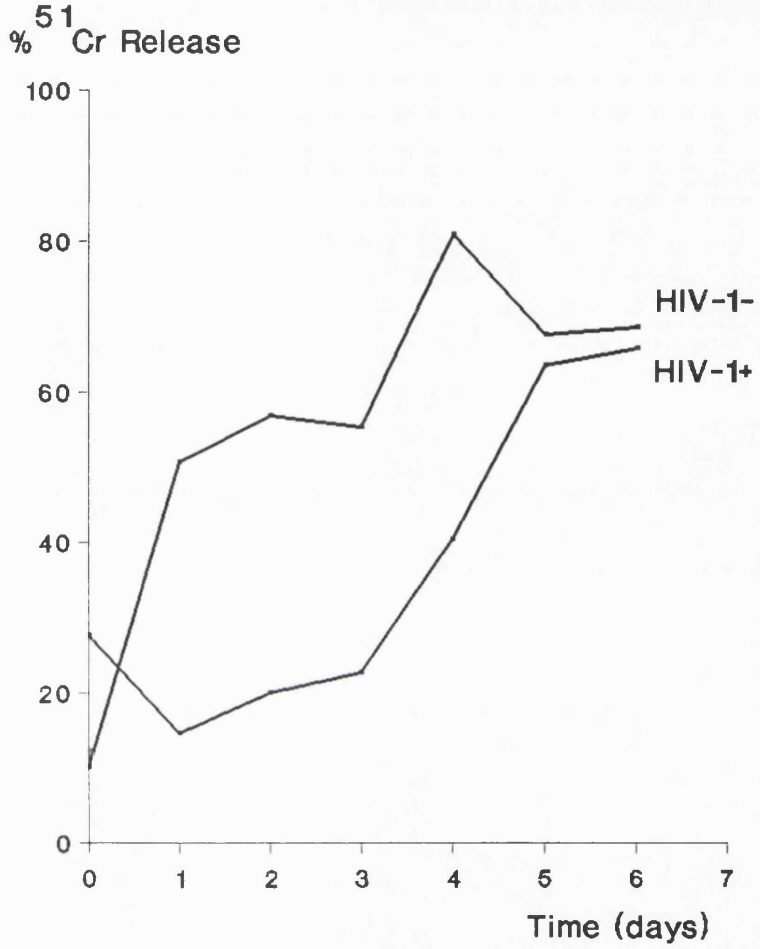


Figure 8.2 Time course of cytolytic activity after stimulation *in vitro*

PBMC from one HIV-1$^-$ and one HIV-1$^+$ individual were cultured in the presence of anti-CD3 plus rIL-2 and the cytolytic activity measured daily using the LDC assay against P815 target cells. The percentage cytotoxicity at E:T 12:1 is shown.

The cytolytic activity of each well was measured at an equivalent of E:T 12:1 without re-adjusting for cell viability after activation

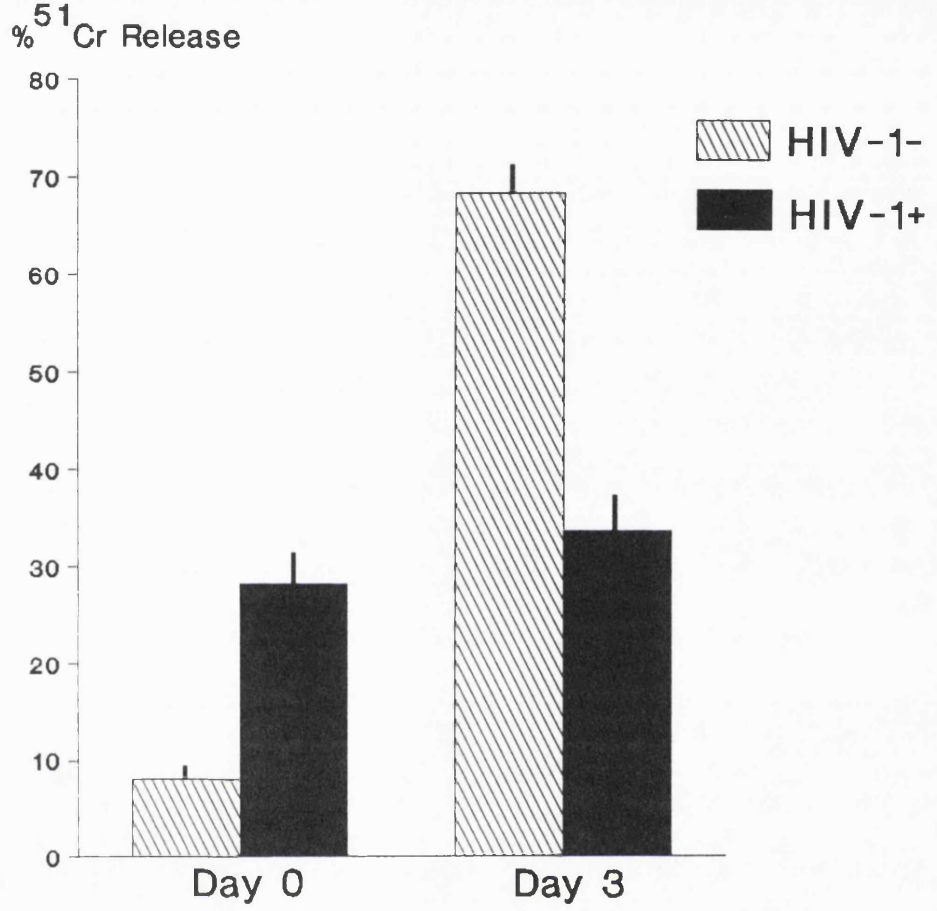


Figure 8.3 A comparison of cytotoxicity before and after activation in vitro

PBMC were depleted of $CD16^+$, NK cells and assayed before and after stimulation with anti-CD3 plus rIL-2 using the LDC assay against P815 target cells. The arithmetic mean ± SEM from 10 $HIV\text{-}1^-$ and 10 $HIV\text{-}1^+$ individuals at E:T 12:1 is shown.

The cytolytic activity of each well was measured at an equivalent of E:T 12:1 without re-adjusting for cell viability after activation

comparable to those seen in the seronegative group (HIV-1$^-$ 58.3±2.3%, HIV-1$^+$ 56.0±1.1%; E:T 12:1).

In conclusion, HIV-1$^+$ individuals have high levels of circulating CTL even in the later stages of HIV-1 disease. These cells are, however, unresponsive and after short-term stimulation *in vitro* this activity is lost due to the death of the responding cells.

HIV-1 specific CTL activity

(i) Freshly Isolated PBMC

To determine if the CTL activity measured in the LDC assay was directed against the HIV-1 virus, 10 HIV-1$^+$ patients were assayed for specific CTL activity in freshly isolated PBMC. Cytolytic killing (>10% at E:T 25:1) was detected in 4 out of 6 asymptomatic patients but was absent in all CDCIV patients tested (n=4). However, CTL activity against P815 target cells in the LDC assay was present in all individuals and was highest in the CDCIV patients. This is illustrated in Fig. 8.4 which shows a representative HIV-1$^+$ patient (A) who has CTL activity against P815 (17%) and HIV-1*gag* (27%) but does not kill the NK target, K562, or the uninfected control cell lines. In contrast, the CDCIV patient shown (B) has activity only against P815 in the lectin dependent system (37%) but has <5% HIV-1 specific killing. No HIV-1 specific CTL were detected when PBMC from HIV-1$^-$ donors were investigated (n=2).

(ii) Lymph Node Suspensions

Using the LDC assay the cytotoxic activity of fresh lymph node suspensions was measured in 6 HIV-1$^+$ individuals. When whole lymph node suspensions were used as effectors, CTL activity was low in all individuals (<5% at E:T 50:1). However, purified CD8$^+$ lymphocytes prepared by negative selection of E$^+$ cells from two individuals did show detectable CTL activity (34.2% & 10.4% at E:T 50:1). An HIV-1*gag* specific CTL assay was then performed using lymph node suspension from one of these individuals both unstimulated and after co-culture with autologous PHA blasts for 5 days (Fig. 8.5). The unstimulated cells showed no HIV-1*gag* specific killing and were cytolytic only against the P815 target cells. However, after co-culture, low levels of HIV-1*gag* specific CTL were detected (13% at E:T

Figure 8.4 HIV-1 specific cytotoxicity in freshly isolated PBMC

PBMC from one HIV-1^+, CDCII (A) and CDCIV (B) individual were assayed for HIV-1*gag* specific cytolytic activity using autologous lymphoblastoid cell lines infected with *vaccinia virus* vectors as described. As a control, cytotoxicity was also determined against both P815 cells using the LDC assay and against the NK sensitive target, K562.

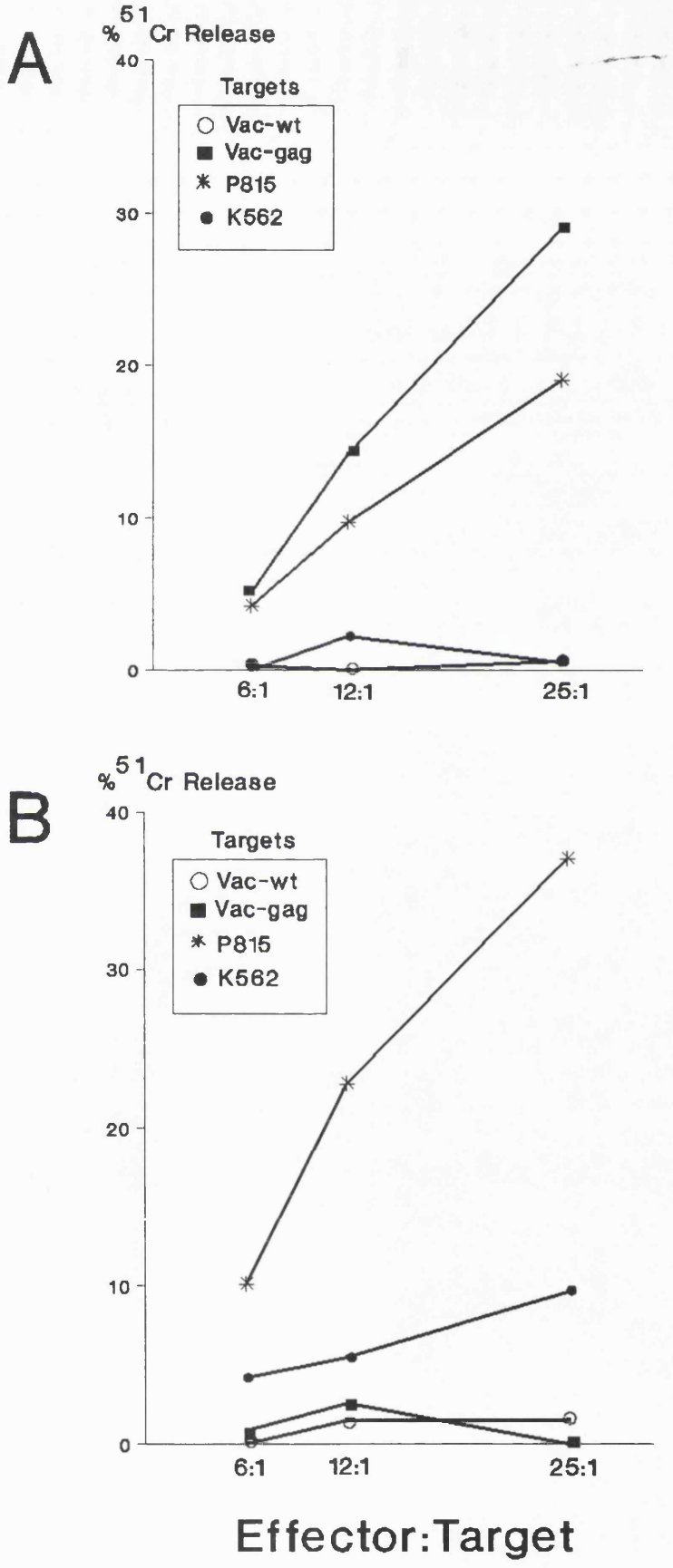
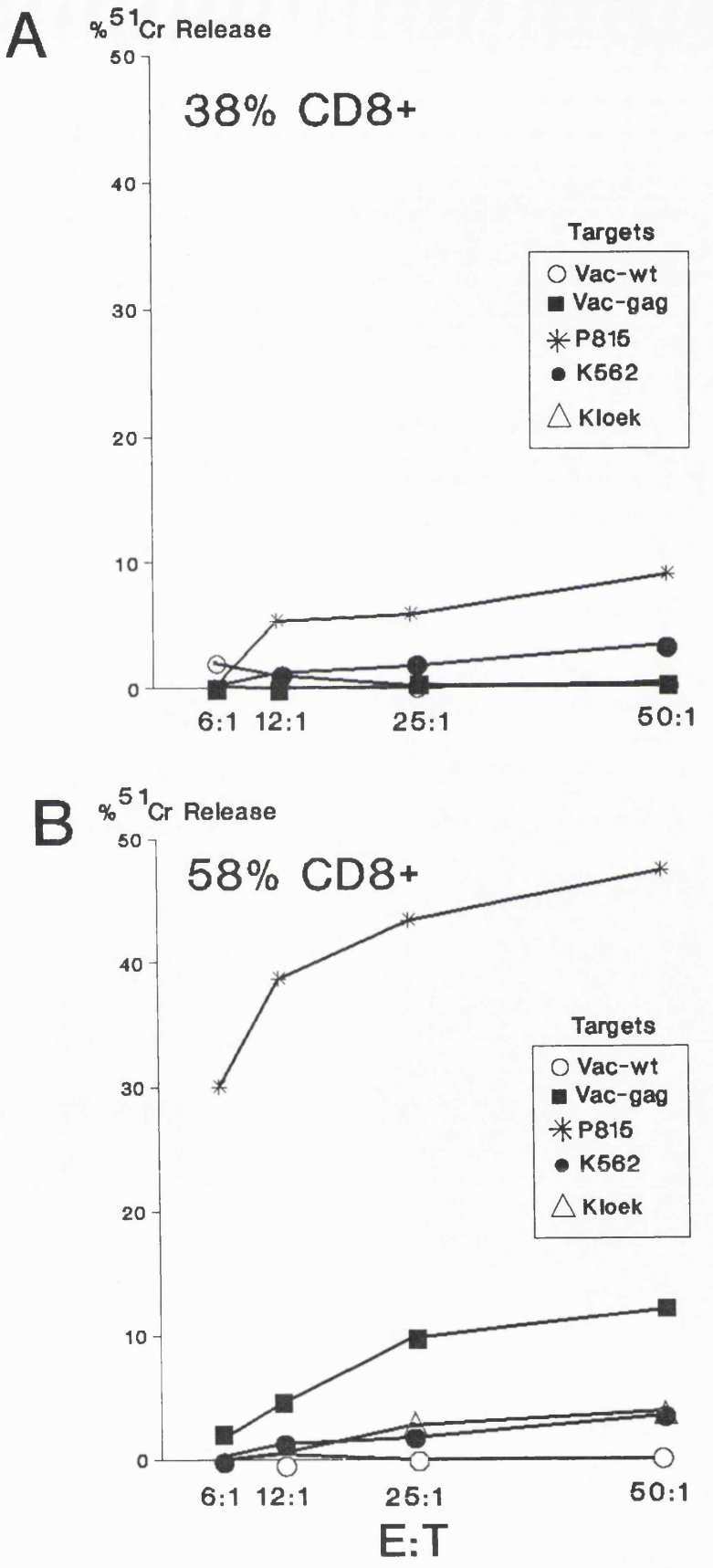

A
% 51 Cr Release
40
30
20
10
0
Targets
Vac-wt
Vac-gag
P815
K562
6:1
12:1
25:1
B
% 51 Cr Release
40
30
20
10
0
Targets
Vac-wt
Vac-gag
P815
K562
6:1
12:1
25:1
Effector:Target

Figure 8.5 HIV-1 specific cytotoxicity in lymph node suspensions

The HIV-1 specific cytolytic activity of lymph node suspensions from one HIV-1^+, CDCIII individual was measured before (A) and after (B) co-culture with autologous PHA blasts. Cytotoxicity against P815 using the LDC assay and the NK and LAK sensitive targets, K562 and Kloek respectively were also measured.


A
% 51Cr Release
38% CD8+
Targets
Vac-wt
Vac-gag
P815
K562
Kloek
50
40
30
20
10
0
6:1
12:1
25:1
50:1
B
% 51Cr Release
58% CD8+
Targets
Vac-wt
Vac-gag
P815
K562
Kloek
50
40
30
20
10
0
6:1
12:1
25:1
50:1
E:T

50:1). Co-culture also greatly enhanced the levels of killing seen using P815 (46% at E:T 50:1) but did not result in high background levels of cytotoxicity against the control target cells, including Kloek, the LAK sensitive target. Therefore, low levels of killing are detected in lymph nodes and this can be increased by stimulation *in vitro*.

The phenotype of CTL in HIV-1 infection

Studies were then performed to identify the T cell subset mediating cytotoxicity in HIV-1 infection. Initial depletion experiments in which the effect of the removal of lymphocyte subsets on cytotoxicity were compared, revealed that $CD8^+$ and $CD45RO^+$ lymphocytes contributed most to the killing detected in the LDC assay (80% and 68% respectively). As it had previously been shown in the MLR system that $CD8^+CD45RO^+$ T cells were the most efficient CTL, this subset was investigated in HIV-1 infected donors (Fig. 8.6). In the absence of NK cells which express CD45RA (Nagler et al 1989), there was a strong positive relationship between the presence of $CD8^+CD45RO^+$ lymphocytes and levels of killing using the LDC assay ($r=0.619$; $p<0.005$; Fig. 8.6) providing indirect evidence that in this system also the $CD8^+CD45RO^+$ subset contains a cytolytic effector population.

The cytolytic activity of purified $CD8^+$ lymphocytes

To further delineate the phenotype of CTL in HIV-1 infection, purified $CD8^+$ lymphocytes were prepared and their cytolytic activity compared with the expression of a variety of markers known to be increased in HIV-1 infection. Highly purified $CD8^+$ populations (>97%) were obtained by positive selection in a single step procedure (Chapter 2) which was used to reduce lymphocyte death or degranulation which might occur over prolonged preparation times. To exclude NK activity a redirected killing assay was used in which anti-CD3 rather than PHA acted as a bridge between target and effector. Again the target used was the cell line P815 which expresses mouse Fc receptor (Dunn & Potter 1975).

Using this assay purified $CD8^+$ lymphocytes from $HIV\text{-}1^+$ individuals showed high levels of killing when compared to the seronegative group ($HIV\text{-}1^-$ $3.2\pm2.2\%$, $HIV\text{-}1^+$ $23.0\pm5.9\%$; E:T 12:1; $p<0.001$). The phenotype of the

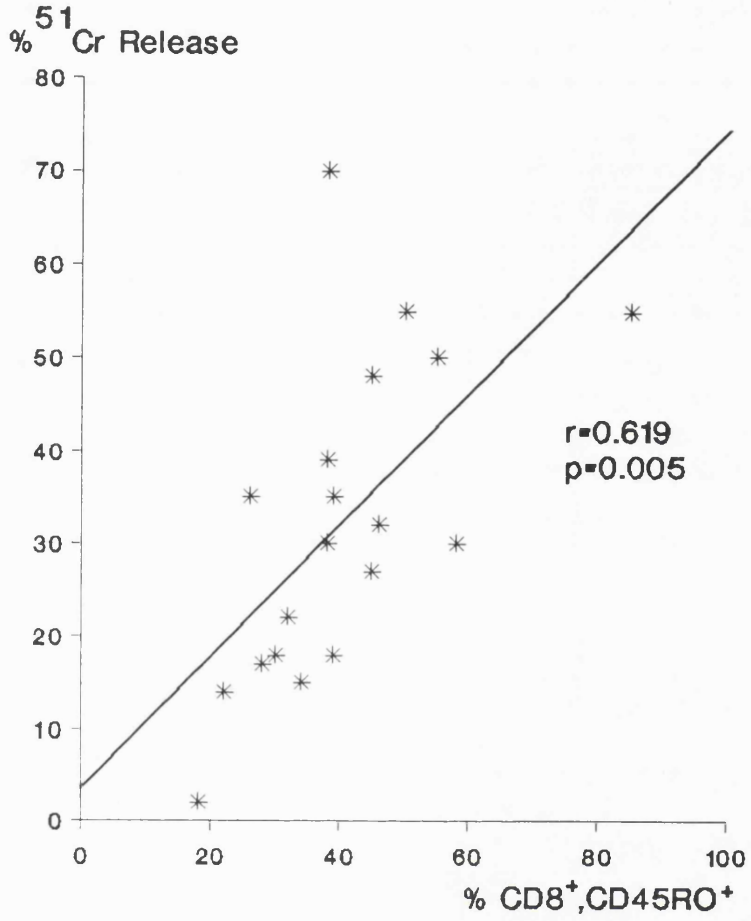


Figure 8.6 The correlation between levels of cytotoxic killing and the expression of CD45RO on $CD8^+$ lymphocytes

PBMC from a range of HIV-1^+ patients were depleted of $CD16^+$, NK cells by complement lysis and the cytolytic activity of the remaining population measured using the LDC assay. These same cells were also investigated for the expression of CD45RO on $CD8^+$ lymphocytes by two colour immunofluorescence. Regression analyses show a significant relationship between these two variables.

$CD8^+$ lymphocyte population was determined with respect to the markers CD45RO, HLA-DR, CD28, Bcl-2 and TIA-1, a cytotoxic granule associated protein. As expected, the expression of CD45RO and HLA-DR on $CD8^+$ cells was increased in HIV-1 infection compared to the control group (CD45RO HIV-1^- 17.9±0.2%, HIV-1^+ 60.8±3.7%; HLA-DR HIV-1^- <5%, HIV-1^+ 48.2±4.9%; $p<0.001$). Similarly, expression of CD57 and TIA-1 were significantly increased (CD57 HIV-1^- 9.3±3.4%, HIV-1^+ 30.0±7.0%; TIA-1 HIV-1^- 25.6±3.8%, HIV-1^+ 65.2±7.9%; $p<0.05$ and $p<0.001$ respectively) while CD28 and Bcl-2 were reduced (CD28 HIV-1^- 84.4±7.2%, HIV-1^+ 38.5±7.9%; Bcl-2 HIV-1^- 85.0±2.1%, HIV-1^+ 62.1±3.9%). Regression analyses of the HIV-1^+ group ($n=18$) revealed a significant correlation between levels of cytotoxicity and the expression singly of CD45RO and TIA-1 ($p=0.005$ and $p=0.02$ respectively; Table 8.2). However, when both markers were combined an even stronger association was noted ($r=0.875$, $p=0.005$; Table 8.2). Although other markers, notably CD57 and HLA-DR have been implicated in CD8 mediated CTL activity, using this analysis method no correlation was found between the proportion of $CD8^+$ cells expressing these markers and the level of cytolyic activity (Table 8.2).

This data suggests that the $CD8^+$,$CD45RO^+$,TIA-1^+ population in the peripheral blood of HIV-1^+ individuals includes lymphocytes with cytolytic activity.

The cytolytic activity of purified $CD8^+CD45RO^+$ lymphocytes

To confirm that the $CD8^+CD45RO^+$ T cell subsets mediated the majority of the cytolytic activity purified $CD8^+CD45RO^+$ and reciprocal $CD8^+CD45RA^+$ lymphocyte subsets were prepared and their cytolytic activity compared using the LDC assay. The levels of killing obtained from purified $CD8^+CD45RO^+$ lymphocytes were consistently higher than either unseparated T cells or the $CD8^+CD45RA^+$ subset. This is illustrated in a representative HIV-1^+ donor in Fig. 8.7 where the cytolytic activity of the T cell population is enriched by the removal of $CD45RA^+$ cells. The $CD8^+CD45RO^+$ lymphocytes mediate approximately twice as much killing as the $CD45RA^+$ subset ($CD45RO^+$ 30.6%, $CD45RA^+$ 17.9%; E:T 12:1) suggesting that the $CD8^+CD45RO^+$ subset are the most potent cytolytic effector cells. However, in this redirected killing assay the $CD8^+CD45RA^+$ subset

Table 8.2 Regression Analyses of Cytotoxicity and Phenotype in $CD8^+$ T Cells[a]

Marker	Correlation Coefficient (r)	Significance Level (p)
$CD45RO^+$	0.663	0.005
$HLA\text{-}DR^+$	0.380	NS
$CD57^+$	0.581	NS
$CD28^-$	0.105	NS
$Bcl\text{-}2^-$	0.485	NS
$TIA\text{-}1^+$	0.783	0.02
$CD45RO^+, Bcl\text{-}2^-$	0.111	NS
$CD45RO^+, TIA\text{-}1^+$	0.874	0.005

[a] The cytolytic activity of purified $CD8^+$ lymphocytes from 18 $HIV\text{-}1^+$ individuals was compared statistically to their expression of the listed markers. Cytotoxicity was determined using a redirected killing assay and an E:T of 12:1 used in the analyses

NS Not significant, $p > 0.05$

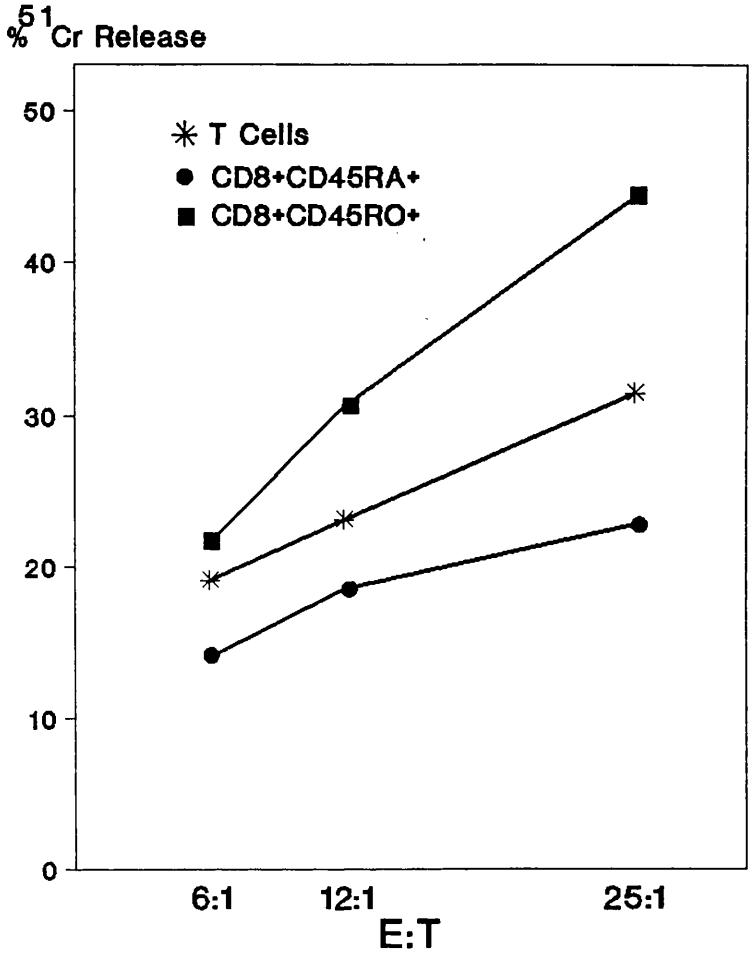


Figure 8.7 The cytolytic activity of $CD8^+$,$CD45RA^+$ and $CD8^+$,$CD45RO^+$ lymphocytes

Enriched $CD8^+$,$CD45RA^+$ and $CD8^+$,$CD45RO^+$ populations were obtained from E^+, T cells by : depletion procedures using CD4 and either CD45RO or CD45RA. These populations were >90% pure after the selection procedure. The cytolytic activity of these subsets was compared to the whole T cell preparation using the LDC assay against P815 target cells. Results from one representative HIV-1^+, CDCII individual is shown (n=4).

was still able to mediate low levels of killing although this might have been due to contaminating NK cells.

The expression of the cytotoxic granule associated proteins TIA-1 and perforin in HIV-1 infection

Cytotoxic killing is thought to be achieved in part through the action of cytolytic granule associated proteins such as perforin (Henkert 1985, Podack & Konisberg 1984) and a recently described protein recognized by the Mab TIA-1 (Anderson et al 1990). As the presence of these molecules could provide important information concerning cytolytic function, their expression was investigated in PBMC and lymphoid tissue from HIV-1^- and HIV-1^+ individuals.

PBMC: The expression of these intracellular markers was measured both in cytospin preparations and by flow cytometry after permeabilisation of the cell membrane (Chapter 6). Both methods gave concordant results, although cytospins were preferred for perforin which was only weakly stained in suspension. After removal of $CD16^+$ NK cells that expressed high levels (>90%) of both TIA-1 and perforin, a small proportion (34.0±4.2%) of $CD8^+$ peripheral blood lymphocytes from HIV-1^- individuals expressed TIA-1 (Table 8.3). Approximately one third of this population (9.4±3.5%) also contained perforin. This trend in which a rather larger percentage of $CD8^+$ cells expressed TIA-1 compared to perforin was maintained in both the HIV-1^+ and AVI patient groups. However, expression of both of these markers was significantly increased in HIV-1^+ (TIA-1 59.6±3.6; perforin 23.0±3.5; $p<0.001$ & $p<0.05$ respectively) and AVI patients (TIA-1 82.1±6.4; perforin 71.8±8.6; $p<0.001$). Interestingly, TIA-1, but not perforin, was also seen in a small percentage (2-8%) of $CD4^+$ lymphocytes and this was not altered in HIV-1 or AVI patients.

To determine the expression of TIA-1 in $CD8^+CD45RO^+$ T cells triple colour FACScan analyses using CD8, CD45RO and TIA-1 were performed on CD16 depleted PBMC. In both HIV-1^- and HIV-1^+ individuals a large proportion of $CD8^+CD45RO^+$ cells contained TIA-1^+ cytotoxic granules (HIV-1^- 39.6±12.0; HIV-1^+ 58.0±5.1; AVI 87.6±4.3; Table 8.3). These are shown as the triple positive (black) population in a representative individual in Fig. 8.8. However, TIA-

or $CD8^+CD45RO^+$

Table 8.3 The Expression of TIA-1 and Perforin in CD8+ Lymphocytes

Part I Peripheral Blood[a]

	n	$CD8^+$ $TIA\text{-}1^+$	$CD8^+,CD45RO^+$[b] $TIA\text{-}1^+$	$CD8^+$ Perforin
$HIV\text{-}1^-$	8	34.0±4.2	39.6±12.0	9.4±3.5
$HIV\text{-}1^+$	24	59.5±3.7*	58.0±5.1	20.9±5.3
AVI	8	82.1±6.5*	87.6±4.3	71.8±8.7*

Part II Lymph Node/Tonsil[c]

	n		$CD8^+$ $TIA\text{-}1^+$	$CD8^+,CD45RO^+$ $TIA\text{-}1^+$	$CD8^+$ Perforin
$HIV\text{-}1^-$	8	GC	ND	ND	ND
		PC	39.5±9.3	39.0±12.0	<2%
$HIV\text{-}1^+$	9	GC	87.6±6.1*	92.7±1.2*	<2%
		PC	79.2±9.6*	86.4±4.8*	<2%

[a] The expression of TIA-1 and perforin in $CD8^+$ lymphocytes in peripheral blood was determined by dual colour IF on cytospin preparations of NK depleted PBMC.

[b] Triple staining for the investigation of $CD8^+,CD45RO^+$ lymphocytes was performed by flow cytometry after permeabilisation of the membrane using acetone/methanol

[c] Normal tonsil sections and $HIV\text{-}1^+$ lymph node sections were investigated using conventional epifluorescence and confocal microscopy as described in the text.

* Significantly increased expression compared to equivalent normal population ($p<0.001$) using Student's t-test.

ND Not determined due to the absence of these cells in normal germinal centres

GC Germinal centre

PC Paracortex

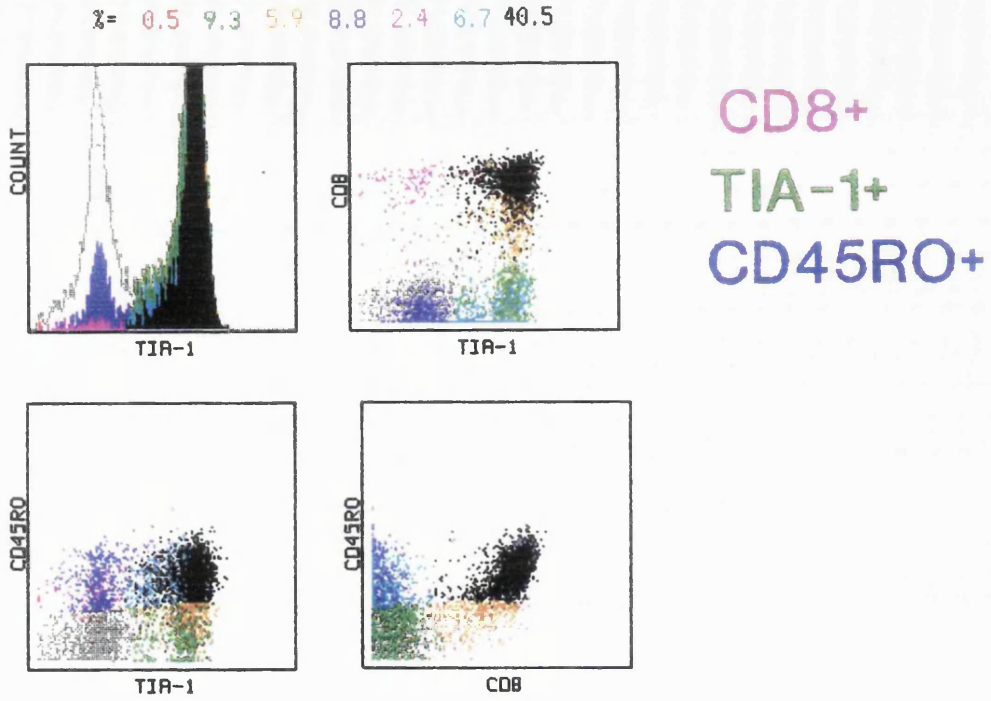


Figure 8.8 Triple colour immunofluorescence study of TIA-1 expression in $CD8^+CD45RO^+$ lymphocytes

PBMC depleted of $CD16^+$, NK cells were membrane stained using indirect immunofluorescence with CD8μ plus GAM-IgM-PE and CD45RO-Biotin plus Strep-Tricolour. After fixation, cells were permeabilised using the acetone/methanol procedure and stained with TIA-1 plus GAM-IgG-FITC. Samples were run on the FACScan, placing an analysis gate around the lymphocyte population. The data was analysed using paint-a-gate. The $CD8^+$,$CD45RO^+$,$TIA\text{-}1^+$ triple positive population appears black. Results from one AVI patient are illustrated.

1 expression was not limited to the $CD45RO^+$ subset as a smaller proportion (30-45%) of $CD8^+CD45RA^+$ cells also contained the protein ($CD8^+CD45RO\text{-}TIA\text{-}1^+$; yellow population Fig. 8.8). Using cell suspensions we were unable to detect perforin outside the $CD16^+$, NK cells. These results may be due to the weak staining of this reagent and the consequent low detection rate in T cells that may express very low amounts of perforin.

The presence of both of these granule associated proteins was also investigated in the $CD28^-$ population shown in chapter 7 to be susceptible to AALD. To achieve this E^+, T cells were depleted of both $CD16^+$, NK cells and $CD28^+$ lymphocytes. The remaining $CD28^-$ lymphocytes were then double stained in cytospin preparations with CD57 and TIA-1 or perforin, a representative example of which is shown in Fig. 8.9. In both $HIV\text{-}1^-$ and $HIV\text{-}1^+$ individuals a large percentage of $CD28^-$ cells expressed both TIA-1 and perforin (TIA-1 27-64%; perforin 20-39%). The $CD57^+$,$CD28^-$ subset was entirely (>95%) positive for both TIA-1 and perforin. In $HIV\text{-}1^+$ individuals, TIA-1 and to a lesser extent perforin were also seen in the $CD28^-$,$CD57^-$ subset (TIA-1 35-44%; perforin 10-15%). However, both of these markers were largely absent (<5%) from this same subset in the $HIV\text{-}1^-$ control group.

Lymph nodes: As shown previously in chapter 6 there is an expansion of $CD8^+$,$CD45RO^+$ lymphocytes in the lymph nodes during HIV-1 infection. These cells infiltrate the germinal centres and are thought to be cytolytic effector cells directed against the follicular dendritic cells (Racz et al 1985, Tenner-Racz et al 1986, Laman et al 1989). The expression of both TIA-1 and perforin was also investigated in tissue sections of normal tonsil and $HIV\text{-}1^+$ lymph nodes. Using confocal microscopy, three colour studies were used to investigate the expression of CD8, CD45RO and either TIA-1 or perforin simultaneously.

In $HIV\text{-}1^-$ individuals $CD8^+$ lymphocytes were not seen within the germinal centres. Small numbers of $CD8^+$ cells were found in the paracortex and $39.5 \pm 9.3\%$ of these expressed TIA-1 (Table 8.3; Fig. 8.10). This occurred primarily in the $CD45RO^+$ subset. The proportion of $CD8^+$ lymphocytes was greatly expanded in lymph nodes from $HIV\text{-}1^+$ donors and large numbers of these were seen to infiltrate the germinal centres. The vast majority of $CD8^+$ lymphocytes in both the

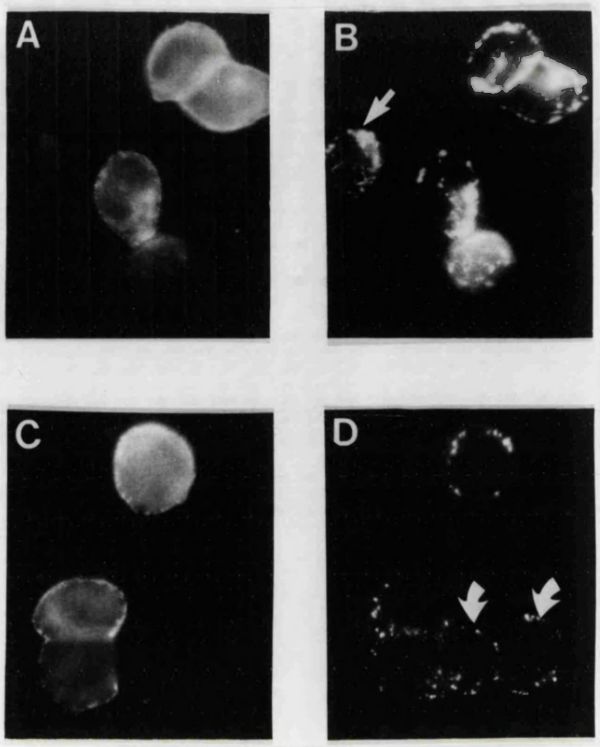
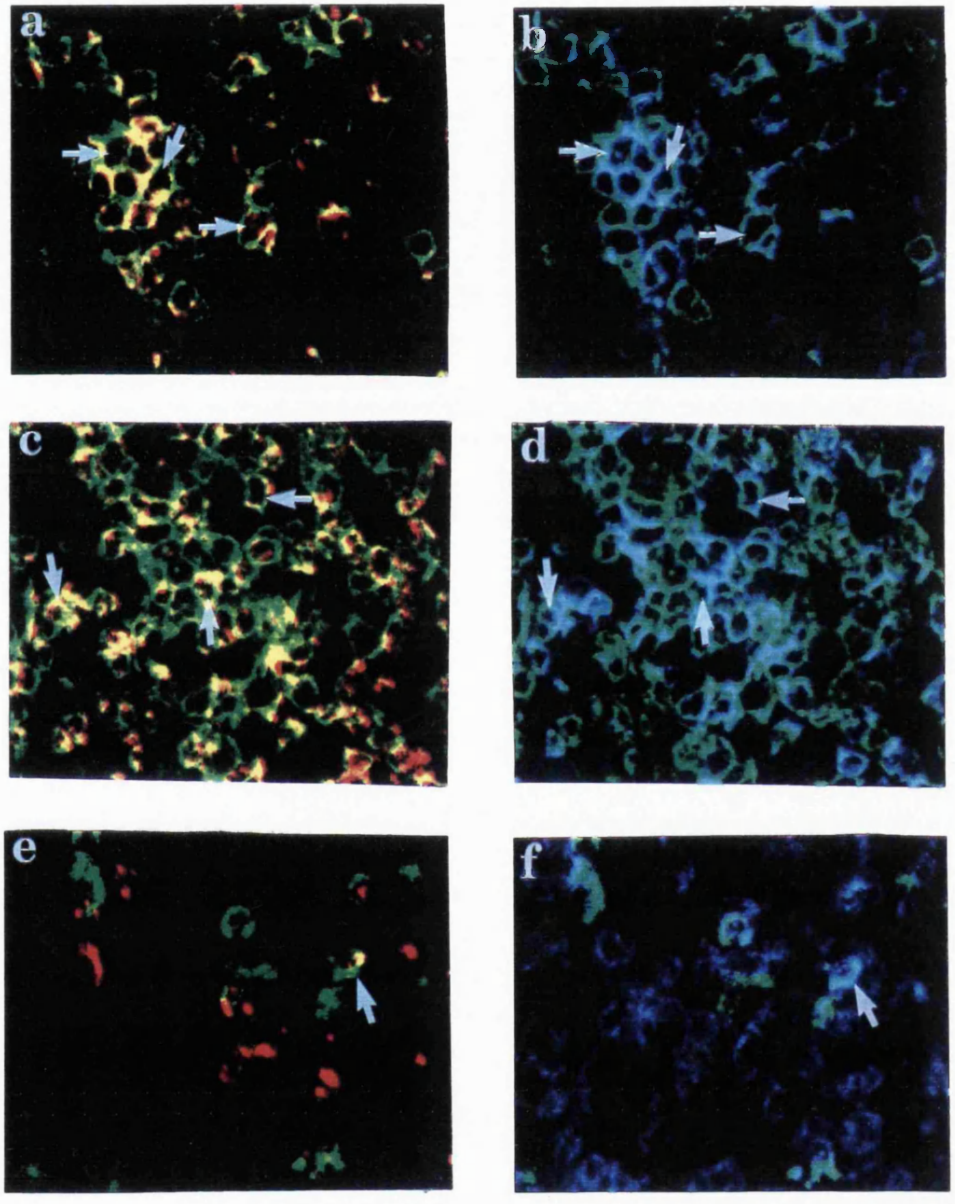


Figure 8.9 The presence of cytotoxic granules in CD28$^-$ lymphocytes

An enriched CD28$^-$ population was prepared from E$^+$, CD16-depleted cells by negative selection with CD28. The remaining CD3$^+$,CD28$^-$ cells were double-stained for the membrane marker CD57 (a,c) and one of the cytotoxic granule-associated proteins; TIA-1 (b) or perforin (d). Note that all CD57$^+$ cells express both TIA-1 and perforin. The arrows indicate the presence of CD57$^-$ cells that also contain the cytotoxic granules. Results from one HIV-1$^+$, CDCII individual are illustrated.

Figure 8.10 The expression of TIA-1 in $CD8^+$,$CD45RO^+$ cells in lymph node sections

Triple colour immunofluorescence studies were performed on tissue sections of lymph node from HIV-1^+, CDCIII individuals and normal tonsil. The triple combinations; TIA-1 plus GAM-IgG1-FITC, CD8μ plus GAM-IgM-LR and CD45RO-biotin plus strep-Cy5 were used and the staining analysed on the confocal microscope. Both germinal centre (a,b) and paracortical areas (c-f) were investigated. The results from one HIV-1^+ (a-d) and one HIV-1^- (e,f) individual are shown. The $CD8^+$ lymphocytes (green) combine with the TIA-1^+ cells (red) to form the yellow staining pattern (a,c,e). The $CD45RO^+$ cells (blue) combine with $CD8^+$ (green) to form the cyan staining pattern (b,d,f). Note that the majority of $CD8^+CD45RO^+$ (cyan) cells in the HIV-1^+ individual also express TIA-1. The arrows indicate the presence of some of these cells.


a
b
c
d
e
f

paracortical and germinal centre areas expressed CD45RO (GC >95%; PC 74.6±3.0%) and TIA-1 (GC 87.6±6.1; PC 79.2±9.6; Table 8.3; Fig. 8.10). Again TIA-1 was restricted to the $CD45RO^+$ subset which was almost entirely $TIA\text{-}1^+$.

Perforin was not detected in $CD8^+$ lymphocytes from either normal tonsil nor $HIV\text{-}1^+$ lymph node. This was not due to the inability of the reagent to stain tissue sections as perforin expression was detected in tissue sections of normal spleen where $CD8^+$ cells in the red pulp are frequently $perforin^+$. Perforin was also absent from cytospin preparations of lymph node suspensions.

In conclusion, in HIV-1 infection high levels of both TIA-1 and perforin are found in $CD8^+$ lymphocytes in peripheral blood, particularly in the $CD57^+$ and $CD28^-$ lymphocytes subsets. In the lymph node, the majority of $CD8^+CD45RO^+$ cells express TIA-1 but do not express perforin. This might be due to the singular absence of $CD8^+CD57^+$ cells from lymphoid tissue.

Discussion

During HIV-1 infection virus specific CTL effectors are present in large numbers in the peripheral blood of asymptomatic individuals (Gotch et al 1990) but decline as patients progress to symptomatic AIDS (Weinhold et al 1988, Hoffenbach et al 1989, Plata et al 1989, Joly et al 1989). Cells of a $CD8^+$ phenotype have also been shown to suppress HIV-1 replication *in vitro* by a non-cytotoxic mechanism and are therefore thought to provide important mechanisms for controlling viral load and maintaining an asymptomatic state (Walker et al 1990, Tsuboto et al 1989). In this study, using an LDC assay, we have shown that $HIV\text{-}1^+$ patients at all stages of the disease show high levels of CTL activity. Thus, although HIV-1 specific CTL are lost with disease progression, large numbers of armed, $CD8^+$ effector cells remain in the circulation. Similar high levels of CTL activity at all stages of HIV-1 infection have also been reported by Vanham et al 1990 and Pantaleo et al 1990 using an anti-CD3, redirected killing assay.

The specificity of the CTL in the peripheral blood of $HIV\text{-}1^+$ patients and the events leading to their generation are unknown. As the LDC assay measures all CTL activity regardless of specificity it is possible that we are measuring the activity of cells that have arisen in response to a secondary viral infection or to the

reappearance of some latent infection. The symptomatic stages of HIV-1 in particular are associated with a number of viral illnesses such as CMV induced retinitis or pneumonitis, shingles (*Herpes zoster*) and EBV related B cell lymphomas. However, in patients with active CMV infections, CMV specific CTL responses were not detected in fresh PBMC (Rook et al 1983) and EBV specific CTL can only be detected after restimulation *in vitro* (Carmichael et al 1993). As with HIV-1 specific CTL, precursor frequencies to other pathogens such as influenza, *m.tuberculosis* and EBV decline with disease progression (Shearer et al 1986, Blumberg et al 1987, Forte et al 1992). It therefore seems unlikely that $CD8^+$ CTL arise in response to a single pathogen.

It is possible that the appearance of $CD8^+$,$CD45RO^+$ T cells with CTL activity in HIV-1 infection results from a general, non-specific stimulation of the immune system, perhaps due to the direct infection of macrophages, or arising from a cytokine imbalance or a perturbation of normal control mechanisms due to the loss of $CD4^+$ lymphocytes. Alternatively, a viral protein or "superantigen" might result in the proliferation of $CD8^+$ cells and the stimulation of CTL as well as the subsequent deletion of selected clones. To investigate these possibilities a number of studies have looked at the distribution of TCR variable (V) region expression of $CD8^+$ lymphocytes from HIV-1^+ patients. Activation with bacterial superantigens such as Staphylococcus enterotoxin B (SEB) is known to preferentially stimulate T cells expressing Vβ8 determinants (White et al 1989) and results in the expansion and subsequent deletion of Vβ8 clones. It was shown by Grant et al 1993 that HIV-1 infected patients with low $CD4^+$ lymphocyte counts had a skewed pattern of TCR-V region representation indicating a selective expansion of particular $CD8^+$ CTL which became more apparent with disease progression. However, a similar study (De Paoli et al 1993) detected a skewed TCR-V region distribution in only 4 out of 66 HIV-1^+ patients suggesting the expansion of $CD8^+$ cells is not selective in the majority of HIV-1^+ patients.

Mitogenic stimulation of normal $CD8^+$ T cells leads to the differentiation of precursor CTL into an effector population and consequently results in an increase in the cytolytic activity measured *in vitro* (Leewenberg et al 1985, Jung et al 1986). In this chapter it was shown that T cell cytotoxicity in HIV-1^+ patients failed to

increase normally after stimulation during the first 5 days but that longer term cultures attained normal levels of killing. This defective generation of CTL in HIV-1 infected individuals was also reported by Gruters et al 1990 after short-term anti-CD3 stimulation and more recently by Watret et al (1993). We now show that this defect is due to the death of the responding cells because when cultures were adjusted for viability, levels of killing in HIV-1^+ individuals returned to normal. Although the reported anti-CD3 induced defect (Gruters et al 1990) was restored by IL-2, this might have been due to the generation of LAK activity by the cytokine (Trinchieri et al 1984; Lanier et al 1985). In our study, $CD16^+$ lymphocytes were removed prior to stimulation and although cultures were supplemented with IL-2, this did not prevent the decrease in CTL activity. Therefore, AALD interferes with the generation of CTL effectors after stimulation *in vitro*.

Based on the expression of cytotoxic granule proteins and comparisons of $CD8^+$ cell phenotype and cytotoxic function, it was concluded that $CD8^+$,$CD45RO^+$ cells that contain TIA-1^+ granules are the CTL effector population in HIV-1 infection. Cells of this phenotype have previously been shown to mediate the most potent CTL activity after MLR stimulation (Yamashita & Clement 1989; Akbar et al 1990). Also, as shown in chapter 4, this subset is greatly expanded in AVI patients, the group now shown to have the highest CTL activity. Triple colour studies on $CD8^+$ cells in HIV-1^+ patients have demonstrated overlapping expression of the markers CD45RO, CD38, HLA-DR and CD57 (Prince & Jensen 1991b, Kestens et al 1992, Levacher et al 1992, Bass et al 1992) all of which have previously been associated with CTL function (*CD45RO* Yamashita & Clement 1989, Akbar at al 1988; *CD38* Ho et al 1993; *HLA-DR* Vanham et al 1990, Pantaleo et al 1990, Ho et al 1993; *CD57* Vanham et al 1990). In chapter 7 these markers were all shown to be increased in the $CD28^-$ population that rapidly dies after stimulation *in vitro*. Also many of these $CD28^-$ and $CD45RO^+$ cells lack Bcl-2 expression and are susceptible to apoptosis. The $CD28^-$ compartment is thus composed of a large proportion of $CD8^+$ effector cells that are short-lived and die after stimulation. These may represent a terminally differentiated $CD8^+$ population as proposed by Gotch et al 1990.

A huge expansion of $CD8^+$,$CD45RO^+$,TIA-1^+ lymphocytes were also seen

in both the germinal centre and paracortical areas of lymph nodes from HIV-1^+ patients. This confirms the findings of Tenner-Racz et al (1993) who showed that the $CD8^+$ cells in the HIV-1^+ lymph node contained TIA-1^+ cytotoxic granules. In this chapter we were able to demonstrate substantial levels of CTL activity in purified $CD8^+$ lymphocytes from two individuals, some of which was directed against HIV-1*gag*. Interestingly, the lymph node histopathology in HIV-1^+ patients with PGL is very similar to that seen in mice infected with lymphocytic choriomeningitis virus (LCMV). Particular isolates of this virus infect macrophages and result in severe immune suppression (Leist, Ruedi & Zinkernagel 1988). It has been shown that $CD8^+$ CTL in these infected animals eliminate LCMV-infected FDC and marginal zone macrophages in the lymph nodes (Odermatt et al 1991). This causes complete destruction of lymphoid follicles and indicates a crucial role for FDC and marginal zone macrophages in maintaining follicular organization. Similarly, sheep naturally infected with the lenti virus *Maedi visna* show an infiltration of $CD8^+$ lymphocytes into the follicles and germinal centre areas of the lymph nodes and are though to play a major part in the pathogenesis of *visna virus* induced lymph node lesions (Watt et al 1992). Thus in HIV-1 infection also, $CD8^+$ CTL could play a critical role in the destruction of follicular structures and contribute significantly to the pathogenesis of AIDS.

CHAPTER 9
DISCUSSION

Qualitative defects in T cell responses after HIV-1 infection are likely to play a key role in the development of HIV-1 disease. Using flow cytometric and microscopic techniques we have shown that poor proliferative responses *in vitro* are due to the death of the stimulated cells. In fact, two forms of cell death were noted. Firstly, a spontaneous cell death that occurred in both HIV-1$^+$ and AVI patients after short-term culture in medium alone. Secondly, an activation associated death that occurred after mitogenic stimulation and was of greater magnitude than the spontaneous cell death (Table 9.1). Cell death of this magnitude has not previously been reported and we propose that this provides a mechanism for the immunodeficiency in HIV-1 infection.

Spontaneous cell death occurred by apoptosis, associated with the loss of Bcl-2 protein expression and was largely prevented by the addition of IL-2 to the cultures. Cell death of this nature was also seen during acute viral infections where T cell apoptosis occurred in Bcl-2$^-$ lymphocytes. Thus, spontaneous cell death bears many similarities to that seen in cytokine dependent cell lines that die after growth factor removal (Vaux et al 1988, Nunez et al 1990) and is almost certainly due to IL-2 deprivation. In direct contrast, AALD could not be prevented by IL-2, was not restricted to Bcl-2$^-$ lymphocytes and did not occur by classical apoptosis. Additionally, this form of lymphocyte death was not noted in AVI patients. Thus, AALD differs markedly from the Bcl-2 dependent apoptotic cell death due to growth factor deprivation and is a separate phenomenon related to chronic HIV-1 infection. Both of these forms of cell death will contribute to the defective proliferative responses described in HIV-1 infection

The spontaneous death of T cells from HIV-1$^+$ individuals was reported previously and shown to occur primarily within the CD8$^+$CD45RO$^+$ lymphocyte subset (Prince & Czaplick 1989, Prince & Jensen 1991a). Similarly, CD45RO$^+$ lymphocyte from acute EBV patients were found to die by apoptosis after short term culture (Uehara et al 1992). In HIV-1 infection, cell death restricted to CD4$^+$ lymphocytes after activation with superantigen was reported by Groux et al (1992). However, in agreement with another study (Meyaard et al 1992) we found that both

Table 9.1 Lymphocyte Death in HIV-1$^+$ and AVI Patients

Spontaneous	AALD
Occurs in both AVI and HIV-1$^+$ individuals	Restricted to HIV-1$^+$ individuals
Associated with the loss of Bcl-2	Bcl-2 independent
Prevented by IL-2	IL-2 independent
Occurs by classical apoptosis	Unknown mechanism
CD28 independent	Occurs in CD28$^-$ T cells

$CD4^+$ and $CD8^+$ subsets died after stimulation with anti-CD3 or PHA.

There is a great deal of evidence that memory responses are lost early in HIV-1 disease. This has been demonstrated *in vivo* as a loss of DTH skin test responsiveness (Reuben et al 1983, Fernandez-Cruz et al 1988) and *in vitro* as a failure to proliferate or release IL-2 after culture with recall antigens (Lane et al 1985, Antonen et al 1986, Hofmann et al 1985). The observation that $CD45RO^+$ T cells in both $CD4^+$ and $CD8^+$ subsets are particularly vulnerable to AALD would support this idea and we would further suggest that the loss of memory responses is an example of cell death rather than unresponsiveness or anergy.

Although both $CD4^+$ and $CD8^+$ T cell subsets are affected by AALD, it is possible that these populations die for different reasons. There is increasing evidence that direct infection of $CD4^+$ T cells or cell lines with HIV-1 causes death by apoptosis (Terai et al 1991, Laurent-Crawford et al 1991, Martin Matear & Vyarkarnam 1994). In addition, cross-linking of CD4 with gp120/anti-gp120 results in the deletion of $CD4^+$ T cells in CD4 transgenic mice *in vivo* (Wang et al 1994a) possibly through a Fas mediated pathway (Wang et al 1994b). Also, in humans, the presence of viral gp120 on the surface of uninfected cells causes modulation of the CD4 molecule and primes the cell for subsequent death upon signalling through the TcR (Banda et al 1992, Theodore et al 1994). Thus, death in $CD4^+$ T cells may be directly related both to the trophism of HIV-1 for CD4 T cells and the ligation of CD4 by virus gp120/anti-gp120 complexes independently of the TcR. However, the $CD8^+$ T cell subset is not infected by the virus *in vivo* and there must be an alternative explanation for death in this population.

There are a number of possible explanations for AALD of $CD8^+$ T cells in HIV-1 infection (Table 9.2). These are discussed below.

(i) Cytokine deprivation

Altered cytokine profiles in HIV-1 infection have been reported by many investigators and might play an important role in the immunodeficiency. Because of the decline in $CD4^+$ T cells, HIV-1 infected individuals suffer a gross deficiency in IL-2 (Creemers et al 1986, Burkes et al 1987) and perhaps other cytokines released by this subset that could greatly influence subsequent activation responses *in vitro*. In

Table 9.2 Possible causes of AALD in T lymphocytes

$CD4^+$	Direct infection with HIV-1
	CD4/gp120 interactions
$CD4^+$ & $CD8^+$	Cytokine deprivation or imbalance
	APC deficiency
	Activation of previously stimulated cells (cells out of G0)
	Inappropriate activation in the absence of a co-stimulus
	Autolysis by CTL
	Viral protein directly interfering with mitosis or cell metabolism

this study, cultures supplemented with IL-2 showed an abrogation of spontaneous cell death but AALD was not prevented, indicating that a deficiency in IL-2 is not responsible for AALD. Also, a mixing experiment in which HIV-1$^-$ and HIV-1$^+$ cells were stimulated together in the same well showed that only HIV-1$^+$ individuals were affected by AALD and that the presence of "normal" cells or factors released by them could not overcome the proliferative defect. Thus, the presence or absence of a cytokine or soluble mediator can largely be excluded as these act in an unrestricted fashion. Nevertheless, as allogeneic cells were mixed, a defect in syngeneic antigen presentation might still occur, particularly in view of the defects in APC function that have been demonstrated in HIV-1 infection (Shannon et al 1985, Petit et al 1988, Knight & Macatonia 1991).

Exogenously added cytokines do however have powerful effects on *in vitro* proliferative responses in HIV-1 infection. For example, it was recently reported that IL-12 can restore defective proliferation and IL-2 secretion to influenza virus or HIV-1 envelope peptides (Clerici et al 1993b). Release of this cytokine in response to *S. aureus* is particularly handicapped in HIV-1 infection and this is related to the direct infection of monocytes with HIV-1 (Chehimi et al 1994). Interleukin-12 is also involved in NK and CTL responses (Chehimi et al 1992, Gately et al 1992) and if added exogenously to PBMC can improve the defective NK activity seen in AIDS (Chehimi et al 1992). Thus, IL-12 deficiency might have a role to play in the immunodeficiency associated with HIV-1 infection and it would certainly be of interest to determine its role in the prevention of spontaneous death and AALD.

(ii) Activation *in vivo*

The activation induced death of mature peripheral T lymphocytes has been reported previously (reviewed in Kabelitz Pohl & Pechhold 1993). In such cases the activation stage of the cell is important in determining the outcome of lymphocyte activation signals. Thus the same stimuli that activate resting cells can trigger death in T lymphocytes reactivated in a non-resting state. For example, using both CD4$^+$ T cell clones and lymph node T cells, Lenardo (1991) demonstrated that culturing T lymphocytes with IL-2 led to the subsequent death of the cells after stimulation with antigen or anti-CD3. This was interpreted to mean that the susceptibility of mature T

cells to TcR induced death is due to the reversal of normal T cell activation where TcR occupancy precedes IL-2 stimulation (Lenardo 1991, Boehme & Lenardo 1993). However, such activation induced cell death can occur following anti-CD3 signalling in the absence of IL-2 (Kabelitz & Wesselborg 1992) and there is no evidence that IL-2 is solely responsible for this primary phase. The second stimulus, which can be a variety of activation signals including antigen, anti-CD3, anti-CD2, PMA plus Calcium ionophore or anti-TcR antibodies (Lenardo 1991, Russell et al 1991, Radvanyi Mills & Miller 1993, Wesselborg et al 1993), is however blocked by Cyclosporin A (Mercep Noguchi & Ashwell 1989) indicating that IL-2 or some other cytokine might be important at this stage. Indeed, using mouse TH1 clones, Liu & Janeway (1990) showed that IFN-γ played a critical role in T cell death after stimulation, however, it did not play any role in anti-CD3 mediated death of other murine TH1 and TH2 clones (Russell et al 1992).

A crucial observation in this study was that levels of Bcl-2 normally decrease after prolonged activation *in vitro* as cells acquired high levels of CD45RO. Thus, after activation and expansion *in vivo*, T cells acquire CD45RO and lose Bcl-2, becoming susceptible to cell death unless they receive the appropriate survival signals. Also, it was shown in EBV infection that levels of Bcl-2 correlated inversely with the appearance of the Fas antigen, which further pushes cells towards apoptosis (Uehara et al 1992). Additionally, $CD45RB^{Low}$ T cells, that arise with progressive rounds of activation and division, have low Bcl-2 and express Fas (Salmon et al 1994). These data provide further evidence for immune stimulation rendering cells more susceptible to cell death. Evidence of *in vivo* stimulation could therefore be of great importance in explaining cell death in HIV-1 infection.

(iii) Activation in the absence of a co-stimulus

The two signal model of lymphocyte activation first proposed by Bretcher & Cohn (1970) predicts that occupancy of the TCR alone is insufficient for optimal stimulation and that co-stimulatory signals provided by accessory molecule/ligand interactions are necessary for optimal proliferation (Meuller Jenkins & Schwartz 1989, Schwartz 1992, Liu & Linsley 1992, Linsley & Ledbetter 1993). Stimulation in the absence of accessory signals causes cells to become unresponsive or anergic.

This is the basis of peripheral tolerance when self-reactive T cells in the periphery come in contact with specific antigen presented by a non-professional APC ie an APC without accessory molecules. To date, the most powerful co-stimulatory signal in T cell activation is provided through CD28 on the T cell combining either with anti-CD28 antibody or with its ligand B7 on APC (reviewed in Lui & linsley 1992). The failure to ligate CD28 is now known to be the tolerogenic event when T cells are stimulated by antigen and chemically modified APC (Harding et al 1992). Similarly, blocking CD28/B7BB1 interactions during antigen/TcR engagement leads to anergy (Tan et al 1993) while co-stimulation through CD28 prevents both allo-antigen specific and Rabies virus specific anergy (Vassiliki et al 1993, Celis & Saibara 1992, Sansom et al 1993).

In mature T cells, anergy is defined as a failure to respond, either by proliferation or cytokine production, to specific antigen. However, such immunological tolerance can also be achieved through deletion as in immature thymocytes. For example, in $CD4^+$ T cell clones ligation of the TcR in the absence of accessory signals leads to cell death rather than unresponsiveness (Liu & Janeway 1990). Deletion as a mechanism of tolerance *in vivo* is seen when mice are injected with superantigen. After an initial clonal expansion, Vβ specific T cells no longer respond to the antigen and are deleted (Webb Morris & Sprent 1990, Kawabi & Ochi 1991).

An important observation in this study was the increased proportion of T cells from HIV-1 infected individuals that lacked expression of CD28. The $CD3^+CD28^-$ T cells belong primarily to the $CD8^+$ subset and after stimulation with a range of mitogens including anti-CD3 or PHA, were unable to proliferate and died. Other groups have also found similar increases in $CD8^+CD28^-$ T cells and have shown that these cells are functionally deficient (Gruters et al 1991, Saukkonen Kornfeld & Berman 1993, Brinchmann et al 1994). The absence of CD28 or down regulation of co-stimulatory molecules, provides an alternative method for the induction of anergy in that even cells meeting antigen presented by a professional APC will be unable to proliferate. There is some evidence for different levels of tolerance (Miethke et al 1994). Unresponsiveness in mature T cells is associated with a decrease in the accessory molecules CD4, CD8 and the TcR (Kisielow et al

1988, Schonrich et al 1991, Rocha & von Boeher 1991) and recently the induction of tolerance in human influenza-specific $CD4^+$ T cell clones was shown to result in decreased mRNA and protein expression of CD28 (Lake et al 1992). Therefore, we could hypothesise that tolerogenic events *in vivo*, perhaps caused by uncontrolled or inappropriate proliferative signals, could result in the down regulation of CD28 such that a second proliferative signal will kill the cell.

(iv) Activation induced autolysis by CTL

One possible explanation for AALD is that after activation *in vitro*, $CD8^+$ T cells kill each other; a form of self destruction that might be envisaged if the immune system had totally broken down. Although there is evidence that activated $CD8^+$ T cells from HIV-1 infected individuals are able to kill uninfected $CD4^+$ lymphocytes in an unrestricted manner, these same cells do not kill Con A-activated $CD8^+$ T cells (Grant Smail & Rosenthal 1994). Indeed, mechanisms are thought to exist which prevent autolysis of CTL (Ding-E Young 1989). Nevertheless, in HIV-1 infected individuals $CD28^-$ and in particular the $CD57^+$, T lymphocytes do contain both perforin and TIA-1^+ cytotoxic granules, indicating that they are highly cytolytic in vivo. Bearing in mind the dominance of $CD8^+CD28^-$ cells in HIV-1 infection it is perhaps not surprising that PBMC and purified $CD8^+$ T cells from HIV-1^+ individuals and AVI patients were found to be highly cytolytic in a redirected killing assay. This activity has also been reported by two other groups who suggested that $CD8^+CD57^+$ or $CD8^+$HLA-DR^+ cells were the CTL effectors (Vanham et al 1990, Pantaleo et al 1990ab). However, in agreement with a number of other groups (Merkenschlager & Beverley 1989, Yamashita & Clement 1989, Akbar et al 1990) we found the greatest correlation between the expression of CD45RO and CTL activity. These discrepancies are not unexpected as these phenotypic markers form overlapping populations within the $CD8^+$ T cells. The observation that $CD45RO^+$ and $CD28^-$ lymphocytes are particularly susceptible to cell death and that subsequent activation *in vitro* decreases cytolytic activity, strongly suggests that within the $CD8^+$ population, effector CTL undergo AALD.

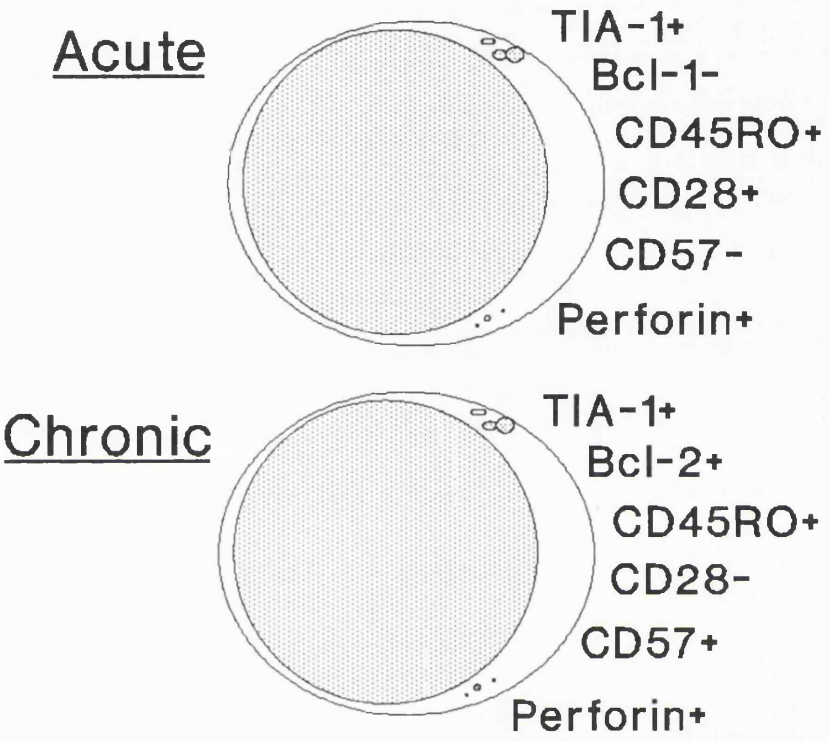
Of these possibilities, the most likely explanation for AALD in the $CD8^+$ T

cell subset is that uncontrolled or inappropriate stimulation *in vivo* causes anergy, including CD28 down-regulation, such that restimulation results in death. Thus evidence of activation *in vivo* could be important in explaining AALD.

Studies on lymphocytes and lymphoblastoid cell lines have shown that infection with HIV-1 or exposure to viral gp120 leads to activation of the cell which cannot then respond normally to further stimuli (Nye, Knox & Pinching 1991). Similarly, there is a great deal of evidence for stimulation *in vivo* induced either directly by the virus or by indirect means. For example, B cells show evidence of polyclonal activation with high levels of immunoglobulin production of low specificity (Seligmann et al 1987). This could arise because of an increase in IL-6 (Chehimi et al 1994) or the action of viral proteins with B cell stimulatory properties (Chirmule et al 1994). Also monocytes and macrophages show evidence of activation (Gendelman et al 1989, Fahey et al 1990) that might be linked either to the binding of gp120 to CD4 or to direct infection with HIV-1 (Wahl et al 1989). Increased serum levels of sCD8, sCD25 and β2-microglobulin have been reported (Fahey et al 1990, Nishinian et al 1991, Pizzolo et al 1992) and are also indicative of immune activation as is the recent report concerning an increase in sCD30 (Pizzolo et al 1994). Similarly, the phenotypic changes that we, and others, have described within the $CD8^+$ T cell population are indicative of immune stimulation *in vivo*. In particular the appearance of large number of cells expressing CD45RO, CD38 or HLA class II all point to this as does the crucial observation of decreased Bcl-2 and CD28 expression.

By comparing AVI and HIV-1^+ patients the phenotypic markers of chronic immune stimulation become more clear. Acute stimulation leads initially to an upregulation of HLA class II and a switch from CD45RA to CD45RO, with a concurrent loss of Bcl-2 protein expression. More prolonged stimulation results in a loss of CD28 followed by the appearance of $CD57^+$ cells (Fig. 9.1). The evidence for this chain of events comes from a number of sources. Firstly, studies on T cells *in vitro* show that short-term activation causes phenotypic changes very similar to those seen in acute infections ie a switch from $CD45RA^+$ to $CD45RO^+$ with large numbers of cells expressing both markers (Akbar et al 1988, Byrne Butler & Cooper 1988, Sanders et al 1989), gain of HLA-DR and loss of Bcl-2 (Akbar et al 1993,

Figure 9.1 Phenotypic markers on CD8 T lymphocytes during acute or chronic viral infections



Akbar et al 1994). Studies on longer term cultures indicate that $CD57^+$ lymphocytes appear only after prolonged stimulation and that IL-2 &/or IL-4 are particularly implicated in their generation (Dupuy d'Angeac et al 1994). While $CD57^+$ T cells are a component of many chronic conditions (Burns, Tsai & Zvaifler 1992, Dupuy d'Angeac et al 1993, Leroy et al 1986) in kidney and liver transplant patients with primary CMV infection the appearance of $CD8^+CD57^+$ cells *in vivo* is preceded by cells with a $CD8^+$,$CD28^-$,$CD57^-$ phenotype (Labalette et al 1993). In addition, the observation that AVI patients do not have large numbers of $CD57^+$ T cells but do have intermediate CD28 negativity, with cells expressing the activation markers CD38 and HLA-DR in both $CD28^+$ and $CD28^-$ populations, implies that loss of CD28 precedes acquisition of CD57. In accordance with the data on apoptosis, the $CD8^+$,$CD28^-$ population were found to have a highly variable Bcl-2 expression and the $Bcl\text{-}2^-$ cells were by no means all $CD28^-$. This suggests that loss of Bcl-2 and loss of CD28, are separate events, linked with acute and chronic stimulation respectively.

It is notable that many of the observations in peripheral blood are reflections of changes within the lymphoid tissue. In particular, there is an expansion of $CD8^+CD45RO^+$ T cells in the paracortex and infiltrating the germinal centres. It seems likely that this population does not arise within the lymph nodes but rather, due to the increased expression of adhesion molecules on this subset, homes to the lymph node. Both blood and lymph node lymphocytes have $CD45RO^+$ T cells with low Bcl-2 expression but this is far more marked within the tissue. Indeed, many of these cells were shown to have fragmented nuclei, indicating they were undergoing apoptosis *in vivo* (Bofill et al submitted). This re-enforces the idea that activated $CD8^+$ T cells home to lymphoid tissue, where, in the absence of survival signals, they apoptose and are removed by the paracortical macrophages. The $CD8^+$ T cells in the node also contain $TIA\text{-}1^+$ cytotoxic granules. The cytolytic activity of $CD8^+$ cells in the blood would indicate that cells in the lymph node have a similar function and yet the absence of $CD57^+$ cells and perforin expression, which is essential for cytolysis (Kagi et al 1994), would suggest they are not. Indeed, cells isolated from lymphoid tissue were not found to be highly cytolytic unless co-cultured with autologous PHA blasts. This suggests that they may already have discharged their

perforin granules or that the most efficient CTL are confined to blood. Based on these observations it seem unlikely that $CD8^+$ T cells in the lymph nodes of HIV-1 infected individuals are actively functioning as CTL *in situ*. Conversely, $CD57^+$ and $CD28^-$ T cell populations are not found within the lymphoid tissue and are primarily a blood-borne population. Cells of this phenotype are however found in lung lavage (Agostini et al 1990) suggesting that they might home preferentially to this tissue.

There is therefore a great deal of evidence to suggest *in vivo* activation in HIV-1 infection but what could be the cause of this? As mentioned previously, superantigens provide one means of oligoclonal stimulation *in vivo* and subsequent tolerance by deletion. A number of bacterial superantigens have been described that are recognized in the context of MHC class II molecules by T cells expressing particular TcR Vβ regions (Marrack & Kapler 1990). Similarly, the Mls antigens encoded by mouse mammary tumour virus and the gag fusion product of murine leukaemia virus (MuLV) are examples of retroviral encoded superantigens that cause activation and subsequent deletion of T cells (Janeway et al 1991, Hugin Vacchio & Morse 1991). In the case of MuLV, the superantigen is thought to be responsible for the murine equivalent of AIDS (Hugin Vacchio & Morse 1991) and it has been proposed that an HIV-1 encoded superantigen might be involved in HIV-1 disease. To detect such a superantigen, investigators have examined patterns of Vβ usage to look for deletions or skewed distribution indicative of superantigens. Although conflicting conclusions were drawn with evidence both in favour (Imberti et al 1991, Dalgleish et al 1992, Dadaglio et al 1994, Rebai et al 1994) and against (Posnett et al 1993, Bahadoran et al 1993, Nisini et al 1994) an HIV-1 encoded superantigen, studies on 9 homozygotic twins discordant for HIV-1 infection did show distinct differences in the Vβ repertoire of the infected twin (Rebai et al 1994). This provides compelling evidence that an HIV-1 encoded superantigen could be involved at some stage of HIV-1 infection, although whether it is responsible for chronic immune stimulation and AALD is unclear.

Alternatively, it has been proposed that immune activation in HIV-1 infection occurs through defective antigen presentation by HIV-1 infected macrophages (Asher & Sheppard 1988). This cell is crucial for the proper function of T cells and

infection with HIV-1 might result in inappropriate activation of T cell proliferation, which if maintained could result in lymphocyte anergy. Also, HIV-1 infected macrophages might have altered cytokine profiles that could act to enhance T cell proliferation. For example, levels of tumour necrosis factor α (TNF-α) are increased in sera from AIDS patients and increased levels are released by monocytes from infected individuals as well as HIV-1 infected monocytoid lines (Lahdevirta et al 1988, Mintz et al 1989, Wright et al 1988, Roux-Lombard et al 1989, Poli et al 1990). This cytokine has previously been shown to stimulate both mitogen and antigen induced proliferation of T cells (Yokota Geppert & Lipsky 1989), while chronic exposure *in vitro* impairs activation through anti-CD3 (Cope et al 1994).

The viral glycoprotein gp120 is also implicated in a mechanism of immune stimulation in HIV-1 infection. Central to this is the dual ability of gp120 to bind CD4 (Dalgleish et al 1984, Klatzman et al 1984) and to mimic MHC (Young 1988, Kieber-Emmons Jameson & Morrow 1989). Gp120 bound to CD4 is likely to interfere with normal CD4/class II interactions (Habeshaw et al 1990). This occurs because gp120 binds to the region on the CD4 molecule that interacts with MHC (Lamarre et al 1989). This might result in inappropriate signalling or the interference of T cell recognition of class II. This is similar to the reaction with allogeneic class II, where subsequent antigen presentation to the TcR by class II is defective, leading to the induction of a chronic allogeneic response similar to experimentally induced graft versus host disease (GvHD; Habeshaw & Dalgleish 1989). In addition, as regions of gp120 have homology with class II, HIV-1 might mimic MHC allo determinants, interacting with and expanding alloreactive T cells again in an allogeneic response (Habeshaw & Dalgleish 1989). In fact, chronic GvHD shares many similarities with HIV-1 infection (reviewed in Ferrara & Deeg 1991) making this an attractive hypothesis.

The question remains as to why spontaneous and activation associated mechanisms of cell death exist. One reason may be that they provide a means of controlling or limiting potentially damaging cells. For example, if a virus is itself non-pathogenic, then the immune response may be more damaging to the host than the initial infection. If this response is effective in eliminating the virus then effector T cells should be cleared as soon as possible to limit damage to the host. The

problem then arises when the response is insufficient to clear the virus, which may home to some immunologically privileged site, and a chronic infection ensues that continues to elicit an immune response. One example of this was seen in mice given overwhelming infection with LCMV. A high dose of LCMV into immunocompetent mice led to a transient CTL response that disappeared, leading to a chronic infection and immunosuppression in the animals (Roost et al 1988). The reason for this appears to be that reactive T cells were expanded rapidly and died resulting in a form of clonal depletion such that no LCMV specific CTLp were left (Moskophidis et al 1993). This scenario seems unlikely in HIV-1 infection. Firstly, there is no evidence that primary infection involves high doses of HIV-1, although a viral superantigen might result in a similar outcome. Secondly, the control of viraemia following primary infection coincides with the appearance of the HIV-1 specific immune response (Pantaleo et al 1994) which, in the case of CTL, can be detected throughout the asymptomatic stage (Weinhold et al 1988, Hoffenbach et al 1989, Pantaleo et al 1990a) suggesting that at this level clonal deletion is not operating. The continual presence of antigen might however lead to a rapid turnover of cells which, if demand exceeded supply, could cause a form of clonal exhaustion.

Virus associated immune deficiency is not unique to HIV-1 infection but is also seen in other, particularly acute, viral infections. For example, it was described some time ago that individuals with acute measles infection did not develop DTH skin reactions against tuberculin (Razvi & Welsh 1994) and it is now clear that lymphocytes from measles infected humans and animals fail to proliferate to a range of stimuli including lectins, anti-CD3 and recall antigens (Rouse & Horohov 1986, Whittle et al 1978, Saron et al 1990). Similarly, during acute VZV or EBV infections, patients show decreased proliferative responses both to viral antigens (Cauda et al 1987) and mitogens (Perez-Blas et al 1992) and patients with chronic *Hepatitis B virus* infection show decreased IL-2 release to PHA or anti-CD3 (Anastassakos et al 1988). Also, during acute LCMV infection in the mouse T cells fail to proliferate or release IL-2 in response to Con A or anti-CD3 (Jacobs & Cole 1976, Saron et al 1990). This was recently found to be another example of AALD which could not be reversed by the addition of IL-2 (Razvi & Welsh 1993). We and others have also shown that T cells from acutely infected individuals are activated *in*

vivo and have lost Bcl-2 expression (Tamaru et al 1993, Akbar et al 1993). It seem likely therefore that the immune response to viral infections involves the expansion of T cells that are subsequently poised to undergo cell death upon TcR or mitogen stimulation.

The importance of cell death in AIDS pathogenesis

In this study we have clearly demonstrated the vulnerability of both $CD4^+$ and $CD8^+$ T cells from HIV-1 infected individuals and their propensity to die after culture *in vitro*, however, the question remains whether this *in vitro* phenomenon is relevant *in vivo*. The decline in $CD4^+$ T cells is the hallmark of AIDS but $CD8^+$ T lymphocytes do not decline and are in fact increased, suggesting that the death of both subsets *in vitro* does not occur *in vivo*. However, we have also demonstrated that the majority of $CD8^+$ T cells in the lymph node lack Bcl-2 expression and are thus poised for death. Also, there is evidence of high levels of apoptosis in HIV-1^+ lymph nodes at all stages of the disease (Ameisen 1994, Bofill et al submitted). Thus both $CD4^+$ and $CD8^+$ subsets are likely to be susceptible to cell death *in vivo* but only $CD4^+$ T cells decline. This might be envisaged if $CD4^+$ T cells encountered specific antigen more often or if the rate of replacement of $CD8^+$ cells was quicker than $CD4^+$. There is some evidence for the latter as after whole body irradiation of primates infected with SIV, $CD8^+$ T cells return rapidly to normal levels while there is prolonged depression of $CD4^+$ (Ameisen 1994). Also, HIV-1 infection of severe combined immunodeficient SCIDhu mice reconstituted with human foetal thymus selectively causes profound depletion of $CD4^+$ thymocytes (Bonyhadi et al 1993). This suggests a rapid turnover of $CD8^+$ T cells in HIV-1 infection that may be enhanced by antigen independent mechanisms. For instance, expansion of $CD8^+$ T cells outside the thymus has been described using IL-4 (Sieling et al 1993) or combinations of IL-2, IL-6 & TNF-α (Unutmaz et al 1994).

Does the death of $CD4^+$ and $CD8^+$ T cells directly contribute to AIDS pathogenesis? To answer this question it is perhaps useful to refer to primate models of pathogenic or non-pathogenic lentiviral infections. Rhesus macaques can be experimentally infected with viral strains of SIV*mac* that induce AIDS or viral recombinant molecular clones of SIV that do not cause disease (Desrosiers 1990,

Kestler et al 1990). Also, chimpanzees infected with HIV-1 do not develop AIDS although they can be infected with the virus (Johnson et al 1993). In both of these models, abnormal activation induced cell death of $CD4^+$ T cells was observed only in the SIV model leading to AIDS while death within the $CD8^+$ T cells was seen in both SIV models (Estaquier et al 1994). Thus, cell death involving $CD4^+$ T cells is more closely related to AIDS pathogenesis than death in the $CD8^+$ population which may be an indirect consequence of immune stimulation. Nevertheless, activated $CD8^+$ T cells may themselves contribute to the pathogenesis of the disease. The number of $CD8^+$ T cells with a primed phenotype is greatly increased in the lymph node during HIV-1 infection. As well as containing TIA-1^+ cytolytic granules, these $CD8^+$ T cells also contain mRNA for IFN-γ (Emilie et al 1990, Emilie et al 1994) which is found in increased levels in the serum of HIV-1 infected patients (Fuchs et al 1989, Canessa et al 1992). It has been shown that excess IFN-γ induces apoptosis in T cells (Liu et al 1990) and medullary thymocytes (Groux et al 1993) indicating that this cytokine might contribute to pathogenesis. In addition, although we were unable to demonstrate perforin with in the $CD8^+$ T cells in the lymph node, other investigators using *in situ* hybridisation techniques have found abundant perforin mRNA in HIV-1^+ lymph nodes (Devergne et al 1991, Emilie et al 1994). Thus it is possible that HIV-1 antigens on the FDC attract and stimulate HIV-1 specific CTL that then destroy the FDC and contribute to follicular lysis. This has a parallel in mice made immunodeficient through infection with LCMV. Immunosuppression in these animals correlates with the finding that LCMV, like HIV-1, can replicate within macrophages and APC (Odermatt et al 1991, Mimms & Tosolini 1969) that are not destroyed by the virus but rather by LCMV specific $CD8^+$ T cells. In this infection, removal of $CD8^+$ T cells prevents both the pathology and the immune suppression despite virus persistence (Leist Reudi & Zinkernagel 1988, Odermatt et al 1991). Lymph nodes from these animals show histological changes very similar to those seen in HIV-1 infection (Odermatt et al 1991) suggesting that the lysis of HIV-1 infected APC by $CD8^+$ CTL could be crucial in AIDS pathogenesis. In support of this is the observation that chimpanzees infected with HIV-1 do not have monocytotropic variants of the virus and do not get AIDS.

Additionally, $CD8^+$ T cells may contribute to AIDS through the lysis of

autologous, uninfected $CD4^+$ T cells and there is indeed evidence that $CD8^+$ T cells are able to kill autologous $CD4^+$ T cells in an unrestricted fashion (Grant Smail & Rosenthal 1994). Finally, the extent of AALD in $CD8^+$ T cells from HIV-1 infected individuals is in itself evidence of its importance, indicating that it might lead to disease by affecting the maintenance of immunological memory and the renewal of effector T cells (Ameisen & Capron 1991).

Conclusions and additional studies

(i) After short term culture *in vitro* T lymphocytes from HIV-1^+ individuals die and this cell death provides a possible mechanism for the immunodeficiency seen in HIV-1 infection.

(ii) There are two forms of cell death; a spontaneous death that occurs both in chronic HIV-1 infection and during acute viral infections and an activation induced death that occurs only in HIV-1 infection.

(iii) Spontaneous cell death occurs by apoptosis in Bcl-2^- T cells and can be prevented by exogenous IL-2. Thus it resembles cell death due to growth factor deprivation.

(iv) AALD is not restricted to Bcl-2^- T cells but occurs in $CD45RO^+$ and $CD28^-$ lymphocytes and cannot be prevented by IL-2. Thus it resembles forms of cell death seen in mature T cell hybridomas or previously activated cells.

(v) The absence of CD28 suggests that the lack of a second signal during activation could cause cell death.

(vi) The cells that die are highly cytolytic indicating that they may be activated *in vivo*.

(vii) The $CD8^+$ T cells that infiltrate the lymph nodes in HIV-1^+ patients have low Bcl-2 expression and also contain TIA-1. These may represent an activated population that selectively homes to lymphoid tissue where it may contribute to AIDS pathogenesis.

This project has raised a number of important issues that merit further investigation:

(i) Although AALD was not limited to Bcl-2$^-$ T cells, the role of the *bcl-2*-related genes, *bcl-X and bax* as well as other death genes eg *cmyc*, *p53* or *fas* is unknown and it would certainly be of interest to more fully examine the death pathway.

(ii) As certain cytokines, particularly IL-12, have been shown to modulate proliferative responses in HIV-1 infected individuals, it would be important to test its effect on AALD.

(iii) A number of recent studies have noted that activation induced cell death occurs in HIV-1 infection and that this could be of great importance in HIV-1 disease. However, in such studies both spontaneous death and AALD are occurring and it may be important to distinguish between the phenomena which may have different pathogenic roles. A study of Bcl-2 expression versus AALD might be useful to dissect both phenomena.

(iv) Although we were able to show that CD28$^-$ T cells died, it is possible that co-stimulation through other molecules might save the cells. For instance it would be interesting to investigate the expression and co-stimulatory function of CD5 and CD6 in HIV-1 infection.

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APPENDIX I

ABBREVIATIONS

AALD	Activation Associated Lymphocyte Death
AIDS	Acquired Immune Deficiency Syndrome
ARC	AIDS Related Complex
AVI	Acute Viral Infections
CD	Cluster of Differentiation
CDC	Center for Disease Control
CMV	Cytomegalovirus
Con A	Concanavalin A
CTL	Cytotoxic Lymphocytes
EBV	Epstein Barr Virus
FCS	Foetal Calf Serum
FITC	Fluoresceine Isothiocyanate
FSC	Forward Scatter
GAM	Goat anti-mouse
GC	Germinal Centre
HIV-1	Human Immunodeficiency Virus Type I
HLA	Human Lymphocyte Antigen
IF	Immunofluorescence
IL-2	Interleukin-2
IL-2R	Interleukin-2 receptor
LAK	Lymphokine Activated Killer
LDCC	Lectin Dependent Cellular Cytotoxicity
LGL	Large Granular Lymphocyte
LN	Lymph Node
LR	Lissamine Rhodamine
LCMV	Lymphocytic Choriomeningitis Virus
MFI	Mean Fluorescence Intensity
MPC	Molecules Per Cell
NK	Natural Killer

PBMC	Peripheral Blood Mononuclear Cells
PC	Paracortex
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PGL	Persistent Generalised Lymphadenopathy
PHA	Phytohaemagglutinin
PMA	Phorbol myristate acetate
PWM	Pokeweed Mitogen
SEB	Staphylococcus enterotoxin B
SSC	Side Scatter
Strep	Streptavidin
TcR	T cell Receptor
TH	T Helper
VZV	Varicella Zoster Virus

APPENDIX II

PUBLICATIONS

Lymphocyte activation in HIV-1 infection. I. Predominant proliferative defects among CD45R0+ cells of the CD4 and CD8 lineages

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Objectives and design: The proliferative defects of CD4 and CD8 cells taken from 474 HIV-1-seropositive individuals during various stages of disease were quantitated. Phytohaemagglutinin (PHA) and soluble anti-CD3 were used in optimal mitogenic concentrations in the presence of recombinant interleukin-2 (rIL-2) and conditioned medium, and the proliferation of cells from HIV-1-seropositive donors was assessed in co-culture with HIV-1-seronegative cells in order to exclude effects of cytokine deficiency. Defects within the CD45RA+ ('unprimed') and CD45R0+ ('primed') T-cell populations were also investigated.

Methods: Quantitative immunofluorescence and double and triple labelling in flow cytometry were performed for (1) CD25 (IL-2 receptor α chain) expression, (2) lymphocyte and T-cell survival, and (3) blast transformation and proliferation — in relation to the original input of cells for each subpopulation.

Results: T cells from normal and HIV-1-seropositive donors were CD25+ at day 1. In HIV-1-seropositive patients a variable number of CD4 and CD8 lymphocytes failed to further increase CD25, and died as a sign of activation-associated lymphocyte death (AALD). Forty-two per cent of asymptomatic subjects, including 32% of those with CD4 cell counts $> 400 \times 10^6$/l, showed a poor blast transformation (< 30% blasts). Cells from HIV-1-seropositive donors showed poor blast responses when co-cultured with HIV-1-seronegative cells; both CD4 and CD8 cells were handicapped. In asymptomatic HIV-1-seropositive people T cells with the CD45R0+ RA– ('primed') phenotype were three to five times more vulnerable to AALD than the CD45RA+ RO– ('unprimed') cells. In patients in Centers for Disease Control and Prevention (CDC) disease stage IV both CD45R0+ and -RA+ populations were severely affected.

Conclusions: This is the first quantitative analysis to demonstrate that in HIV-1 infection mitogen-stimulated CD45R0+ ('primed') T cells preferentially die upon activation. Both the CD4 and CD8 lineages are affected, as seen in animal models of graft versus host disease. AALD may explain defects of immunological memory. The analysis of AALD may be a suitable assay for studying whether antiviral drugs influence the proliferative responses of lymphocytes.

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Keywords: Immunodeficiency in HIV infection, AIDS, lymphocyte transformation, interleukin-2 receptor, mitogens, immunological memory, CD4, CD8.

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Introduction

Studies with a variety of stimuli, including specific antigens [1–5] and mitogens such as pokeweed (PWM) [3,5–8], phytohaemagglutinin (PHA) [3,7,9–11] and anti-CD3 [9,12,13], have shown that the defects of proliferative responses in asymptomatic HIV-1-seropositive individuals can vary, but are frequently severe even before blood CD4 levels fall below 400×10^6/l. These studies mostly assessed proliferative responses in bulk assays such as thymidine uptake, but some also quantitated the numbers of responding cells undergoing blast transformation and expressing interleukin-2 (IL-2) receptor α in response to different stimuli [3,10,14]. It appears that T cells from HIV-1-seropositive individuals are more defective than would be expected on the basis of the number of cells containing HIV-1 provirus [10,12,15]. Furthermore, double- and triple-colour flow cytometry [16] studies reveal that both CD4 and CD8 populations exhibit poor mitogen responsiveness in HIV-1 infection [13] and that these defects remain when CD4 and CD8 cells are co-cultured [10,12].

We wished to extend these investigations in three aspects. First, our objective was to precisely quantitate responses by absolute cell counting in a large HIV-1-seropositive cohort following optimal stimulation with PHA and anti-CD3 antibodies in the presence of recombinant (r) IL-2 and conditioned medium, in order to estimate the importance of intrinsic T-cell defects. This is a complementary approach to investigating cell populations in isolation when cytokine deficiency or lack of helper effects also contribute to poor function [13,17,18]. Culture conditions were further optimized by stimulating CD4 and CD8 cells from HIV-1-seropositive asymptomatic individuals together with HIV-1-seronegative normal lymphocytes in mixed cultures.

Second, we wished to document the time-course of the development of cellular defects during the activation cycle. To achieve this, the sequential events studied were IL-2 receptor α (IL-2Rα) expression, blast transformation, DNA synthesis, and the absolute numbers of surviving blast cells and dividing cells [19–21].

Third, the features of normal 'unprimed' ('naive') and 'primed' ('memory') T cells have recently been clarified [20,21]. 'Unprimed' cells exhibit CD45RA, the higher molecular weight isoforms of CD45 antigens, while 'primed' T cells exhibit CD45R0, the lower molecular weight isoform [20–23]. We assessed the performance of 'unprimed' and 'primed' T-cell populations in cultures taken from HIV-1-seropositive individuals. This is an area of acute interest in the pathology of HIV-1 infection. Recall responses to soluble antigens, a feature of 'primed' T cells, are damaged particularly early during HIV-1 infection [1–5], leading to hypotheses about preferential defects of memory T cells [2,24–26]. Nevertheless, previous attempts to substantiate this idea by phenotypic analysis, using CD29 antibodies [24,26–28] that react with only subsets of CD45R0+-activated 'primed' populations [29], have been inconclusive.

We found that under optimal activation for both 'unprimed' (CD45RA+) and 'primed' (CD45R0+) T cells, large proportions of lymphocytes from asymptomatic HIV-1-seropositive subjects undergo an abortive process of activation-associated lymphocyte death (AALD), and that this outcome cannot be reversed by rIL-2 or by co-culturing the cells with normal HIV-1-seronegative lymphocytes. In cultures from HIV-1-seropositive donors AALD occurs in both CD4 and CD8 cells, and preferentially in the CD45R0+ population. Thus, in early stages of HIV-1 infection, the most remarkable finding is not so much the absence of memory cells, as suggested on the basis of CD29 expression [24–28], but the defective activation of 'primed' CD45R0+ lymphocytes. In the development of AIDS, however, all T-lymphoid lineages are compromised and only a few residual T cells proliferate normally, in agreement with previous data on defective T colony-forming cells [12,30,31].

Subjects and methods

Subjects

An HIV-1-seropositive cohort (474 individuals including 350 homosexual men, 74 haemophiliacs and 50 other individuals) was grouped according to absolute CD4 counts [32]. Only one mid-course value per individual was included. The 77 HIV-1-seronegative controls included 30 homosexual men [33], seven women attending a clinic for sexually transmitted diseases, 30 haemophilics and 10 volunteers. An overlapping cohort (215 HIV-1-seropositive individuals) was grouped by the Centers for Disease Control and Prevention (CDC) disease classification [34] and compared with 57 HIV-1-seronegative controls.

Tests for HIV-1 antibody and antigen

Wellcozyme HIV recombinant assay (Wellcome Diagnostics, Dartford, England, UK), HIV-1 enzyme immunoassay (EIA; Abbott Diagnostics, Maidenhead, England, UK) and Serodia HIV particle agglutination test (Mast Diagnostics, Bootle, England, UK), were performed by the Virology Department of the Royal Free Hospital (London, UK) to establish serological status. HIV p24 antigen was detected by enzyme-linked immunosorbent assay (ELISA) in culture supernatants (DuPont UK, Stevenage, England, UK), and by immunofluorescence (IF) in the cytoplasm of mononuclear cells. Smears were air-dried, fixed for 30 min in acetone and incubated with 1 μg/ml anti-p24 antibody (Dako Ltd, High Wycombe, England, UK), followed by affinity-purified goat anti-mouse immunoglobulin (Ig) fluores-

cein isothiocyanate (FITC; Southern Biotechnology Associates, Birmingham, Alabama, USA). HIV-1-infected line C2688 was the positive control. The phenotype of p24+ cells was detected using phycoerythrin (PE)-labelled anti-CD4 (clone RFT4) and anti-CD8 (clone RFT8).

Lymphocyte purification

Mononuclear cells obtained on Ficoll–Hypaque from heparinized venous blood were rosetted by sheep red blood cells into T- and natural killer (NK) cell-rich (E+, CD2+) and non-T (B plus monocytic) fractions [21]. CD4 or CD8-rich and CD45RA– and CD45R0-rich populations were prepared from this E+ fraction by negative selection on sheep anti-mouse Ig-coupled magnetic beads (Dynabeads; Dynal Ltd, New Ferry, England, UK). The subsets were 92–98% positive for CD4, CD8, CD45RA and CD45R0 [35].

Lymphocyte stimulation

Mononuclear cells obtained from heparinized venous blood within 1–14 h of withdrawal were incubated as triplicates of 200 µl cultures in 96-well microplates or in flat-bottomed vials of 1.2 ml volume [21]. The initial lymphocyte number was adjusted to 1×10^6/l in RPMI-1640 with 10% fetal calf serum (FCS), 30% conditioned medium, and 1 ng/ml of rIL-2 and antibiotics. Since sera from patients with AIDS can inhibit lymphoblast transformation [36], and in our preliminary studies suppressive serum factors were occasionally identified in HIV-1-seropositive patients with low responses, autologous sera were avoided. FCS was selected on the basis of optimal support for mitogenic and low background stimulation. Mitogenicity was similar in cultures containing 10% FCS or 10% autologous serum.

The two mitogens used were PHA-P (used at 1 µg/ml optimal concentration; Wellcome Reagents Ltd, Beckenham, England, UK) and anti-CD3 (used at 0.5–2 µg/ml; UCHT1 or OKT3, American Type Culture Collection, Rockville, Maryland, USA) [37]. Thymidine incorporation (TdR uptake) was measured at 68–72 h using ^{3}H-thymidine of intermediate specific activity (^{3}H-TdR; Amersham International, Amersham, England, UK). Cultures were harvested at different times for phenotypic analysis.

Assessment of lymphocyte activation with flow cytometry

Lymphoblasts were identified by forward- (FSC) and side-scatter (SSC) (Fig. 1) on FACScan (Becton Dickinson, Oxford, England, UK). Beads (7.2 µm; FCSC Standards, Becton Dickinson), were added to a known concentration (5×10^5/ml) in order to facilitate cell counting in a fixed culture volume. The blast transformation or blastogenesis was evaluated in two ways. First, after 72 h of activation with mitogens the blast counts were expressed as percentages of the initial 1×10^6 lymphocytes/ml. This is referred to as total lymphocyte performance (Figs 1 and 2). Individuals were grouped as normal (> 70%), poor (30–70%) and very poor (< 30%) performers. In the second set of experiments T-blast subset counts were expressed as percentages of the corresponding starting T-cell or T-subset concentrations. This test is referred to as T (subset) performance (Figs 3 and 4). A Cytoron-Absolute counter (Ortho Diagnostics, High Wycombe, England, UK) that obtains blast counts in fixed volumes of medium without a need for beads has recently been introduced (Fig.1).

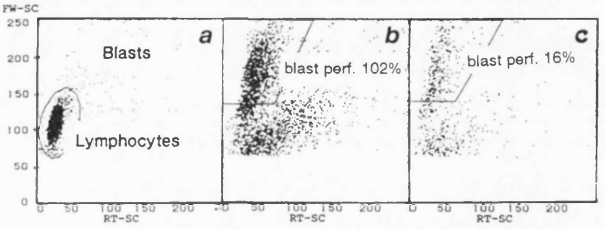

Fig. 1. Setting of gates ('blasts') on Cytoron for analysing the numbers of blasts among fresh lymphocytes (a) and in cultures stimulated with phytohaemagglutinin (PHA) for 3 days (b, c) from HIV-1-seronegative donors (a, b) and an HIV-1-seropositive patient (c). The use of an absolute counter can facilitate the counting of a given volume (0.1 ml) of culture, and the 'blast'/ml value is determined. This is divided by the lymphocyte input (10^6/ml) to give the lymphocyte performance: 102% blasts/input in the HIV-1-seronegative (b) and 16% in the HIV-1-seropositive sample (c).

Double and triple labelling was performed [16] with antibodies conjugated to FITC, PE and biotin plus Streptavidin TXRD–PE TANDEM (Southern Biotechnology Associates, Birmingham, Alabama, USA), or streptavidin-Tricolor (CALTAG; Bradsure Biological Ltd, Market Harborough, England, UK). The antibodies were CD4–PE (clone RFT4; [32]), CD8–PE, CD8–FITC and CD8–biotin (clone RFT8; [32]), CD3–FITC (clones OKT3, MEM-57 and UCHT1; [37,38]), CD45RA–FITC and CD45RA–PE (clone Sm130; [21]), CD45R0–biotin (clone UCHL1; [21]) and CD25–FITC (clone RFT5; [39]).

Experiments mixing mononuclear cells from normal HIV-1-seronegative and asymptomatic HIV-1-seropositive patients differed from previous experiments [40] in three aspects: (1) the donors were histo-incompatible for HLA-A2 to distinguish proliferating cells from normal and HIV-1-seropositive individuals; the powerful mitogenity of PHA and anti-CD3 masked any alloproliferation; (2) conditioned medium and rIL-2 were added at the start of culture; (3) asymptomatic individuals in CDC stage II, rather than patients with AIDS [40], were studied. The surviving cells from HIV-1-seropositive individuals were identified in cultures containing admixed cells from HLA-A2-disparate normal donors using double IF (anti-HLA-A2-biotin plus streptavidin-FITC with either CD4–PE or CD8–PE) and triple analysis (CD4–PE/CD8–FITC/HLA-A2–biotin). Anti-HLA-A2 antibody (clone NA2.1) was kindly donated by Prof. A. McMichael (John Radcliffe Hospial, Oxford, England, UK). The total num-

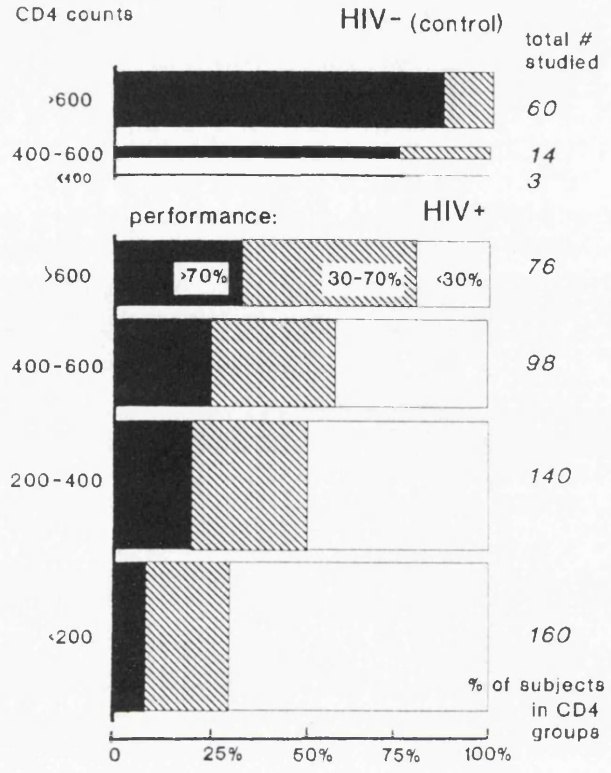

Fig. 2. Lymphocyte performance in phytohaemagglutinin-stimulated cultures from 77 HIV-1-seronegative and 475 HIV-1-seropositive donors. The percentages are blasts/lymphocyte input as shown in Fig. 1: > 70% (■), 30–70% (▨) and < 30% (□). The subjects are grouped by CD4 cell count in blood (> 600, 400–600, 200–400 and < 200 × 10^6/l). The width of bars is proportional to the number of people tested in each group (shown on the right).

bers of T cells in the anti-CD3-stimulated cultures were determined by a mix of CD5–PE and CD7–PE [38].

The amounts of (IL-2Rα) expressed on T cells after varying periods of stimulation were determined in a semiquantitative assay [41]. Cells were incubated with saturated amounts of CD25 antibody (10 µl culture supernatant per 10^6 cells; clone RFT5 reactive with IL-2Rα chain (55 kD [39]) and stained with affinity-purified and FITC-labelled goat anti-mouse Ig (AP–GaM–FITC), applied at 1 µg/ml saturating concentration. Microbeads coated with known amounts of mouse IgG, such as 0, 10, 40, 80, 400 and 1000 × 10^3 molecules per cell (m.p.c.), were stained with AP–GaM–FITC to construct a standard curve for mean fluorescence intensity (MFI) on FACScan at log setting, in order to estimate CD25 antigen expression on activated lymphocytes above 5 × 10^3 m.p.c. (autofluorescence interferes with quantitation below this value). We determined the percentage positivity of CD25+ cells, followed by the MFI values on the gated CD25+ cells.

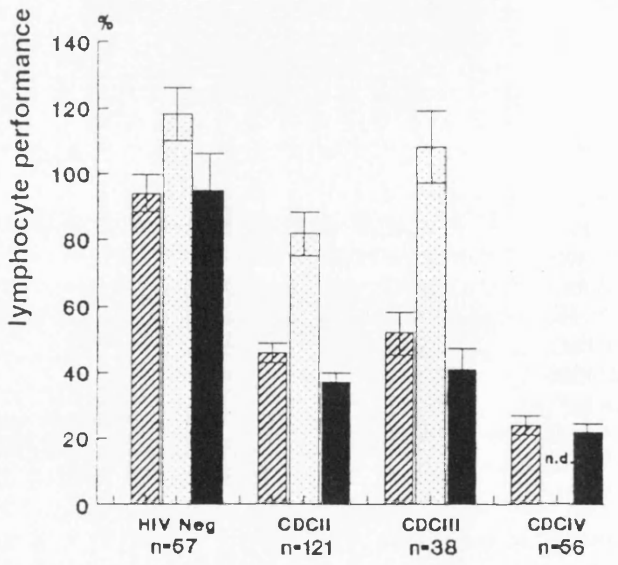

Fig. 3. T-cell subset performance in phytohaemagglutinin-stimulated cultures from 57 HIV-1-seronegative and 215 HIV-1-seropositive donors. The percentages are blasts/CD3 T-cell input (▨), CD4 blasts/CD4 lymphocyte input (□) and CD8 blasts/CD8 lymphocyte input (■) assessed within Centers for Disease Control and Prevention (CDC) disease stages II, III and IV, as shown. The bars are means ± s.e.m.; n.d., not determined because there were too few CD4+ cells to read.

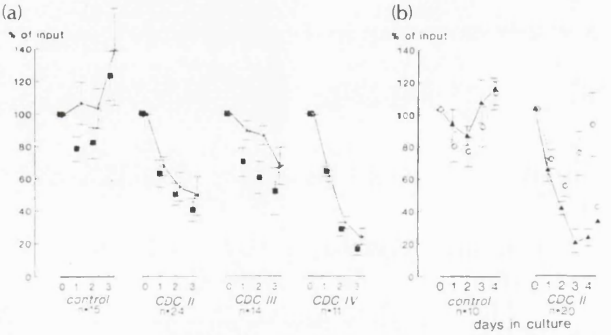

Fig. 4. Time-course of the numbers of CD4 (*) and CD8 (■) cells recovered from phytohaemagglutinin (PHA)-stimulated cultures per the number of CD4 or CD8 cells placed into the culture at day 0. (b) Time-course of CD45RA (○) and CD45R0 (▲) recovery per the numbers of RA or RO cells placed into culture. The cultures were stimulated with PHA for varying periods as shown. All viable cells were counted and the numbers of people tested in each Centers for Disease Control and Prevention (CDC) stage are indicated.

Assessment of lymphocyte activation by microscopy

DNA synthetic blasts were observed by adding bromodeoxyuridine (BrdU) to the cultures for 4 h before harvesting and staining the BrdU+ cells in smears [19]. Cytochalasin-B (5 µg/ml; Sigma, London, UK) was added to cultures from 48 h onwards in order to determine the number of proliferating cells [20]. After 72–84 h of activation some T-blasts, also referred to as T-colony-forming cells [30,31], start to proliferate rapidly. These cells form multinucleated blasts, when arrested by cytochalasin-B. These were counted

in smears after incubation with anti-CD4 and anti-CD8 followed by rabbit anti-mouse Ig peroxidase and cytochemical development.

Statistics

Hypothesis tests for comparison between groups were performed using unpaired t-test. All P values shown are two-sided. A Pearson correlation coefficient was used to assess the correlation between continuous measures. The error bars refer to ± 1 s.e.m.

Results

Lymphocyte activation by PHA and anti-CD3

In cultures stimulated by PHA and conditioned medium T cells expressed CD25 on day 1 (23–33 h), underwent blast transformation on day 2 (31–54 h) and initiated DNA synthesis a few hours later (50–82 h). Cultures activated by anti-CD3 were 8–14 h slower and the ensuing proliferation was asynchronous. The first mitoses were seen by 68–72 h, and other blasts first divided much later (> 120 h). In control cultures a modest increase in cell numbers was first detectable by 72–96 h.

There was a severe but variable drop in the numbers of recovered lymphoid cells in PHA and anti-CD3-stimulated cultures from HIV-1-seropositives: by day 3 less than 50% of input could be recovered (Table 1). Although the correlation between blast transformation and TdR uptake was good (r = 0.92), the former discriminated better between values from HIV-1-seronegative control and HIV-1-seropositive cultures (E. Medina and M. Bofill, unpublished data).

Table 1. Survival of lymphoid cells from HIV-1-seropositive subjects in cultures incubated with mitogen*.

	No. subjects	Mean ± s.e.m. PHA	Mean ± s.e.m. Anti-CD3
Control	20	97.2 ± 4.2	82.7 ± 4.5
CDC stage II/III	24	45.8 ± 3.9	39.9 ± 3.0
CDC stage IV	16	24.2 ± 6.2	14.8 ± 5.5

*Recovery of the total number of cells ($\times 10^4$/ml) including activated lymphoblasts in cultures stimulated for 72 h with the mitogen shown plus conditional medium and recombinant interleukin-2 (rIL-2). Control cultures contained rIL-2 but no mitogens were added. The original lymphocyte input at 0 h was 100 × 10^4/ml. PHA, phytohaemagglutinin; CDC, Centers for Disease Control and Prevention.

The blast cell responses in PHA and anti-CD3-stimulated cultures from 40 HIV-1-seropositive and 20 normal individuals were compared. At day 3 in the HIV-1-seropositive group the blastogenesis was lower than in the controls (33.5 versus 90.0%, $P < 0.001$), irrespective of the mitogen used. The higher numbers of blasts seen in PHA versus anti-CD3 cultures in the HIV-1-seronegative group (97.2 ± 4.2 and 82.8 ± 4.5), the HIV-1-seropositive CDC stage II (45.8 ± 3.9 and 39.9 ± 3.0) and CDC stage IV groups (24.2 ± 6.2 and 14.8 ± 5.5, respectively) indicate that PHA is a slightly stronger mitogen than soluble anti-CD3, even in the presence of conditioned medium and rIL-2. Nevertheless, in each individual the mitogenicity of the two agents yielded concordant results (r = 0.89; $P < 0.0001$).

Only one of the 50 (2%) HIV-1-seropositive patients studied at the start of the cultures had p24+ cells (0.08% of cells) in the smears analysed. At day 3 in PHA cultures 37% contained 0.02–1.1% p24+ blast cells. Seventy per cent of cultures showed 2–20% p24+ cells when propagated for > 6 weeks. On two occasions these included CD8 blasts. The numbers of actively HIV-1-synthetic cells in our cultures are therefore in line with the low numbers of cells positive for HIV-1 provirus in the blood of HIV-1-seropositive patients studied by sensitive quantitative polymerase chain reaction (PCR) [15].

Lymphocyte proliferative defects in asymptomatic HIV-1-seropositive patients

Blastogenesis in cultures stimulated with PHA for 3 days was related to the original lymphocyte input (Fig. 1). Results were grouped according to the CD4 cell counts (Fig. 2). The HIV-1-seropositive subjects (74 haemophiliacs, 350 homosexuals and 50 others) were compared with 77 HIV-1-seronegative controls. HIV-1-seropositive patients with normal (> 600 × 10^6/l) or near normal (400–600 × 10^6/l) counts CD4+ cells showed a significantly poorer performance than HIV-1-seronegative control cultures with similar CD4 cell counts ($P < 0.001$). Only 31% of the HIV-1-seropositive group with a CD4 cell count > 600 × 10^6/l showed a good performance, while 21% showed a very poor (< 30%) performance. Eighty-two per cent of HIV-1-seronegative controls showed a performance > 70%. There was a significant further deterioration of blastogenesis with decreasing (> 600, 200–600 and < 200 × 10^6/l) CD4 cell counts in HIV-1-seropositive patients. The values between the 400–600 and 200–400 × 10^6/l groups were, however, not significantly different: the former group already had a low functional status with 50% of patients revealing < 30% performance (Fig. 2).

Since PHA and anti-CD3 are primarily T-cell mitogens, the blastogenesis was also expressed per original T-cell input (Fig. 3). Again, the poor blast transformation was confirmed in asymptomatic patients (CDC stage II; 121 subjects), as well as in the group with persistent generalized lymphadenopathy (PGL; CDC stage III; 38 subjects); the mean T-cell blastogenesis was 45 and 51%, respectively, with a further decrease in CDC stage IV to 26% (56 patients; Fig. 3). The corresponding mean normal values for blasts per T-cell input in 57 HIV-1-seronegative controls were approximately 95%.

Both CD4 and CD8 cells perform poorly

The time course was investigated in anti-CD3-stimulated cultures by analysing the recovery of CD4 and CD8 subsets in 64 subjects (Fig. 4a). In 15 controls both CD4 and CD8 cells survived well, with a 20% drop in CD8 count at days 1–2; by day 3 the counts started to increase. In contrast, in CDC stage II (24 subjects) both CD4 and CD8 populations had declined to 50% by day 2. CD8 counts appeared to decline more than CD4 counts in CDC stage III (14 subjects), but there was wide individual variation. In the CDC stage IV (11 subjects) both subsets declined rapidly to < 30% recovery.

The blastogenesis among CD4 and CD8 T cells during a 3-day PHA stimulation was measured by selective gating and by relating the blast counts to the corresponding input of CD4 or CD8 cells at the start of culture (Fig. 3). Both lineages were affected in HIV-1-seropositive donors, but CD8+ cells appeared to be more seriously damaged. In the HIV-1-seronegative control cultures the PHA-stimulated CD4+ blast values at day 3 were 118% of the CD4 input. The HIV-1-seropositive CDC stage II yielded significantly lower CD4 blast counts per CD4 input (78%; $P < 0.01$), while the PGL group (CDC stage III) still had near-normal values (103%), indicating that the remaining CD4 cells transformed fairly well. At the same time CD8 blastogenesis dropped from the 95% control values to as low as 37 and 40% in both CDC stage II and III, respectively. Blastogenesis in the CDC stage IV was approximately 18–20%, reflecting the poor performance of the CD8+ cells, which predominate in these patients.

Poor lymphocyte performance in the presence of normal CD4+ cells

The powerful mitogenicity of PHA and anti-CD3, which activates T cells, masks allogeneic mixed lymphocyte reaction, which stimulates only 3–7% of lymphocytes. For this reason blood mononuclear cells from paired HLA-A2 histo-incompatible normal donors and HIV-1-seropositive patients were mixed in equal numbers and stimulated with PHA or anti-CD3 for varying times. Six donors and 12 asymptomatic HIV-1-seropositive individuals were analysed in 15 paired experiments (Table 2). In a representative experiment shown in Fig. 5, the HLA-A2+ cells were from an HIV-1-seronegative donor, and the HLA-A2– cells from an asymptomatic HIV-1-seropositive donor. In the unstimulated cultures the HLA-A2+/A2– ratio remained approximately 55% throughout the 5-day culture (data not shown). In PHA-stimulated cultures the initially balanced ratio changed: within 3–5 days the A2– (HIV-1-seropositive) contribution declined to 27 and 18%, respectively. At day 3 the A2– decline was attributable to cell death, since the bulk proliferation of A2+ cells had not yet started. There was no indication that the cells in HIV-1-seropositive donors significantly suppressed the proliferation of control blasts at any time during the 3–5 day culture period (Table 2).

These experiments also confirmed the demise of the CD8 populations in HIV-1-seropositive donors (Fig. 4a). Because of different CD4:CD8 ratios in the blood samples, the CD8 cell input from the HIV-1-seropositive (A2–) subject was higher (68%) than that from the HIV-1-seronegative (A2+) donor (32%). However, during the 5-day culture, A2– CD8+ cells declined to 23%, whereas the proportion of A2+ CD8+ cells increased to 77%. These population shifts reflect the cell death of the HIV-1-seropositive and the proliferation of the HIV-1-seronegative populations within the same tissue culture environment during the 5-day culture period.

Table 2. Quantitation of mitogen responses in mixed cultures of HLA-A2 histo-incompatible cells from HIV-1-seronegative controls and HIV-1- seropositive donors*.

Mitogen	No. subjects	CD4 count ($\times 10^6$/l) of HIV-1+ donors	Mean ± s.e.m. HIV-1–	HIV-1+	Mixed† Expected	Found
Recovery at day 3						
Phytohaemagglutinin	4	200–400	115 ± 15	60.7 ± 14	88 ± 11	81 ± 21
	2	117–200	122 ± 130	51 ± 57	86 ± 94	90 ± 80
Anti-CD3	5	200–400	99.9 ± 10.1	53 ± 19	76 ± 14	71 ± 12
	4	127–200	107.1 ± 7.1	41 ± 7.9	74 ± 7.3	61 ± 10
Recovery at day 5						
Anti-CD3	5	200–400	150 ± 6.1	70.2 ± 14‡	110 ± 11	91 ± 21
	4	127–200	161 ± 17	41.1 ± 8.8‡	102 ± 13	91 ± 12

*HIV-1-seropositive donors were asymptomatic (Centers for Disease Control and Prevention stage II). Cell suspensions of 10^6 lymphocytes/ml from HIV-1-seronegative and HIV-1-seropositive populations were mixed in equal numbers. The T-cell proportions were 70%, and the T (CD5 + CD7), CD4 and CD8 cells were followed from each donor by double or triple staining with anti-HLA-A2 (see Subjects and methods). †The expected values are the sums of 50% counts of the pairs grown separately at 10^6/ml starting concentration. Differences between expected and observed values are not significant ($P = 0.15$). ‡The HLA-A2 labelling showed that cells from HIV-1-seronegative donors declined in numbers (see Fig. 5).

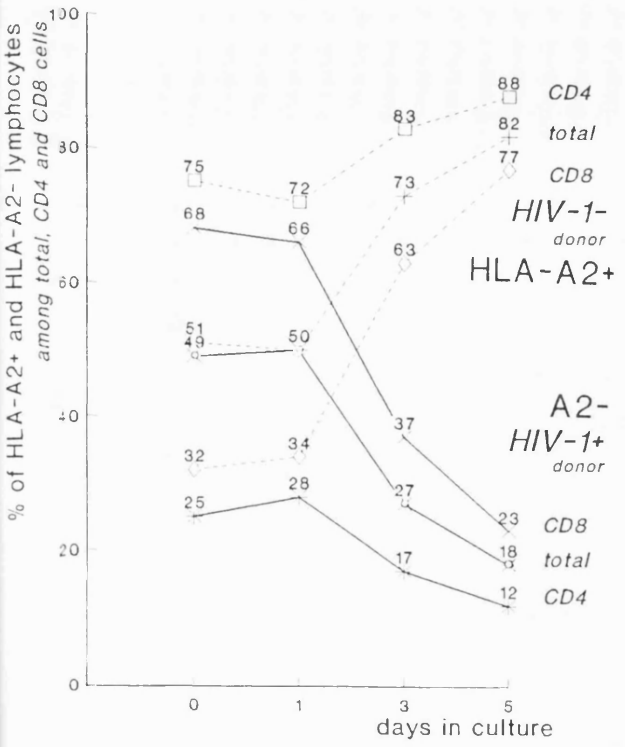

Fig. 5. Representative mixing experiment using HLA-A2 histoincompatible lymphocytes from an HIV-1-seropositive donor (HLA-A2−) and an HIV-1-seronegative donor (HLA-A2+) set up in equal numbers (50%) and incubated in the presence of phytohaemagglutinin, recombinant interleukin-2 and conditioned medium for various periods, as shown. The percentage values of HLA-A2+ (□, +, △) and HLA-A2− cells (X, ○, ·) within the CD3 (+, ○), CD4 (□, ·) and CD8 (△, X) populations are shown. In addition to the shifts seen in cell proportions the absolute cell numbers also increased between days 3–5, mainly due to the proliferation of the then dominant HLA-A2+ cells (not shown).

CD45R0+ cells from HIV-1-seropositive donors proliferate poorly

Two types of experiments documented the different behaviour of PHA-stimulated CD45RA+ ('unprimed') and CD45R0+ ('primed') populations obtained from HIV-1-seronegative controls and HIV-1-seropositive individuals. First, purified CD45RA+ or CD45R0+ lymphocytes were admixed to 10% E rosette-negative non-T cells and stimulated by PHA in the presence of rIL-2 and conditioned medium for varying periods (Fig. 4b). Cell recovery was high (80%) in the cultures of CD45RA+ and RO+ from HIV-1-seronegative donors. However, when taken from HIV-1-seropositive asymptomatic donors the PHA-stimulated cultures of CD45RA+ and CD45R0+ cells behaved differently. By day 2 the proportion of CD45RA+ cells had decreased to 55%, but by day 4 it rapidly recovered to 90%. These cells were actively proliferating, as documented by the multinucleated blasts present in cytochalasin-B cultures. In contrast, by day 2 many CD45R0+ cells had died and the recovery by days 3–4 was as low as 20% (Fig. 4b). In cytochalasin-B cultures only occasional blast cells formed multinucleated blasts.

Second, the generation of CD45RA+ and -RO+ lymphoblasts was measured in unseparated lymphocyte cultures during PHA stimulation (Table 3). During such activation CD45RA+ blasts unidirectionally switch to CD45R0 as a sign of 'priming' [21], but at day 3 still express residual CD45RA on their membranes, since they have only just started to display CD45R0 (RA+ RO±). At the same time, activated CD45R0+ blasts remain CD45RA− (RO+ RA− [21]). Thus the proportion of RA+ RO± blasts was related to the original input of RA+ 'unprimed' cells, and those of RO+ RA− blasts were shown as the percentage of the RO+ 'primed' lymphocyte input. RA+ RO± blasts showed a high blastogenesis (> 80%), in cultures from HIV-1-seropositive donors in CDC stages II and III, while RO+ RA− blasts revealed a poor blastogenesis (35%). Although both populations were affected in HIV-1-seropositive donors in CDC stage IV RO+ RA− blasts showed lower values than the RA+ RO+ blasts (Table 2).

Table 3. Performance of CD45RA+ and CD45RO+ blast cells in phytohaemagglutinin-stimulated cultures from HIV-1-seropositive donors*.

	No. subjects	Mean ± s.e.m. CD45RA+RO±†	Mean ± s.e.m. CD45RO+RA−
HIV-1−			
Controls	10	119.5 ± 20.8	94.6 ± 11.0
HIV-1+			
CDC stage II and III	19	81.9 ± 10.8‡	35.2 ± 5.6‡
CDC stage IV	10	44.4 ± 9.6	23.8 ± 4.7

*Blastogenesis was measured in the CD45RA+ and CD45R0+ subsets. The values are the percentage of blasts with RA+RO± or RO+RA− phenotypes related to the input of RA+ or RO+ T cells, respectively. †Mononuclear cells were cultured and harvested at 72–74 h, when CD45RA+ T cells have not yet fully 'switched' their phenotype to CD45R0+. These blasts are referred to as CD45RA+RO±. ‡Significant difference between the CD45RA+RO± and CD45R0+RA− subsets at a $P < 0.01$ level. CDC, Centers for Disease Control and Prevention.

Abortive activation of CD4 and CD8 populations from HIV-1-seropositive donors

Our final objective was to determine when HIV-1-related changes first manifest during the T-cell activation cycle. CD25 (IL-2Rα) positivity in PHA cultures was analysed on CD3 cells (Fig. 6) and on the CD3/CD4 and CD3/CD8 subsets. By day 1 similar CD25 expression was induced on T cells from HIV-1-seronegative and HIV-1-seropositive donors (Figs 6b and f). During days 2–3 the HIV-1-seronegative donor cells progressed to CD25+ blast stage (Fig. 6d), while in cultures from HIV-1-seropositive patients a variable proportion, and frequently the majority, of CD25 cells died (Fig. 6h).

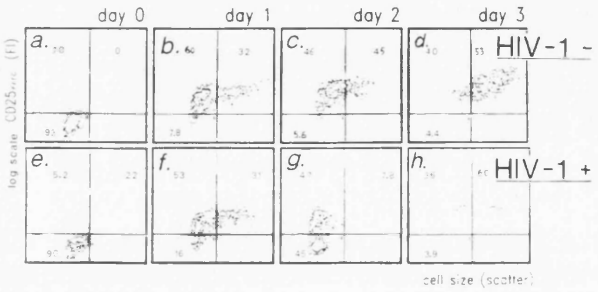

Fig. 6. Time course of CD25 expression in phytohaemagglutinin-stimulated cultures of T cells from an HIV-1-seronegative donor (a–d) and an asymptomatic HIV-1-seropositive donor (e–h). The cells were stained for CD3, with phycoerythrin (PE), and CD25 with fluorescein isothiocyanate (FITC), and the forward cell scatter (x axis) versus CD25 positivity (y axis; log display) of the gated CD3–PE population is shown. The numbers inside the quadrants refer to the relative percentages of cells and not to their absolute numbers (but see Table 4). Similar observations were made when CD4–PE and CD8–PE cells were gated (not shown).

These changes were quantitated in 50 subjects (Table 4). There were only few CD25+ cells in the positive gate in the unstimulated blood of HIV-1-seronegative controls (2.1 ± 0.2%; see Subjects and methods). In similar samples from HIV-1 seropositive donors (CDC stages II/III and IV) the values were 2.4 ± 0.2 and 2.9 ± 0.3%). These few lymphocytes expressed low amounts of CD25 close to the detection limit of our test.

In PHA cultures set up at 10^6/ml lymphocyte concentration with 75% T cells (75×10^4/ml) IL-2Rα expression was induced during the first 24 h on most T cells. The numbers of CD25+ T cells recovered in the control, CDC stage II/III and stage IV groups was 72.5, 56.7 and 37.7×10^4/ml, respectively. These activated T cells expressed moderate amounts of IL-2Rα (MFI: 62.8–82.6 in Table 4, corresponding to 5–6.5 × 10^3 m.p.c.) The number of CD25+ blasts in the control cultures remained the same between 24–72 h (80.7×10^4/ml at 72 h). In the CDC stage II/III groups however, the values at 48 and 72 h (48.4 ± 6.0 and 42.9 ± 9.6 × 10^4/ml, respectively) were heterogeneous: cultures with good blastogenesis (> 70% recovery in Fig. 2) retained high numbers of CD25+ blasts throughout, while those with poor performance (< 30% recovery) lost most CD25+ cells. Fewer CD25+ cells were seen in the CDC stage IV group (27.5×10^4/ml). Nevertheless, the lymphoblasts in all groups, including the residual surviving blasts in the CDC stage IV group, had similar MFI (330–338, corresponding to 55–67 × 10^3 m.p.c.) showing that IL-2Rα display on the surviving activated cells was approximately 10 times higher at 72 than at 24 h.

Between 72–96 h in all three groups T blasts proliferated and multiplied, on average, by a factor of 1.80, 1.56 and 1.56, to reach the values of 144, 72.5 and 39.7×10^4/ml in the control, CDC stage II/III and CDC stage IV groups, respectively. This was accompanied by a further minor increase in MFI (Table 4).

Discussion

This study quantitated disorders of lymphocyte activation in optimally stimulated cultures from HIV-1-seropositive donors at different disease stages. Conditioned medium and rIL-2 were added at the start, and in a series of experiments normal lymphocytes from HIV-1-seronegative donors were also cocultured with cells from HIV-1-seropositive donors. We found that normal cells are unable to reconstitute these HIV-1-related defects, and that transmissible suppressor effects do not appear to play a significant role. Similar conclusions have been reached in another kind of mixing experiment using cells from patients with advanced AIDS [40]. Under these conditions, therefore, the poor responses to PHA and anti-CD3 are attributable to intrinsic disorders of lymphoid cells taken from an immunosuppressed donor. Our observations do not deny the importance of IL-2 defi-

Table 4. The absolute numbers and mean fluorescence intensity of CD25+ (interleukin-2 receptor α) T cells in phytohaemagglutinin (PHA)-stimulated cultures from HIV-1-seronegative and HIV-1-seropositive donors.

	No. subjects	Hours (mean ± s.e.m.)				
		0	24	48	72	96
Absolute number of CD25+ cells (× 10^4/ml)*						
HIV-1–	15	2.1 ± 0.21	72.5 ± 2.69	80.1 ± 3.3	80.7 ± 4.0	144 ± 8.2
CDC stage II/III	22	2.4 ± 0.18	56.7 ± 3.36	48.4 ± 6.0	42.7 ± 9.6	72.5 ± 13.4
CDC stage IV	13	2.9 ± 0.26	37.7 ± 4.25	31.5 ± 4.3	27.5 ± 6.6	39.7 ± 9.6
Mean fluorescence intensity†						
HIV-1–	15	32.7 ± 1.05	82.6 ± 6.23	260 ± 23.4	387 ± 36.3	437 ± 36.6
CDC stage II/III	22	32.1 ± 1.40	77.4 ± 4.10	227 ± 17.5	388 ± 29.4	502 ± 39.0
CDC stage IV	13	31.8 ± 1.96	62.8 ± 5.61	159 ± 18.3	330 ± 40.6	414 ± 54.7

*The absolute numbers of CD3+ CD25+ cells (× 10^4/ml) recovered in cultures stimulated for varying periods, as shown in PHA cultures. The starting cell concentrations were 100 × 10^4 containing 75 ± 10^4 T cells. †The mean fluorescence intensity values were measured on the gated CD25+ cell populations, ignoring the CD25– cells. CDC, Centers for Disease Control and Prevention.

ciency as a contributing element [9,12,13,17,18], but show that it is an additional factor (see below).

In our studies approximately 30–80% of T cells from asymptomatic HIV-1-seropositive donors failed to transform when incubated with mitogens, and these proportions are five- to 100-fold higher than the numbers of cells that harbour HIV-1 provirus [15,26] and develop p24 positivity during longer-term cultures (see above and [12]). In addition, our observations confirm and extend previous studies in establishing that both CD4 and CD8 lymphocytes are severely handicapped. This CD8 malfunction, originally described in patients with AIDS [5,29,42] has been shown during asymptomatic HIV-1 infection in mixed populations following PHA activation [10], as well as when enriched CD8 cells were stimulated with anti-CD3 [13] and anti-CD3 plus rIL-2 [12]. While the loss of thymidine uptake in cultures of separated CD4 and CD8 populations in these previous studies was of the same magnitude, in our study the blast transformation of CD8 lymphocytes was significantly more handicapped than that of the residual CD4 cells (Fig. 3), confirming the severity of the secondary abnormalities in HIV-1 infection [13,43].

HIV-1-associated proliferative defects were described soon after the discovery of HIV-1, when it was shown that the inoculation of HIV-1 into cultures of mitogen- and antigen-stimulated normal and cloned CD4 lymphocytes caused a block in proliferation that preceded mature virion production [44–46]. Other experiments, however, indicate that the lymphocyte defects are neither restricted to CD4 lymphocytes nor even seen in HIV-1 infection alone. Similar phenomena were observed in murine GvHR models elicited by major histocompatibility complex class I/II disparate parental cells injected into non-irradiated F1 recipients [47] or by H-2-identical bone marrow plus T cells given to irradiated H-2-identical mice mismatched for multiple donor loci [48]. Three weeks after initiating GvHR, T cells from the spleen of GvHR mice displayed normal levels of CD3 but responded poorly to stimuli such as anti-CD3, concanavalin [47] or PHA [48], despite coculture with normal F1 accessory cells [47]. In this model both CD4 and CD8 GvHR T cells proliferate poorly: although IL-2Rα expression could be induced at 12 h, IL-2R+ cells were no longer detectable between 24 and 48 h. Again, cytokines added to these cultures did not restore full proliferative activity, and mixing experiments did not demonstrate suppressive effects [47].

What are the events that may contribute to the lymphocyte defects seen in these activated cultures? Previous studies have reported decreased [5,7,10,17,30,42,49], normal [12] or increased IL-2Rα expression [50] on stimulated T cells from HIV-1-seropositive patients. Our findings in Table 4 reconcile these findings. On the first day IL-2Rα display is induced on most T cells in all HIV-1-seropositive groups, albeit in smaller proportion in AIDS patients. On days 2–3, however, a variable number of T cells, including many IL-2Rα+ cells, die despite the addition of rIL-2R to the medium and the concomitant expression of IL-2Rβ on most of them (N. Borthwick and G. Janossy, unpublished data). This phenomenon is referred to as AALD.

It has recently been suggested that misprogramming of lymphocytes in HIV-1 infection leads to their programmed cell death (PCD), also referred to as apoptosis [51–54]. AALD is, however, not fully explained by apoptosis for the following reasons. During the acute phase of viral infections caused by Epstein–Barr virus, varicella zoster virus and HIV-1 circulating blood CD8 lymphoblasts increase in number and express lower than normal levels of bcl-2 protein; when incubated *in vitro* in the absence of IL-2 most of these cells undergo classical apoptosis with signs of DNA degradation, peripheral chromatin condensation associated with low scatter but intact membrane antigens [55]. Apoptosis has been documented in small proportions of cells during the asymptomatic chronic phase of HIV-1 infection [53,54], and the death of at least some of these lymphocytes appears to be preventable by rIL-2 [56]. Interestingly, the proportions of these apoptotic cells are only marginally increased by stimulation with a powerful mitogen such as anti-CD3. When a sensitive nick translation method was used to detect apoptic cells in cultures incubated overnight without mitogens 10% of lymphocytes (13% of CD8 cells) were apoptotic, and during anti-CD3 stimulation 13% (18% of CD8 cells) were positive [54]. The facts that in asymptomatic HIV-1-seropositive patients the proportion of bcl-2– T cells are only increased by approximately 20% above the normal values seen in healthy individuals, but are much lower than the proportions of bcl-2– T cells in an acute viral infection [55] are in line with these observations (N. Borthwick, unpublished data).

It is likely that the appearance of apoptotic cells is explained by IL-2 starvation *in vitro* [57], particularly if the samples have been taken from patients with recent episodes of acute viral infections [55]. In marked contrast, we have shown that during the asymptomatic stage of HIV-1 infection powerful mitogens such as PHA and anti-CD3 induce AALD in larger proportions (30–80%) of T cells, despite the availability of rIL-2 in the cultures. Nevertheless, in the presence of weaker mitogens such as PWM or rIL-2 plus FCS the stimulation processes in the culture are slower. When cultured in this way the majority of small lymphocytes (> 60%) remain viable for longer than 5–6 days — at least until a proportion develop IL-2Rα and die (E. Miedema and M. Bofill, manuscript in preparation). Thus, the number of T cells undergoing AALD is dependent upon the stimuli used. Anti-CD3, a mitogen that mimics activation processes generated by specific antigens [37], is a suitable agent to investigate this phenomenon.

The mechanisms of AALD is unknown. However, during the asymptomatic stages of HIV-1 disease large subsets in CD8 and probably also in CD4 populations develop phenotypic and metabolic features that are known to be associated, during thymocyte development, with cell death and vulnerability [58,59]. These features include the co-expression of CD45R0 and CD38 surface molecules and particularly low levels of 5′-nucleotidase [59]. Although immature cells with these features are normally restricted to the thymus, abnormal terminal differentiation may induce similar changes and metabolic vulnerability in the activated T-memory cell populations in chronic viral infections.

We feel that our observations about the particular vulnerability of CD45R0+ 'primed' T-cell populations at an early asymptomatic stage of HIV-1-infection are important for two main reasons. First, our findings are in agreement with other studies that demonstrate the poor survival [56,60] and low clonogenicity of HLA–DR+ CD8 cells [61] leading to the deterioration of HIV-1-specific cytotoxicity. These populations are likely to correspond to the CD45R0+ 'primed' CD8 cells. At the same time the HLA–DR– CD8 population is still capable of proliferative and cytotoxic responses — but essentially devoid of HIV-1-specific precursors of cytotoxic effector cells [62]. The latter CD8 subset corresponds to the CD45RA+ 'unprimed' CD8 subset [23]. Nevertheless, the CD45R0+ CD8 cells represent a different cohort than the cells identified by the 4B4 antibody of the CD29 cluster [29]. The CD29+ cells are sometimes referred to as 'memory cells' [24,25,28]. When studied in normal and HIV-1-seropositive blood, CD29+ lymphocytes constitute only 71.4 and 58.3% of CD45R0+ CD8 cells, respectively (M. Bofill and E. Medina, unpublished data), and it is likely that CD45R0+ cells that are prone to AALD no longer express the CD29 differentiation antigen.

Second, the selective quantitative assessment of the blast transformation and AALD within the CD45R0+ population is a convenient method that can be applied to the analysis of 'primed' CD4 and CD8 populations. Such assays may have strong prognostic significance. Other lymphocyte stimulation assays may also acquire prognostic significance, due to the detection of the functional defects amongst 'primed' T lymphocytes. Among the stimulants used *in vitro*, soluble antigens [63], pokeweed [64] and insolubilized anti-CD3 antibody at low density [64,65] have been shown to predominantly activate CD45R0+ T cells. The same assays provide early and sensitive laboratory indicators for early disease progression during asymptomatic stages of HIV-1 infection [1,2,6,25,66,67]. These prognostic indicators are independent of CD4 counts [67].

In conclusion, AALD involving the CD45R0+ 'primed' cells appears to be an important mechanism contributing to the early deterioration of immunological memory of HIV-1 infection. This phenomenon probably occurs *in vivo*, and explains the relevance of other immunological tests currently in diagnostic use. In a recent seminal study Prince *et al.* [68] showed positive correlation between activated CD8 lymphocytes and serological markers such as high serum levels of soluble CD8, IL-2Rα and β_2-microglobulin, which are likely to derive from 'primed' CD8 cells undergoing AALD and exhibiting a higher turnover *in vivo*. The investigation of these issues may not only provide prognostic tests but also help elucidate the biochemical mechanism(s) involved in AALD and the analysis of how antiviral immunosuppressive and immunopotentiating drugs affect lymphocyte survival and function under the chronic stress of HIV-1 infection.

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The Significance of Low bcl-2 Expression by CD45RO T Cells in Normal Individuals and Patients with Acute Viral Infections. The Role of Apoptosis in T Cell Memory

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Summary

The bcl-2 gene product has been shown to prevent apoptotic cell death. We have now investigated the bcl-2 protein expression by resting and activated mature T cell populations. Freshly isolated $CD45RO^+$ T cells within $CD4^+$ and $CD8^+$ subsets expressed significantly less bcl-2 than $CD45RO^-$ ($CD45RA^+$) T cells ($p < 0.001$). When $CD45RA^+$ T cells within both $CD4^+$ and $CD8^+$ subsets were activated in vitro, the transition to CD45RO phenotype was associated with a decrease in bcl-2 expression. Patients with acute viral infections such as infectious mononucleosis caused by Epstein-Barr virus infections or chickenpox, resulting from varicella zoster virus infection, had circulating populations of activated $CD45RO^+$ T cells which also showed low bcl-2 expression. In these patients, a significant correlation was seen between low bcl-2 expression by activated T cells and their apoptosis in culture ($r = 0.94$, $p < 0.001$). These results suggest that the primary activation of T cells leads to the expansion of a population that is destined to perish unless rescued by some extrinsic event. Thus the suicide of $CD45RO^+$ T cells could be prevented by the addition of interleukin 2 to the culture medium which resulted in a concomitant increase in the bcl-2 expression of these cells. Alternatively, apoptosis was also prevented by coculturing the activated T lymphocytes with fibroblasts, which maintained the viability of lymphoid cells in a restinglike state but with low bcl-2 expression. The paradox that the $CD45RO^+$ population contains the primed/memory T cell pool yet expresses low bcl-2 and is susceptible to apoptosis can be reconciled by the observations that maintenance of T cell memory may be dependent on the continuous restimulation of T cells, which increases their bcl-2 expression. Furthermore, the propensity of $CD45RO^+$ T cells to extravasate may facilitate encounter with fibroblast-like cells in tissue stroma and thus be an important additional factor which promotes the survival of selected primed/memory T cells in vivo.

The bcl-2 proto-oncogene and its product have been shown to control the survival of both normal and malignant cells (1). The *bcl-2* gene was first identified in most follicular B cell lymphomas at the breakpoint of the translocation between chromosomes 14 and 18 (2, 3). It was subsequently shown that in lymphoid environments the low expression of the bcl-2 gene product is associated with the selection or deletion of cells (4, 5). Thus, the expression of bcl-2 by proliferating B cells in the germinal centers of LNs is low (5, 6). Similarly, immature cortical thymocytes undergoing selection are $bcl\text{-}2^-$ (1). The product of the human *bcl-2* gene has been shown to block programed cell death or apoptosis (1, 7, 8), and an induced increase in bcl-2 expression rescues appropriate B cells or thymocytes from this suicide (5, 6, 9). In addition, in transgenic mice with upregulated bcl-2 there is a prolongation of secondary immune responses (10, 11). These observations, taken together, suggest that factors that alter bcl-2 expression are important in the development of both memory B cells and thymocytes (10, 12).

Although compelling data demonstrate that the bcl-2 gene product may have a role in lymphoid selection, no data is available on the changes of the bcl-2 protein during peripheral T cell activation and/or development. Clearly, the investigation of bcl-2 expression by T cells before and after im-

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mune stimulation in vivo is of considerable interest as a possible way in which activated T cells may be selected for survival. It has been shown that T lymphocytosis, especially within the $CD8^+$ subset, is induced by acute viral infections and these cells are activated and enter the proliferative cycle in vivo (13–15). However, $CD4^+$ and $CD8^+$ cell numbers rapidly return to normal levels on disease resolution even though specific antiviral memory T cells persist in vivo (16). This suggests that mechanisms that determine if activated T cells survive or perish regulate the balance between the generation of a memory population and the reestablishment of cellular homeostasis after activation in vivo.

Recent studies have demonstrated that various phenotypic and functional changes parallel the differentiation of T cells from an unprimed to a primed/memory state (17–19). The most discriminating markers for unprimed and primed T cells in humans, the CD45RA and CD45RO antibodies, are directed to the high and low molecular weight isoforms of the leukocyte common antigen, respectively (19, 20). In this study, we have investigated the expression of bcl-2 protein by resting and activated subpopulations of T cells from normal individuals and patients with acute viral diseases caused by EBV and varicella zoster virus (VZV),[1] and related the bcl-2 expression to the LCA display on these cells. Our findings demonstrate that there is a decrease in bcl-2 protein expression in T cells after activation which leads to apoptosis unless they are rescued by appropriate factors, in analogy with the selection of B cells in germinal centers (5, 6). Thus, apoptosis can be prevented by the addition of exogenous IL-2, which maintains the activated T cells in cycle and induces the reexpression of bcl-2. In contrast, tissue stromal cells such as fibroblasts can also prevent the occurrence of apoptotic death in activated T cells, but enable them to attain a restinglike state with low bcl-2 expression. These data also provide clues into the interplay between mechanisms which may firstly lead to the persistence of T cell memory in humans, yet at the same time enable the homeostatic balance of total T cell numbers to be maintained after immune activation in vivo.

Materials and Methods

Patient and Control Samples. Heparinized venous peripheral blood was obtained from 10 patients with either acute infectious mononucleosis (seven males and three females; mean age 25 yr: range 19–45 yr) or with varicella zoster infections (six males and four females; mean age 31 yr: range 20–40 yr) who were admitted to the Infectious Diseases Unit at Coppett's Wood Hospital within 10 d after the onset of symptoms. Blood was also obtained from a male patient with acute HIV-1 infection who had p24 antigen in the serum before developing anti-p24 antibody. In addition, normal blood was obtained from 10 healthy laboratory personnel and medical students (five males and five females; mean age 28 yr: range 21–45 yr) and normal tonsils were obtained at elective surgery after antibiotic therapy. LN biopsies from HIV-1–infected individuals were frozen and analyzed in cryostat sections.

[1] *Abbreviations used in this paper:* MGG, May-Grunewald-Giemsa stain; PI, propidium iodide; VZV, varicella zoster virus.

Antibodies and Cytokines Used in the Study. The CD45RA (SN130; IgG1) and CD45RO antibodies (UCHL-1; IgG2a, generously provided by Professor P. C. L. Beverley, Imperial College Research Fund, London, UK) were previously shown to react with unprimed and primed T cells, respectively (20). CD4 (RFT4; IgG1) and CD8 (RFT8; either IgG1 or IgM) antibodies were used to identify and/or isolate helper and suppressor/cytotoxic subsets of T cells, respectively (21). A CD3 antibody (MEM-57; IgG1) was kindly provided by Dr. V. Horejski (Czechoslovak Academy of Sciences, Prague, Czechoslovakia) (22). The IgG1 antibody reacting with the bcl-2 gene product by recognizing a 26-kD protein (23) was obtained from Dr. D. Y. Mason (Nuffield Department of Pathology, Oxford, UK), and Dako Ltd. (High Wycombe, Bucks, UK). In this study, this protein will henceforth be referred to as bcl-2. Ig isotype–specific FITC or PE-conjugated affinity-purified goat anti–mouse second layer antibodies (Southern Biotechnology Associates, Birmingham, AL) were used at pretitrated optimal concentrations. Recombinant human IL-2 was kindly provided by Dr. Max Schreier (Sandoz Pharma Ltd., Basel, Switzerland) and recombinant GM-CSF was obtained from British Biotechnology Ltd. (Abingdon, Oxon, UK).

Preparation of Lymphocyte Subsets. $CD2^+$ cells were prepared by E-rosetting from Ficoll-Hypaque (Nycomed, Oslo, Norway) separated PBMC as previously described (24). CD4, CD8, CD45RA, and CD45RO subsets of $CD2^+$ cells were prepared by immunomagnetic bead depletion (Dynal Ltd., Wirral, UK) as described in detail elsewhere (25). Only negatively selected subsets were used in any of the assays. The subsets prepared in this way were regularly 90–95% positive for the CD45RA or CD45RO phenotype and 94–98% CD4 or CD8 positive.

Fibroblast/Lymphocyte Coculture. Human embryonic lung fibroblasts grown in 24-well plates (Falcon Labware, London, UK; Becton Dickinson, Ltd., Oxford, UK) in RPMI-1640 medium supplemented with L-glutamine, benzyl-penicillin, streptomycin (Gibco Ltd., Paisley, UK) and 10% of fetal bovine serum (FBS; Flow Laboratories Ltd., Rickmansworth, UK) were maintained in a humidified atmosphere containing 5% CO_2. The fibroblasts were used as confluent monolayers between passages 8–19. These cell lines were mycoplasma free as shown by Hoescht staining and an RNA probe (Laboratory Impex, Middlesex, UK). Every 3–4 d, half of the spent growth medium in fibroblast cocultures was replenished.

Lymphocyte Activation. T cell subsets were activated with 1 μg/ml PHA (Wellcome Ltd., High Wycombe, Bucks, UK) in the presence or absence of IL-2 (2 ng/ml; 26). Cells were harvested from replicate cultures at various times for phenotypic analyses. To establish IL-2–dependent cell lines, $CD4^+$ or $CD8^+$ T cells were first activated by PHA and IL-2 (2 ng/ml) for 6 d in bulk in the presence of 10% autologous adherent cells. The cells were then washed and resupplemented with IL-2 every 3–4 d. After 3–4 wk, the cells were reactivated with PHA in the presence of autologous adherent cells and recultured with IL-2 which was replenished every 3–4 d as before. To prevent overcrowding, the concentration of these cells was periodically readjusted. The acute withdrawal of IL-2 resulted in a rapid decrease in viability and increase in cell death by apoptosis as described (27).

Cell Staining. PBMC or T cell subsets were stained in suspension, as smears after cytocentrifuge preparation or in histological sections. First, membrane staining was performed and followed by membrane permeabilization to allow for cytoplasmic staining (28). The membrane markers used were CD3, CD4, CD8, CD45RA, and CD45RO antibodies and isotype-specific second layers conjugated to FITC (Southern Biotechnology Associates). The cells were fixed with 0.3–0.4% paraformaldehyde in PBS for 2 min,

washed with PBS containing 0.2% azide and 0.2% BSA, and permeabilized in 500 μl of ice-cold 1:1 acetone methanol. This mixture was then incubated on ice for 15 min and washed twice with PBS plus azide before adding pretitrated optimal amounts of bcl-2 antibody. After washing, goat anti-mouse IgG conjugated to PE was added and incubated for 15 min at room temperature. The specificity of the method has been established by analyzing suspensions of tonsil cells containing surface(s) IgD^+ B lymphocytes ($bcl\text{-}2^+$) and $CD38^+$ germinal center blast cells ($bcl\text{-}2^-$). The cells were finally washed and fixed with 4% paraformaldehyde. The negative control antibodies for bcl-2 staining consisted of isotype-matched unreactive antibody followed by identical second layer labeling as above. The expression of bcl-2 on lymphocyte subsets was investigated by two-color immunofluorescence on a FACScan® (Becton Dickinson & Co.) and compared with similar staining performed in cytocentrifuge preparations. These methods have been described in detail elsewhere (29, 30). Double fluorescence staining was performed on acetone-fixed cryostat sections from tonsils and LN biopsies of HIV-1–infected individuals as described previously (31). After rehydration, CD8 and bcl-2 antibodies were added for 45 min, and after washing FITC or tetramethyl rhodamine isothiocyanate conjugated subclass-specific second layers were added in the presence of 10% normal human serum to inhibit nonspecific binding. After 45 min at room temperature, the sections were washed and then examined by fluorescence and confocal microscopy as above.

The Enumeration of the Absolute Number of Lymphocytes/Lymphoblasts. The Cytoron absolute (Ortho Diagnostic Systems, Ltd., High Wycombe, Bucks, UK) is a flow cytometer which allows the enumeration of absolute lymphocyte numbers identified by fluorochrome-labeled antibodies. Cells were fixed in 0.05% paraformaldehyde and gated on forward and 90° side scatter. The absolute number of fluorescent cells was then determined within the lymphoid gate. Dead cells, identified by their forward and side scatter profiles, were excluded from further analysis (32).

Detection of Apoptosis. Apoptosis was measured by four methods. First, the cleavage of DNA into oligonuclosomal fragments was tested as described previously (33). Briefly, 10^6 cells from normal or virally infected individuals were snap frozen in liquid nitrogen and the pellets were resuspended in 20 μl of 10 mM EDTA, 50 mM Tris/HCl buffer (pH 8) containing 0.5% sodium lauryl sarkosinate (BDH, Lutterworth, Leics, UK) and 0.5% mg/ml proteinase K (Pharmacia Biotechnology Ltd., Milton Keynes, UK). After incubation at 50°C for 60 min, RNase A stock solution (10 μl of 0.5 mg/ml; Sigma Chemical Co., Poole, Dorset, UK) was added, incubated for 1 h at 50°C, and the samples were then heated to 70°C. EDTA (10 μl of 10 mM) containing 1% low melting point agarose (Sigma Chemical Co.), 0.25% bromophenol blue, and 40% sucrose was mixed with each sample. This mixture was loaded onto a 2% agarose gel containing 1× TBE buffer (90 mM Tris/borate, 1 mM EDTA) containing 250 ng/ml ethidium bromide (Sigma Chemical Co.) before electrophoresis (80 V, 1.5 h).

Second, the proportion of apoptotic cells present in cultures was also determined in cytocentrifuge preparations by their morphology, by chromatin condensation, nuclear fragmentation, and by a decrease of the nuclear/cytoplasmic ratio after May-Grunewald-Giemsa staining (MGG; see Fig. 3 *F*). Third, these cells were studied by electron microscopy. Fourth, the apoptotic cells were identified by double-color fluorescence technique using surface labeling in conjunction with nuclear labeling with propidium iodide (PI) after permeabilization with paraformaldehyde at a final concentration of 0.5% in PBS for 2 min (32). This technique identified a PI-reactive apoptotic population that was smaller in size than resting viable PI^- lymphocytes, but was distinct from debris and the nonviable cells exhibiting an increased 90° scatter. Similar proportions of apoptotic cells were detected by the three morphological methods.

Statistics. The results were analyzed by the Student's *t* test and by linear regression analysis.

Results

The Expression of bcl-2 by T Cell Subsets. PBMC isolated from 10 normal individuals and analyzed for bcl-2 expression within the $CD3^+$ T cell cohort revealed that the majority (mean 80%) expressed bcl-2. A minor $bcl\text{-}2^-$ population was, however, consistently found in each individual tested (Table 1) and these cells were observed mainly in the CD4 subset (Fig. 1 *B*). The $CD45RO^+$ T cells expressed less bcl-2 than both the whole $CD3^+$ ($p < 0.001$) and also the $CD45RO^-$ T cell subsets ($p < 0.001$; Table 1, Fig. 1). Single cell analyses revealed that the difference between the bcl-2 expression between $CD4^+$ and $CD8^+$ subsets in normal individuals was due to the greater numbers of $CD45RO^+$ cells within the former subset. The minor $CD8^+$, $CD45RO^+$ T cell population in normal individuals

Table 1. *The Expression of bcl-2 by T Cell Subsets from Normal and Virally Infected Individuals*

Groups	*n*	CD3	CD4	CD8	$CD45RO^+$	$CD45RO^-$
Normal	10	80 (73–84)*	82 (63–95)	83 (68–93)	63 (53–73)§	89 (78–96)
VZV	6	53 (40–67)**	70 (52–82)‡	65 (42–89)¶	45 (28–48)¶	63 (51–72)**
EBV	7	55 (38–67)**	71 (48–82)‡	49 (31–80)**	44 (16–68)‖	67 (46–89)**

* The proportion of $bcl\text{-}2^+$ cells within PBMC T cell subsets analyzed by two-color immunofluorescence. The results represent the mean percentage (and range) of $bcl\text{-}2^+$ cells in the different subsets as shown.
‡ Not significantly different to normal as analyzed by the Student's *t* test.
§ The proportion of $bcl\text{-}2^-$ cells is significantly higher among normal $CD45RO^+$ T cells than within the total CD3 population ($p < 0.001$).
‖ Significant change from normal ($p < 0.02$).
¶ Significant change from normal ($p < 0.01$).
** Significant change from normal ($p < 0.001$).

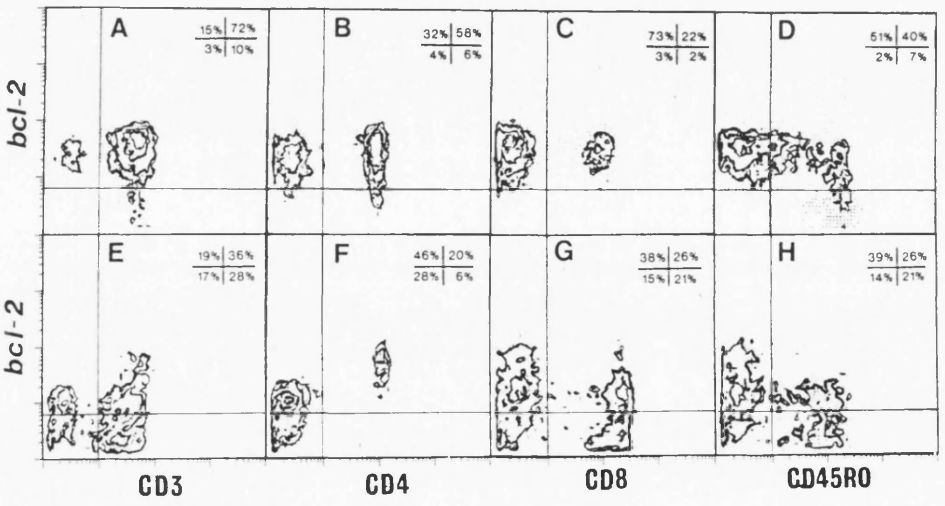

Figure 1. The expression of bcl-2 by T cell subpopulations from a normal individual (*A–D*) and a patient with acute EBV infection (*E–H*). PBMC were analyzed for coexpression of bcl-2 with other T cell markers by double-color immunofluorescence (5,000 cells in the lymphoid gate). The percentages shown represent the proportion of cells in each quadrant of the fluorescence gates, set using negative control antibodies. Within the $CD8^+$ subset, only the brightly stained cells ($CD3^+$) were analyzed whereas the CD8 dim ($CD3^-$, $C16^+$, $CD56^+$) NK cells were excluded.

also included cells with low bcl-2 expression (data not shown). A bimodal distribution of bcl-2 on normal $CD45RO^+$ T cells was consistently found. Cells expressing high levels of CD45RO showed low bcl-2 expression and vice versa (Fig. 1 *D*).

When the bcl-2 expression of T cells from normal individuals and patients with acute viral infections were compared, it was found that the $bcl\text{-}2^-$ subsets within the $CD3^+$ T cell population significantly expanded in both EBV and VZV infected individuals ($p < 0.001$; Table 1). Previous studies have already indicated that these patients have increased numbers of $CD45RO^+$ T cells within the $CD8^+$ subset (14), a finding which agrees with our own observations shown here. There was significantly decreased bcl-2 expression in both $CD8^+$ and $CD45RO^+$ subsets of virally infected as compared with normal patients, which largely accounted for the decrease of bcl-2 expression within the $CD3^+$ population (Table 1; see representative EBV patient in Fig. 1, *E–H*). It was noted that the $CD3^-$ subset in both VZV and EBV patients also expressed lower levels of bcl-2 than normal individuals (representative experiment in Fig. 1 *E*). The $CD3^-$, $bcl\text{-}2^-$ cells are $CD19^+$ and of the B-lineage (data not shown). When the bcl-2 reactivity of $CD45RO^+$ ($CD45RA^-$) PBMC from EBV and VZV patients was analyzed, it was found that they expressed significantly lower levels of bcl-2 than the $CD45RO^-$ ($CD45RA^+$) PBMC population, an observation which was also apparent in normal individuals (Table 1 and Fig. 1 *H*) and which was confirmed when these subsets were analyzed within purified T cells instead of PBMC populations (data not shown). The $CD45RO^-$ PBMC cells from normal individuals however, express significantly higher levels of bcl-2 than those from EBV and VZV patients probably because of the presence of $bcl\text{-}2^-$ B cells that are localized within the $CD45RO^-$ subset in these

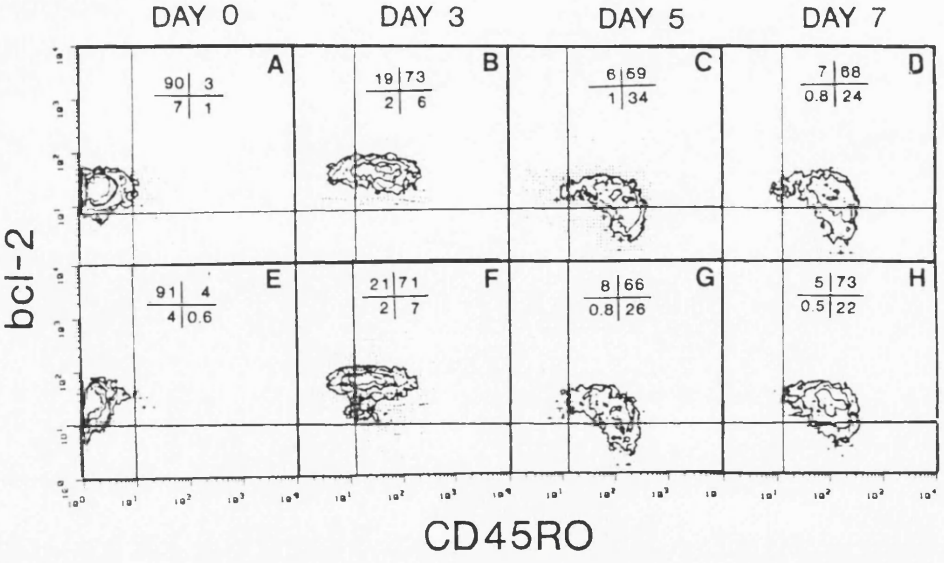

Figure 2. The changes in bcl-2 expression after PHA activation of $CD4^+$, $CD45RA^+$ (*A–D*) and $CD8^+$, $CD45RA^+$ cells (*E–H*) for various periods of time (day 0–7) in comparison with the appearance of CD45RO reactivity (5,000 cells in the lymphoid gate). For details see Fig. 1.

patients but not in the normal patients (Fig. 1, *A* and *E*). The low bcl-2 expression in the CD45RO$^-$ PBMC population could also be due to activated T cells in transition from a CD45RA$^+$ (CD45RO$^-$) to a CD45RA$^-$ (CD45RO$^+$) phenotype that may have lost their bcl-2 expression before acquiring CD45RO reactivity. However, the kinetics of the loss of bcl-2 and gain of CD45RO reactivity by CD45RA$^+$ T cells after activation render this latter possibility unlikely (see Fig. 2).

The Expression of bcl-2 by Normal T Cells during Activation In Vitro. The difference in bcl-2 expression between normal CD45RO$^+$ and CD45RO$^-$ T cell populations suggested that changes in the expression of this protein may occur as a result of T cell differentiation linked to recent priming. These observations were confirmed *in vitro* by activating purified T cells with mitogens such as PHA followed by the analysis of bcl-2 expression. CD4$^+$, CD45RA$^+$ (Fig. 2, *A–D*), and CD8$^+$, CD45RA$^+$ (Fig. 2, *E–H*) populations were isolated and activated with PHA in order to observe the changes in bcl-2 expression in parallel with the transition from CD45RA$^+$ to CD45RO$^+$ expression. Before activation, both the isolated CD4$^+$ and CD8$^+$ populations were <4% CD45RO$^+$ (95% CD45RA$^+$) and >95% bcl-2$^+$ (Fig. 2, *A* and *E*). After 3 d of stimulation with PHA, 79% of the CD4$^+$ cells and 75% of the CD8$^+$ T cells started to express CD45RO (Fig. 2, *B* and *F*, respectively) but the majority of CD4$^+$ (93%) and CD8$^+$ (86%) T cells still remained bcl-2$^+$. After 5 d of stimulation, 94% of the CD4$^+$ and 93% of the CD8$^+$ T cells expressed CD45RO (Fig. 2, *C* and *G*) and a bimodal distribution of bcl-2 developed in both CD4$^+$ and CD8$^+$ subsets (34 and 26% bcl-2$^-$, respectively; Fig. 2, *C* and *G*). These results have also been confirmed in cytocentrifuge preparations stained for bcl-2 in conjunction with other lymphocyte markers (data not shown). The bcl-2$^-$ cohort was still observed after 7 d of activation (Fig. 2, *D* and *H*).

The Association of Low bcl-2 Expression with Apoptosis in T Cells. In cytocentrifuge smears of normal tonsil cell suspensions stained for bcl-2 heterogeneity was observed (Fig. 3 *a*). The large blast cells (CD38$^+$ germinal center B cell blasts) were bcl-2$^-$ whereas the majority (>95%) of small lymphocytes were bcl-2$^+$ (Fig. 3 *a*). In comparison, normal PBMC populations did not contain blast cells and were >80% bcl-2$^+$ (Fig. 3 *c*). When the tonsil suspensions were activated for 48 h by PHA plus IL-2, T cell blasts developed and still showed strong expression of bcl-2$^+$ (Fig. 3 *b*). bcl-2 was reduced in T cell blasts in longer term cultures (>84 h). Freshly isolated PBMC populations from patients with EBV and VZV infections included many activated T cell blasts that expressed low bcl-2 (Fig. 3 *d*) suggesting that in the patients in vivo these T cells were activated for longer periods (Fig. 2). We next investigated if the low expression of bcl-2 by T cells from EBV and VZV infected patients reflected their

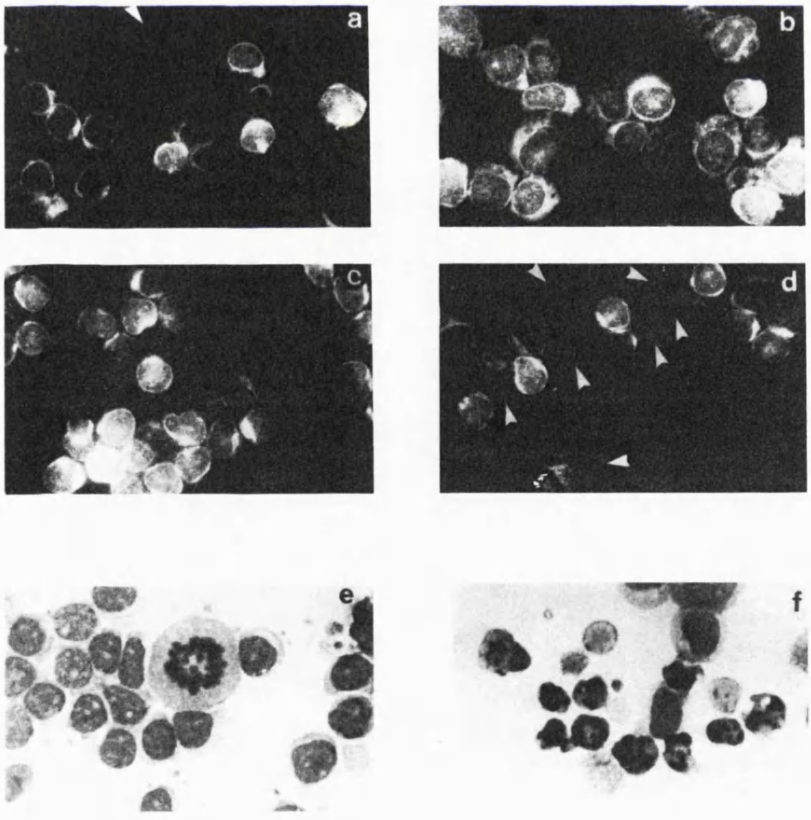

Figure 3. Bcl-2 expression of lymphocytes in cytocentrifuge smears were prepared from suspensions of tonsil before (*a*) and after activation with PHA and IL-2 (2 ng/ml) for 24 h (*b*), and from the fresh PBMC of a normal (*c*) and an EBV-infected donor (*d*). (*White arrowheads*) Blasts with low bcl-2 expression. The morphology of the lymphoid cells from the same EBV donor is also shown in MGG preparations that were freshly isolated (*e*) or cultured for 24 h in the absence of IL-2 (*f*).

susceptibility to suicide by an apoptotic process. To document these changes, adherent cells were removed from the PBMC populations before culture to prevent the clearance of apoptotic cells by phagocytosis (34). In many individuals, a proportion of lymphoid cells freshly isolated from patients with EBV and VZV infections showed characteristic hallmarks of apoptosis. The numbers of these cells were increased after 24 h of culture in the absence of any stimuli (Fig. 3 *f*). In normal individuals, apoptotic cells were rare either before or after 24 h of PBMC culture (data not shown). The presence of apoptotic cells in PBMC cultures of patients with viral infections was confirmed by DNA electrophoresis where oligonucleosomal DNA fragmentation of PBMC was observed in EBV and VZV infected but not normal individuals (Fig. 6). The apoptotic morphology of these cells was also formally confirmed by electron microscopy (data not shown).

Another direct confirmation of apoptosis at the single cell level was provided by double-color immunofluorescence using PI together with surface marker labeling (32). With this method we could confirm that the majority of PI-stained cells in patients with viral infections were amongst the $CD8^+$ and $CD45RO^+$ cells (Fig. 4, *c* and *d*). The proportion of PI-labeled cells correlated closely with the proportion of $CD8^+$, $CD45RO^+$ cells in apoptosis as determined by morphology. The PI-labeled cells were virtually unreactive with other markers such as CD4, CD16, and CD45RA.

The PI^+ and PI^- populations were further investigated by their scatter characteristics. The PI^- population (Fig. 4 *b*, *L*) fell in the lymphocytic and blastic gate as expected (Fig. 4 *a*) and the PI^+ population (Fig. 4 *b*, *A*) was smaller than normal lymphocytes (Fig. 4 *a*) and consisted of condensed apoptotic lymphocytes which, however, still showed intact membrane labeling. The fact that in the same samples healthy blast cells of $CD8^+$, $CD45RO^+$ phenotype and dividing cells were also present (Fig. 3 *e*) indicated that the activation/expansion and death by apoptosis were occurring simultaneously within the $CD8^+$,$CD45RO^+$ population of T cells in these patients. Normal individuals showed virtually no PI reactivity in T cell subpopulations (data not shown).

We next investigated if in EBV and VZV infection the numbers of T cells with low bcl-2 expression correlated with the cells undergoing apoptosis. The fresh cells were first investigated for bcl-2 expression by flow cytometry. The cells were cultured for 24 h and analyzed in cytocentrifuge smears for morphological signs of apoptosis developing during the incubation period. The correlation between the proportion of bcl-2^+ cells in the fresh samples and the presence of apoptotic cells in the 24-h samples is shown in Fig. 5. There were significantly more bcl-2^- and apoptotic cells in VZV and EBV patients as compared with normal uninfected samples ($p < 0.001$ in both cases; Fig. 5). Furthermore, there was a strong positive correlation between the proportion of bcl-2^- cells and apoptosis in both the EBV ($r = 0.99$, $p < 0.001$) and VZV groups ($r = 0.73$, $p < 0.02$) analyzed separately or when the normal and patient groups were combined ($r = 0.94$, $p < 0.001$). A patient with acute HIV infection also showed low bcl-2 expression and increased apoptosis when compared with normal controls. This correlation between low bcl-2 expression and apoptosis was also found if the extent of DNA fragmentation seen after 24 h of culture was compared with the number of cells with low bcl-2 expression, before culture, as shown in a representative experiment (Fig. 6).

The Prevention of Apoptosis by IL-2 and Coculture on Fibroblasts. The previous observations suggested that low bcl-2 expression in $CD45RO^+$ populations of EBV and VZV infected individuals was associated with the propensity for suicide. We investigated next the ability of IL-2 to prevent apoptosis in these cultures. The addition of IL-2 to PBMC from these patients at the initiation of cell culture for 24 h significantly reduced the proportion of apoptotic cells from 39 to 8% of the cultured populations ($p < 0.001$), whereas the addition of GM-CSF had no significant effect (Table 2). As the survival of activated T cells can also be promoted by fibroblasts (35), we investigated if the apoptosis of PBMC from patients with viral infections could be prevented by fibroblast coculture for 24 h. We found that fibroblast coculture significantly reduced the proportion of apoptotic cells

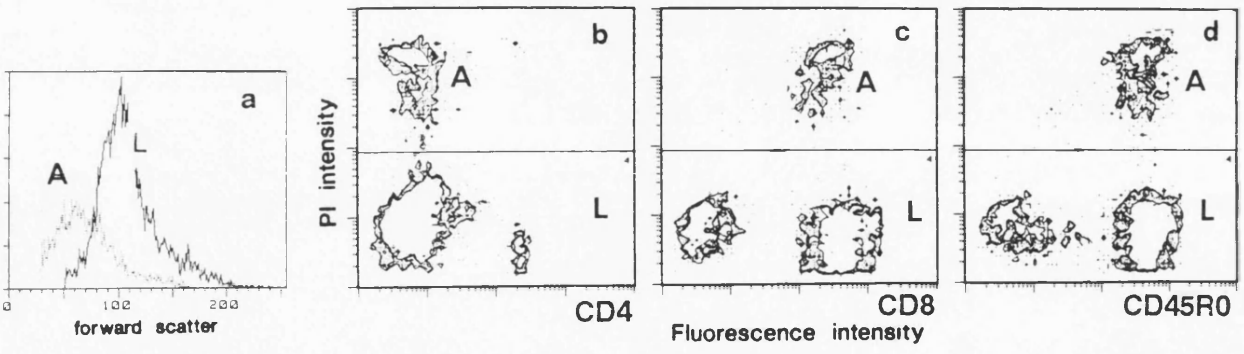

Figure 4. The identification of apoptotic cells by staining with PI in monocyte-depleted lymphocyte suspensions taken from an EBV donor and incubated for 24 h in the absence of IL-2. The PI^+ apoptotic cells (*A* in *b*) are smaller than lymphocytes (*L*) when studied by forward scatter (*a*) and are $CD4^-$ (*b*), $CD8^+$ (*c*), and $CD45RO^+$ (*d*). The percentage of PI-reactive cells showed a close agreement with the proportion of apoptotic cells identified by MGG morphology.

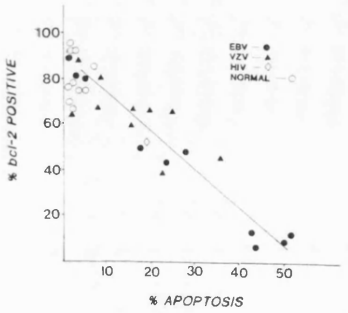

Figure 5. The association of low bcl-2 expression with apoptosis in EBV and VZV patients. PBMC were depleted of adherent cells and stained for bcl-2 and analyzed by flow cytometry (*y-axis*). Samples of the same populations were also cultured for 24 h in medium without any stimulus and the percentages of apoptotic cells among 1,000 cells were counted by two observers in smears stained with MGG (*x-axis*). Samples from normal blood (○) and from patients with acute EBV (●), VZV (△), and HIV-1 infections (◇) are shown.

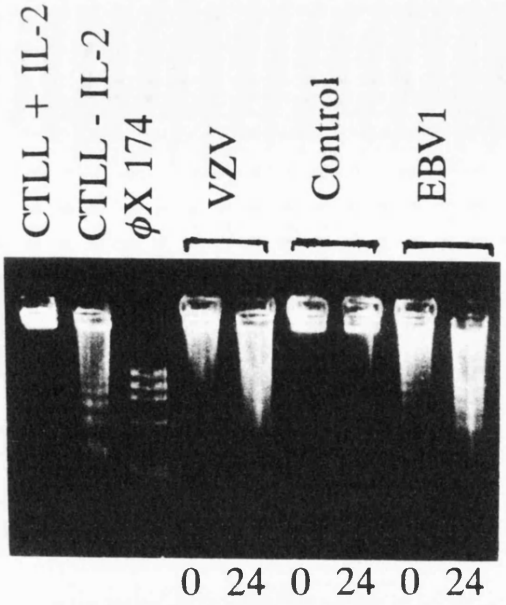

Figure 6. Fragmentation of DNA in lymphocytes from patients infected with EBV and VZV. PBMC samples from normal, VZV, and EBV patients were depleted of adherent cells and analyzed either before (*0* h) or 24 h after incubation. EcoRI-treated $\phi\chi174$ DNA were included as markers, and the controls included samples of an IL-2–dependent CTLL line cultured for 24 h in the presence or absence of IL-2.

from 39 to 16% ($p < 0.001$; Table 2) and substantially enhanced the number of viable cells recovered (data not shown).

To further investigate ways by which apoptosis may be prevented in activated T cells, we first established IL-2–dependent CD4$^+$ and CD8$^+$ T cell populations from normal individuals (see Materials and Methods). The use of these cell lines enabled the generation of large numbers of apoptotic T cells on IL-2 withdrawal, for study when required. These cells were CD45RO$^+$. The CD8$^+$ lines were highly cytotoxic in a lectin-mediated cytotoxic assay, and these blasts were phenotypically and functionally comparable to the T cells seen in the patients with acute viral disease. When IL-2 was removed from these cells, apoptosis was evident as soon as 24 h and greatly increased by 48–72 h (Fig. 7 *c*). The apoptotic changes were readily documented by both morphology and oligonucleosomal fragmentation (data not shown), indicating that the T cell line could be used to reproduce the apoptosis seen in cultured PBMC from EBV and VZV infected patients.

We next investigated whether fibroblasts could also prevent apoptosis in these cell lines after IL-2 removal and also studied whether or not this increased survival was associated with changes in the bcl-2 expression of these cells. The cells in IL-2–supplemented cultures were large and blastlike on day 6 (Fig. 7 *g*) and the mean forward scatter (MFS), a measure of cell size, was 157 in this population. The CD8$^+$ T cells cultured on fibroblasts for 6 d were smaller (Fig. 7 *e*; MFS 123). These cocultured T cells were, however, still marginally larger than the freshly isolated resting population (Fig. 7 *c*; MFS 123 vs MFS 110; data not shown). The results ob-

Table 2. *The Prevention of Apoptosis in EBV-infected Patients by IL-2 and Fibroblasts*

Expt.	Control*	IL-2	Fibroblasts	GM-CSF
1	61‡	15	23	ND
2	43	6.1	16.9	44
3	33.2	1.3	10.7	34.8
4	20.5	9.8	14.7	19.3
MEAN ± SEM	39.4 ± 8.5	8.1 ± 2.9§	16.3 ± 2.6§	32.7 ± 6.9‖

* Monocyte-depleted PBMC from EBV patients were cultured for 24 h alone, with fibroblast monolayers, or in the presence of 2 ng/ml of IL-2 or GM-CSF, respectively.
‡ Percentage of cells in apoptosis was determined by morphology in cytocentrifuge preparations.
§ Significantly different from control as determined by the Student's *t* test ($p<0.001$).
‖ Not significantly different from normal.

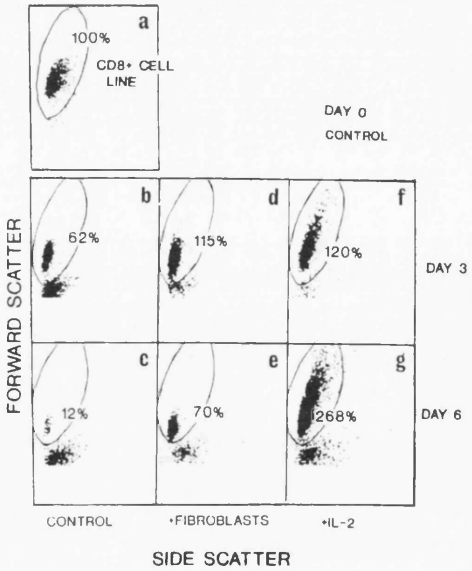

Figure 7. The prevention of apoptosis in an IL-2-dependent CD8+ T cell line by cocultivation on fibroblasts. IL-2-dependent lines (*a*: day 0 control) were cultured without IL-2 (*b* and *c*) on fibroblasts (*d* and *e*) or with IL-2 (2 ng/ml; *f* and *g*), and their absolute numbers were counted on the Cytoron and shown as percent surviving cells. The representative results from one of five experiments are shown. Similar results were also obtained with IL-2-dependent CD4+ T cell lines.

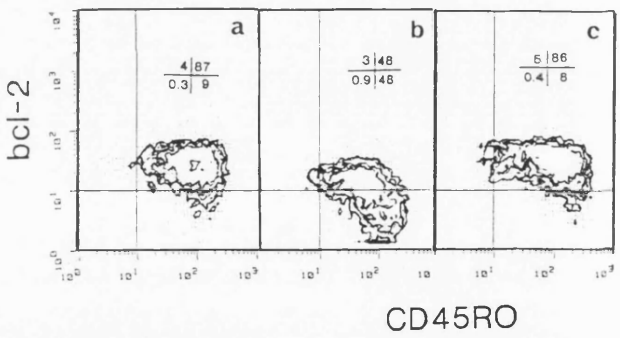

Figure 8. The changes of bcl-2 expression (*y-axis*) in correlation with CD45RO positivity (*x-axis*) in an IL-2-dependent CD8+ T cell line when these cells are cultured for 6 d in the presence of IL-2 (*a*) or fibroblasts (*b*). The bcl-2 expression of strongly CD45RO+ viable lymphocytes in the fibroblast cocultures dropped to low levels during the 6-d cultures (*b*). When these cells were recultured for 48 h in the presence of IL-2 (2 ng/ml; *c*) the bcl-2 expression returned to high values. Cells in similar cultures kept without IL-2 exhibit poor viability (data not shown, but see Fig. 7 *c*).

tained for both CD4+ and CD8+ cell lines were similar. When cells were cultured for 6 d in the presence of IL-2, >91% of the population remained bcl-2+ (Fig. 8 *a*). In control samples cultured for 6 d without IL-2, only 12% of the original cellular input could be recovered in the viable gate (Fig. 7*c*) and these residual cells remained bcl-2+ (77%; data not shown). If the CD8+ T cells were cultured for 6 d on fibroblasts after IL-2 withdrawal, 70% of the original cells remained viable despite their lower bcl-2 expression in 50% of cells (compare Figs. 7 *e* and 8 *b*). These findings suggest that fibroblasts maintain the survival of T cells in a bcl-2 low state.

We then investigated whether in the absence of fibroblasts these cells may undergo apoptosis. After 6 d the CD8+ T cell line which was cocultured with fibroblasts was removed from the fibroblast monolayers and recultured in the presence or absence of IL-2. After a further 48 h in the presence of this cytokine, >90% of these cells regained bcl-2+ expression (Fig. 8 *c*) and the cell recovery remained high (>95%), excluding the possibility of the selective death of bcl-2− cells. In the absence of IL-2, however, all cells perished within 96 h.

The Expression of bcl-2 by LNs of Normal and HIV-infected Individuals. The possibility that the bcl-2 expression by T cells from virally infected patients is an artefact in vitro has been excluded by documenting the presence of bcl-2−, CD8+ cells within LN populations from patients with viral infections. There was strong expression of bcl-2 in >90% of T cells in normal tonsil tissue. These bcl-2+ cells included CD4+ T cells localized inside the germinal centers (data not shown) as well as the CD8+ lymphocytes within the paracortical areas (Fig. 9, *a* and *b*). A different pattern was observed in the LNs of patients infected with HIV (Fig. 9, *c* and *d*). Extensive infiltrates of CD8+ cells were seen and the majority (60–80%) of these CD8+ T cells expressed CD45RO (31). A large proportion (30–60%) of these CD8+ T cells had reduced or negative bcl-2+ expression (Fig. 9, *c* and *d*). The pattern of bcl-2 reactivity in the B cells was, however, similar to that found in normal individuals revealing bcl-2 positivity in the B lymphocyte corona and low bcl-2 expression by B blasts inside the germinal center (data not shown).

Discussion

Previous studies have established the role of bcl-2 in the regulation of cell survival associated with the selection and/or deletion of germinal center B lymphoblasts (5, 6, 10) and cortical thymocytes (4, 9, 12). In these lymphoid organs, the loss of bcl-2 is linked with a short life span whereas the upregulation of this protein results in the relative longevity of these recently generated cells. The bcl-2 protein has been shown to block apoptosis which enables the disposal of unwanted cells to occur (1, 7, 8). Nevertheless, the role of this protooncogene product has thus far remained uncharted in the regulation of life span and memory development in mature T cell populations despite the fact that, when stimulated by antigen, these cells also undergo differentiation (18–20).

In histological studies it has already been established that the majority of T cells in the paracortical areas are bcl-2+ (4, 29). We now show that not all circulating T cells are bcl-2+, but that a minor bcl-2−, CD3+ population is present in the blood. In normal individuals these cells are primarily of a CD4+, CD45RO+ primed phenotype, although the relatively small CD8+, CD45RO+ population in these individuals also shows low expression of this protein. As CD45RA+ T cells have been shown to develop CD45RO as a consequence of activation (17–20), these

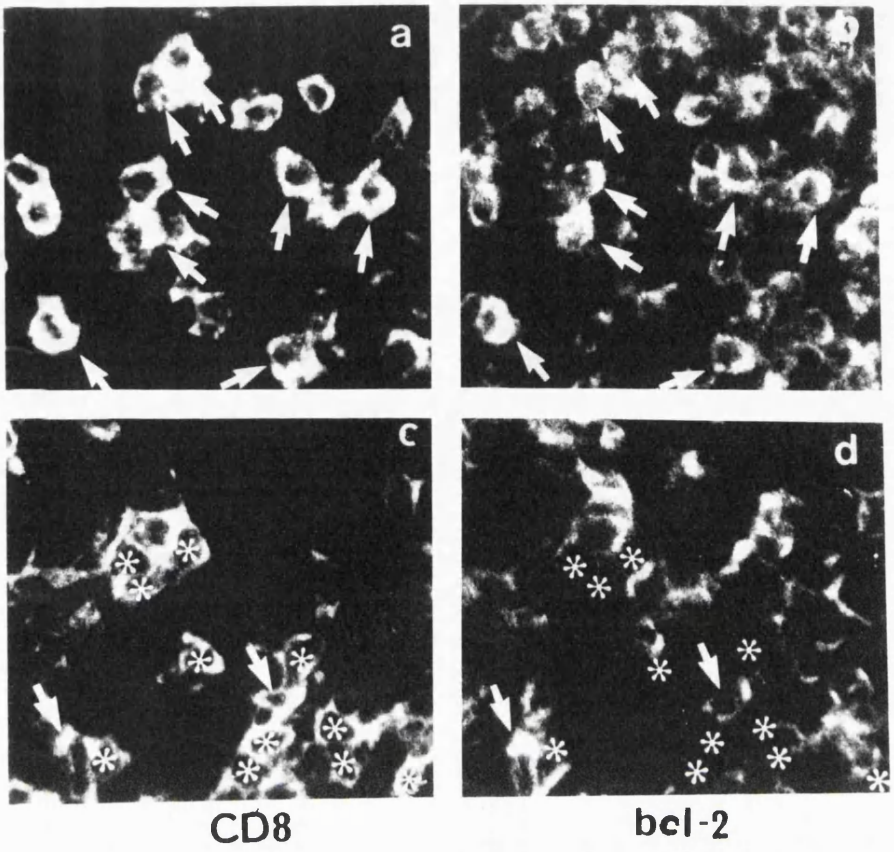

Figure 9. The CD8$^+$ cells in normal tonsil (*a*) mostly express bcl-2 (*arrows*, *b*) whereas CD8$^+$ cells in HIV-1–infected LN (*c*) frequently lack this protein (*d*, *). These sections were stained with double immunofluorescence for CD8 (*a* and *c*) and bcl-2 (*b* and *d*) and the same areas (*a* and *b* and *c* and *d*) were photographed with selective filters.

findings indicate that bcl-2 might be downregulated during the activation process. We investigated this possibility in two ways. First, patients with recent acute viral infections were studied to determine whether activated T cells, developing in vivo, lose their bcl-2. Such patients, particularly those who have EBV and VZV infections, have expanded circulating T cell populations expressing HLA-DR and CD45RO (14, 36). Second, we isolated CD45RA$^+$ lymphocytes within both CD4 and CD8 subsets which were stimulated with PHA in 7-d cultures in order to investigate changes in bcl-2 expression in parallel with the transition from a CD45RA to a CD45RO phenotype in vitro. These studies clearly establish that the bcl-2 downregulation is associated with the development of CD45RO$^+$ cells after activation and that these bcl-2$^-$, CD45RO$^+$ cells include a subset of both CD4 and CD8 lymphoblasts. Nevertheless, in viral infections, the CD45RO$^+$, CD8$^+$, bcl-2$^-$ subset is predominant in the circulation, but low bcl-2 expression can also be demonstrated in a much smaller cohort of primed CD4$^+$ T cells when sensitive single cell methods are used in these patients (14).

It has been well documented that the T cell lymphocytosis associated with both EBV and VZV infections is transient as the absolute number of circulating T lymphocytes and the relative proportions of CD4$^+$ and CD8$^+$ cells return to normal upon resolution of the disease (13–15, 37). This suggests a rapid clearance of the majority of activated T blasts in vivo. Indeed, the apoptotic death of these T cells has been demonstrated by both morphology and DNA cleavage (36, 38). A balance must exist between cell death and survival, however, as immunological memory is retained after these infections and a higher cytotoxic precursor frequency of EBV and VZV specific T cells is found after primary infection (13, 16). We now propose that bcl-2 regulation may play a pivotal role in this balance, and that apoptosis is a major mechanism for the removal of unwanted T cells after resolution of viral disease.

Programed cell death or apoptosis, a suicide pathway resulting in endonuclease activation and the subsequent cleavage of DNA into nucleosomal fragments (39), is important in physiology, e.g., in metamorphosis, embryogenesis, and tissue atrophy where the restriction of cell numbers is essential (7). Apoptosis may also play a part in the life cycle of mature T cells because activated T cells and T cell lines perish by this process if IL-2 is removed (27). We have shown that the apoptosis of activated T cells from EBV and VZV patients is correlated with their reduced bcl-2 expression. The association of apoptosis with low bcl-2 expression is, however, not disease specific, as it was found in all acute viral infections studied including EBV and VZV, as well as the single case of acute HIV-1 infection studied. It is of interest that in normal individuals the Fas antigen, a marker associated with apoptosing cells, is elevated on CD45RO$^+$ as compared with CD45RA$^+$ T cells, and that there is a further increase of Fas on the CD45RO$^+$ lymphoblasts in EBV-

infected individuals (36). Thus, the relationship between bcl-2 and Fas expression appears to be reciprocal on CD45RO$^+$ T cells, and it would be of importance to determine if both molecules have roles in the same or different pathways leading to apoptosis.

Our data would suggest that after T cell activation in vivo, the expanded bcl-2$^-$ population is destined to perish unless these cells are rescued from apoptosis. This situation is analogous to the rescue of bcl-2$^-$ apoptosis-prone germinal center B cells by surface Ig and CD40 ligation and also by the presence of cytokines such as IL-4 (5, 6). The downregulation of bcl-2 as a result of both T and B cell activation provides a means by which an expanded lymphoid pool, arising as a result of immune stimulation, can be decreased, thus enabling the reestablishment of cellular homeostasis.

We have described two mechanisms that prevent the apoptosis of activated T cells. First, the continued presence of IL-2 maintains these cells in an activated state with elevated expression of bcl-2. Second, the interaction of T cells with a soluble fibroblast–derived factor enables these cells to return to a restinglike state but with low bcl-2 expression. In both these situations, the continued presence of IL-2 or the fibroblast factor is required and the T cells rapidly apoptose (at least in vitro) if either is removed.

Previous reports have already demonstrated that the coculture of apoptosis-prone leukocytes such as neutrophils (40), eosinophils (41), leukemic cells (30), and activated T cells (35) with monolayers of fibroblasts can prevent apoptosis and maintains the viability of these cells. The mechanism by which fibroblasts promote the survival of leukocytes, some of which are apparently bcl-2$^-$, is unknown at present. Fibroblasts produce a wide array of cytokines, including TGF-β, GM-CSF, IL-1β, IL-6 (42) and also extracellular matrix proteins such as collagen, vitronectin, and fibronectin (43), all of which may be potential candidates for promoting T cell survival. Other mechanisms, apart from IL-2 and fibroblasts, may also be required for the survival of activated T cells in vivo and enable these cells to return to a resting state. One such mechanism may be the engagement of the CD28 costimulatory pathway, in analogy with the prolongation of survival of germinal center B cells by the crosslinking of surface Ig together with CD40 ligation (6).

One of the crucial questions to be answered is how the proportion of activated T cells destined for either apoptotic death or for survival after an immune response is determined in vivo. One possibility is the competition for survival factors by activated T cells (7) and these factors fall into two broad categories. The first category of signals promote survival by maintaining the activated cells in cycle. In contrast, the second group are those produced by stromal cells such as fibroblasts which enable the cells to return to rest. At the end of an immune response, limiting concentrations of the first set of factors such as antigen and/or cytokines would ensure that only the most efficient cells, i.e., those with the greatest affinity for antigen or the most efficient signal transduction pathways obtain sufficient stimuli to remain in cycle and survive (7). Some of the activated T cells, however, may be induced to return to rest by stromal factors but limiting amounts of these factors will, once again, only promote the viability of the most competitive cells. The competition between activated T cells will on the one hand enable homeostasis to take place after immune activation, as the cells that do not obtain these factors will perish, and on the other permit the retention of the most competent primed cells.

Many studies have shown that T cell memory resides within the CD45RO$^+$ subset (17–20). It may therefore seem paradoxical that CD45RO$^+$ T cells express low bcl-2 and are destined for an apoptotic death. It has been shown, however, that although memory to an antigenic encounter may persist for decades, the average life span of human T cells is less than 2 yr (44). The unexpectedly short T cell life span, together with the observations that recall responses to antigen reside within a cycling population of CD45RO$^+$ cells (17, 44, 45) have suggested that T cell memory may persist as a consequence of the continual stimulation of a previously primed population (17). This hypothesis is supported by the demonstration that T cell memory responses in vivo are dependent on the presence of the original priming antigen (46). When placed in the context of our current data, this indicates that mechanisms that keep primed CD45RO$^+$ T cells in cycle will elevate the bcl-2 in these cells, promote their survival, and thus allow for the persistence of a dynamically generated memory population. Alternatively, in the absence of reactivation, the propensity of CD45RO$^+$ T cells to extravasate (45, 47) will facilitate their encounter with stromal fibroblasts and secure their viability until subsequent antigenic reencounter. This latter observation has implications for autoimmune disorders where fibroblast–lymphocyte interactions may become aberrant (35).

Collectively, our data suggest a scheme by which the changes in expression of the bcl-2 gene product by activated cells play a pivotal role in the balance between T cell death and survival after activation. The rescue of bcl-2$^-$ primed T cells by various factors enables the generation of a dynamic primed/memory T cell population, yet also allows for the homeostatic maintenance of T cell numbers after immune stimulation in vivo.

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Lymphocyte activation in HIV-1 infection. II. Functional defects of CD28– T cells

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Objectives and design: The expression of the accessory molecule CD28 was compared in various populations of T and natural killer (NK) cells from HIV-1-negative and HIV-1-positive individuals and correlated with activation using mitogens *in vitro*.

Methods: Multiparameter flow cytometric analysis using combinations of CD3 CD28 and other markers was performed together with absolute cell counting in peripheral blood. Blast transformation and proliferative responses were also quantitated using the $\text{Cytoron}_{\text{absolute}}$ after stimulation with phytohaemagglutinin (PHA) and anti-CD3. CD28– cells were also purified to confirm the observations.

Results: In HIV-1-negative individuals >90% of CD3+ T cells were CD28+ and responded to stimulation, while CD3– CD16+ CD57+ NK-like cells were CD28– and failed to respond. In HIV-1-positive individuals the expression of CD28 was greatly reduced and the proportion of CD3+CD28– T cells expanded. CD8 lymphocytosis was caused entirely by the accumulation of CD28– T cells and many of these expressed activation markers human lymphocyte antigen-DR, CD38 and CD45RO on their membrane and molecules such as TIA-1 and perforin, associated with cytolytic function, in their cytoplasm. The strong positive correlation ($r = 0.66$) between the lack of CD28 expression and the poor proliferation from HIV-1-positive individuals was confirmed by demonstrating that only CD28+ cells transformed into lymphoblasts and proliferated. Although the CD28– including CD3+ T cells transiently expressed CD25 (interleukin-2Rα), they did not undergo blastogenesis or activation measured by bromodeoxyuridine uptake and died after 3–4 days in culture. These observations were confirmed in costimulation experiments with anti-CD2 and anti-CD28.

Conclusion: In HIV-1 infection activated CD3+CD28– T cells accumulate but are unresponsive to mitogens and anti-CD28. These cells appear to represent terminally differentiated effector cells which fail to respond to further stimuli because of the absence of a CD28 second signal.

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Keywords: Immunodeficiency in HIV infection, AIDS, lymphocyte transformation, costimulatory molecules, CD4, CD8, CD28, perforin

Introduction

Individuals with HIV-1 infection experience severe immunodeficiency even before overt CD4+ lymphocyte depletion develops. The proliferative responses of T-lymphocytes are impaired when investigated with mitogens such as anti-CD3 and phytohaemagglutinin (PHA) or specific antigen [1–3]. These defects might be caused by apoptosis of some CD4+ and CD8+ lymphocytes [4]. Furthermore, the activation of lymphocytes from HIV-1-positive donors leads to cell losses that do not show typical features

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of apoptosis [5]. These functional defects are likely to be associated with changes in lymphocyte populations. In particular, CD8+ lymphocytes that are characterized by the co-expression of CD45RO, human lymphocyte antigen (HLA)-DR and CD38 accumulate [6–10]. There is also an increase in the number of CD57+ T lymphocytes that are distinct from typical natural killer (NK)-like cells because they co-express CD3 [11,12].

The importance of costimulatory molecules in T-lymphocyte activation is well documented [13]. One such molecule, CD28, is of particular interest because it strongly facilitates activation via the T-cell receptor complex (TCR) or the CD2 molecule without being mitogenic on its own [14–16]. The engagement of CD28 is currently the strongest known 'second signal' in human T-lymphocyte activation [13]. One of the natural ligands for CD28, B7/BB1 [17] is expressed on antigen-presenting cells (APC) including activated monocytes and B cells [18–20], suggesting a role for CD28 in the interaction with APC [20]. The potentially close functional relationship between the TCR and CD28 system is indicated by the expression of CD28 antigen, among peripheral blood lymphocytes (PBL), on >95% of CD4+ and approximately 50% of CD8+ populations [21,22], while among CD3-negative CD16+ NK cells, CD28 is absent [23]. Despite its importance as a costimulatory molecule, in HIV infection CD28 expression has not been fully correlated with the phenotypic shifts of lymphocyte subsets or with functional features such as responsiveness to mitogens or antigens. This is despite the fact that in a small group of HIV-1-infected individuals decreased CD28 expression has been recorded in a population of CD8+ cells [24]. It is not known, however, whether these are NK cells or CD3+ T lymphocytes. In another study of HIV-1-positive haemophiliacs, CD4+ cells showed lower than normal proportions of CD28+ cells [25].

A possible explanation for an increase in lymphocyte vulnerability during HIV-1 infection could be the loss of CD28 antigen on T lymphocyte, leading to the absence of an obligatory second signal during activation. In this study, we have investigated CD28 expression in the various T-cell subsets by three-colour immunofluorescence and compared these results with the blast transformation and proliferation of the same cell populations. During the study it was necessary to investigate the CD28+ enriched and purified CD28– populations in order to confirm the uniformly poor responses of the CD28– cohort to a variety of stimuli *in vitro*.

Subjects and methods

Subjects

The HIV-1-seropositive cohort of 70 individuals attended outpatient clinics at the Royal Free Hospital, London between October 1991 and December 1992 and were grouped according to the Centers for Disease Control and Prevention (CDC) classification [26], where stage II refers to asymptomatic individuals, III to subjects with lymphadenopathy and IV to patients with symptomatic AIDS. The HIV-1-seronegative group were healthy laboratory personnel with a mean age of 31 years. The serological status of individuals was confirmed at the Department of Virology at the hospital, using a Wellcozyme HIV recombinant assay (Wellcome Diagnostics, Dartford, England, UK) in combination with an HIV-1 enzyme immunoassay (EIA; Abbott Diagnostics, Maidenhead, England, UK) and Serodia HIV particle agglutination test (Mast Diagnostics, Bootle, England, UK).

Lymphocyte purification

Venous blood was drawn into preservative-free heparin and mononuclear cells isolated on a Ficoll-Hypaque (Nycomed, Oslo, Norway) gradient. In some cases, peripheral blood mononuclear cells (PBMC) were separated into T-enriched (E+,CD2+) and non-T (B plus monocytic) fractions by neuraminidase treated sheep erythrocytes as described previously [27]. The CD2+ NK cells were removed from the E+ fraction by incubating the cells with saturating concentrations of CD16 [Leu 11b; immunoglobulin (Ig) M, a gift from Drs S. Brown and J.S. Thompson, University of Kentucky, Lexington, Kentucky, USA) and CD57 (Leu7; IgM, American Tissue Typing Collection, Rockville Maryland, USA) and lysing the positive cells, adding pretitrated, optimal concentrations of baby rabbit complement (Serological Reagents Ltd, East Grinstead, England, UK) as described previously [28]. This method yielded >98% pure CD3+ populations [28]. In some experiments CD28+ cells were depleted from PBMC using goat anti-mouse coated magnetic beads (Dynal UK Ltd, Wirral, England, UK [29]).

Lymphocyte activation

Mononuclear cells obtained from heparinized venous blood were cultured in triplicate wells in flat bottomed 96-well microplates (Becton Dickinson Ltd, Cowley, England, UK). Lymphocytes were adjusted to 10^6/ml in RPMI-1640 medium (Gibco Ltd, Glasgow, Scotland, UK) supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin, 100µg/ml streptomycin (Gibco Ltd) and 2mM L-Glutamine (ICN Biomedicals Ltd, High Wycombe, England, UK) and stimulated with 1µg/ml PHA (PHA-P, Wellcome Reagents Ltd, Beckenham, England, UK). The cultures were routinely supplemented with 1 ng/ml recombinant interleukin-2 (rIL-2; provided by Dr Max Schreier, Sandoz Pharma Ltd, Basel, Switzerland). Other cultures were stimulated with a pair of anti-CD2 reagents; OKT11 (ATTC) and GT2-1D7 (Life Technologies Ltd, Paisley, Scotland, UK), both at pretitrated optimal concentrations, plus anti-CD28

(Kolt2 ascites; IgG1) at a final concentration in culture 1 : 5000.

Lymphoblasts developing in the cultures after activation were counted on the $Cytoron_{Absolute}$ (Ortho Diagnostics Ltd, High Wycombe, England, UK), a cell counter that enumerates the absolute number of cells [5]. Proliferative activity was measured at 90–94 h by ^{3}H-TdR uptake (2Ci/mmole, Amersham International, Amersham, England, UK).

Immunofluorescence studies

The expression of CD28 on lymphocyte populations was investigated by double- and triple-colour indirect immunofluorescence (IF) techniques. The monoclonal antibodies (MAb) were used in conjunction with goat antimouse (GAM) Ig coupled to fluorescein-isothiocyanate (FITC), phycoerythrin (PE) and biotin, together with streptavidin-tricolor (CALTAG, Bradsure Biologicals Ltd, Market Harborough, England, UK). MAb used included, CD28 (Kolt2; [30]) and two NK-associated markers CD16 (Leu11b, IgM) and CD57 (HNK-1, IgM). The CD4 (T4-407, IgG2a; Oxoid Ltd, Basingstoke, England, UK) and CD8 (RFT8, IgM) were used to identify helper and suppressor/cytotoxic subsets of T cells, respectively [31]. A CD3 MAb (MEM57, IgG_{2a} and T10B9, IgM) were kindly provided by Drs J. Horejsi, Charles University, Prague and S. Brown and J.S. Thompson, University of Kentucky. The CD45RA (SN130; IgG1) and CD45RO (UCHL1; IgG2a, provided by Prof. P.L.C. Beverley, University College and Middlesex School of Medicine, London) were shown previously to react with unprimed and primed T cells, respectively [27,32]. A CD25-FITC (RFT5, IgG1) was used to detect the IL-2R α-chain. The second layers were GAM-IgG1-PE, GAM-IgG2a-FITC and GAM-IgM-FITC (Southern Biotechnology Associates Inc., Birmingham, Alabama, USA).

The membrane expression of CD38 molecules was determined using a quantitative immunofluorescence indirect assay (QIFIFIT; Biocytex, Marseilles, France; [33]).

Staining for cytotoxic granule-associated proteins was performed on cytospin preparations using the MAb TIA-1 (IgG1; Coulter Immunology, Hialeah, Florida, USA; [34]) and an anti-perforin antibody PA1 (IgG2b; a gift from Prof. B. Dupont, Memorial Sloan-Kettering Cancer Center, New York, New York, USA; [35]). Purified CD28– T cells were double-stained with anti-CD57 plus GAM-IgM-TRITC(SBA) in combination with either TIA-1 or anti-perforin plus GAM-IgG-FITC(SBA).

Bromodeoxyuridine (BrdU) incorporation

Lymphocytes cultured with PHA for 48 h were incubated with BrdU (Sigma Chemical Co. Ltd, Poole, England, UK) at a concentration of 10μmol for 4 h [36]. Cytospin preparations were fixed in acetone for 15 min prior to triple staining with anti-BrdU (Amersham International), GAM-IgG2a FITC (SBA) and membrane markers CD57 labelled with GAM-IgM conjugated to Lissamine rhodamine B (Jackson Immunoresearch Inc., West Grove, Pennsylvania, USA) and CD28-biotin (SBA) plus streptavidin conjugated Cy5 (Biological Detection Systems Inc., Pittsburgh, Pennsylvania, USA). The triple-labelled cells were viewed using a laser scanning confocal microscope (Bio-Rad Laboratories Ltd, Hemel Hempstead, England, UK) equipped with lines at 488, 568 and 647 nm.

Statistical analysis

Unless stated otherwise, the data are presented as arithmetic mean ± SEM. Mean values were compared using Student's t-test for independent means; P values < 0.05 were considered significant. In some experiments linear regression analyses were performed on data to determine the correlation between the two variables.

Results

CD28 expression during HIV-1 disease

Initial studies to investigate the normal distribution of CD28 on lymphocyte subsets revealed that 98 ± 2.4% (n = 10) of CD28+ cells were CD3+ T cells. This constituted about 90% of the total CD3+ population while Ig+ B-cells, CD4+ monocytes and CD3– NK cells, defined by the expression of CD16 or CD57, were negative. The proportion of CD28– cells within the CD16+CD3– and CD57+CD28– NK populations were >99%.

A comparison between CD28 expression in HIV-1-positive individuals and HIV-1-negative controls revealed that the vast majority of CD28+ lymphocytes, as with HIV-1-negative controls, were seen within the CD3+ population. Nevertheless, the proportions and absolute numbers of CD3+ T cells that expressed CD28 were significantly reduced in HIV-1-positive individuals at every stage of disease because of the expansion of a CD3+CD28– population (control, 0.14 ± 0.026; CDC stage II, 0.68 ± 0.13; III: 1.04 ± 0.16; IV, 0.47 ± 0.14 × 10^9/l; $P < 0.005$; Fig. 1).

Most residual CD4+ T cells in the CDC stage II and III groups still expressed CD28. A substantial drop in the proportion of CD28+CD4+ cells ($P < 0.006$; Table 1) could only be detected in the CDC stage IV patients, but because of the decrease in CD4+ cells the overall absolute numbers of CD4+CD28– T cells remained the same during the course of HIV-1 infection (control, 0.05 ± 0.01; CDC stage II, 0.02 ± 0.001; III, 0.01 ± 0.002; IV, 0.05 ± 0.027 × 10^9/l; Fig. 1).

These results suggest that the increased number of CD28– T cells during HIV-1 disease were present in the CD8+ population. The increased CD8 numbers

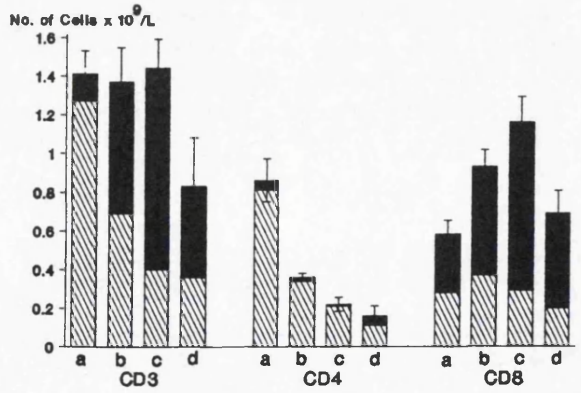

Fig. 1. The contribution of CD28+ (▨) and CD28– (■) lymphocytes within the CD3+, CD4+ and CD8+ subsets from HIV-1-negative controls and HIV-1-infected individuals. Two-colour immunofluorescence was used to determine the percentage of CD28+ and CD28– subsets within the lymphocyte subsets. The total number of CD3+, CD4+ or CD8+ lymphocytes is shown as the total height of the bar while the shaded areas represent the numbers of CD28+ or CD28– cells within each group. A total of 53 individuals were investigated: control (a), 10; HIV-1-positive Centers for Disease Control and Prevention (CDC) stage II (b), 22; stage III (c), eight; stage IV (d), 13.

Table 1. The expression of CD28 on T-cell subsets.

		CD3+CD28+	CD4+CD28+	CD8+CD28+
HIV-1-negative control	10	89.8±0.7	94.3±1.2	48.0±6.4
HIV-1-positive				
CDC II	22	49.8±6.3*	85.3±5.6§	40.1±4.1§
CDC III	8	27.6±8.3*	96.4±6.7§	26.7±6.1†
CDC IV	13	42.9±4.3*	54.9±13.9‡	30.3±4.7†

The percentage of CD28+ lymphocytes within the CD3+, CD4+ and CD8+ T-cell population was determined by two-colour immunofluorescence in HIV-1-negative controls and HIV-1-infected individuals. The data were compared with the control group using Student's t-test; *$P<0.001$; †$P<0.05$; ‡$P=0.006$; §not significant. The values between Centers for Disease Control and Prevention (CDC) stage II, III and IV groups are not statistically significant at the 5% level.

were caused by a selective increase of CD8+CD28– lymphocytes (control, 0.30±0.026; CDC stage II, 0.56±0.07; III, 0.87±0.15; IV, 0.49±0.09×10^9/l; $P<0.003$; Fig. 1). The CD8+CD28+ population remained constant throughout (control, 0.28±0.07; CDC stage II, 0.37±0.04; III, 0.29±0.06; IV, 0.20±0.045). Furthermore, triple-colour studies using CD3, CD8 and CD28 revealed that in HIV-1-negative individuals, 76.8±3.5% (n=10) of CD3+CD8+ lymphocytes were CD28+ and the CD3–CD8+ variants of NK cells were entirely CD28-negative (Fig. 2a and b). In contrast, in HIV-1-positive individuals, the percentages of CD3+CD8+CD28– were increased (control, 25.6±6.6; HIV-1-positive CDC stage II, 64.5±4.9; Table 2) resulting in a dominance of CD28 lymphocytes within the CD8+ T cells.

Phenotypic features of CD28– T cells in HIV-1 infection

Because HIV-1 infection is associated with phenotypic changes in peripheral blood, studies were

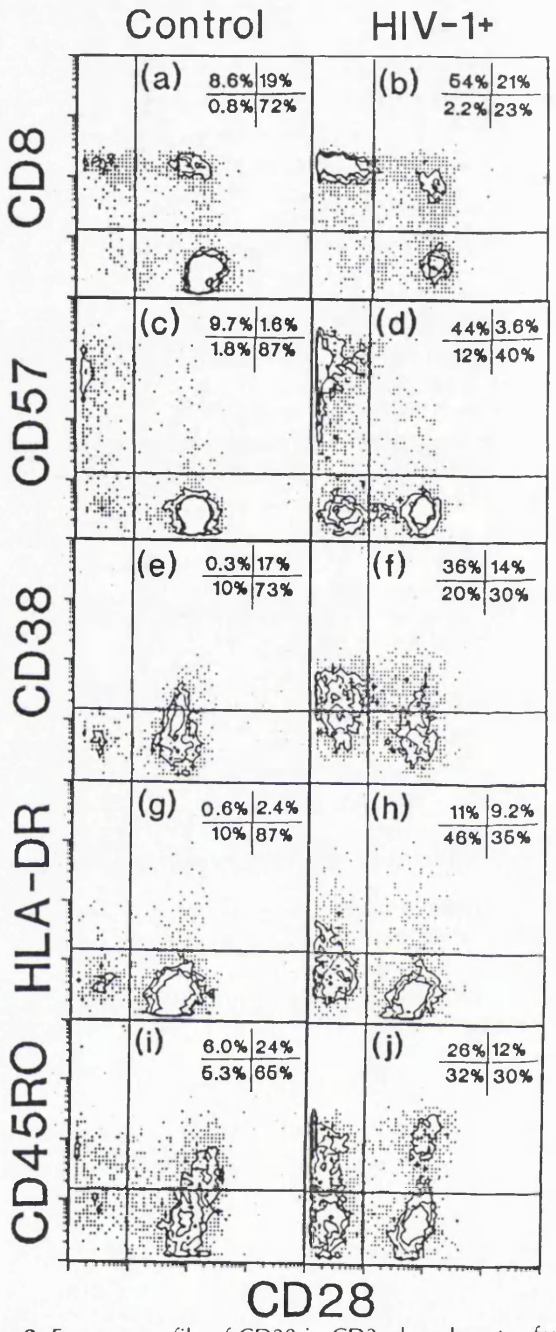

Fig. 2. Facscan profile of CD28 in CD3+ lymphocytes from control and HIV-1-positive individuals. Triple-colour combinations of CD3 and CD28 plus one of CD8, CD57, CD38, human lymphocyte antigen (HLA)-DR or CD45RO were used to illustrate the distribution of CD28– in CD3+ T cells. During data acquisition, a gate was set up around the CD3+ cells and the expression of CD28 investigated. Data from one representative control (a,c,e,g,i) and one HIV-1-positive individual (b,d,f,h,j) are shown.

performed using triple-colour IF to clearly define the rare CD3+CD28– lymphocyte subset in control and the dominant CD3+CD28– cells in HIV-1-infected individuals. These are shown in Table 2 with representative examples in Fig. 2. Using the triple combination CD3, CD28 and CD8 found that in HIV-1-

Table 2. Phenotypic features of CD28– T cells*.

	Part I: Expression of markers within the CD3+CD28– gate†				
	CD8	CD57	CD38	HLA-DR	CD45RO
HIV-1-negative	55.0±12.9	65.4±5.2	<1%	<1%	52.6±10.8
HIV-1-positive	89.8±2.1	54.2±7.1	45.7±2.0	33.9±9.9	57.7±6.0
	Part II: Percentage of CD28– cells within				
	CD3+CD8+	CD3+CD57+	CD3+CD38+	CD3+HLA-DR+	CD3+CD45RO+
HIV-1-negative	25.6±2.8	74.8±4.8	1.6±1.3	ND	10.8±4.2
HIV-1-positive	64.5±4.9	89.4±2.0	45.1±8.0	67.3±2.4	56.5±1.7

The phenotypic features of CD28– T cells were investigated by triple-colour immunofluorescence studies. During data analyses cells were gated either according to CD3+CD28– expression (Part I) or CD3 in combination with either CD8, CD57, CD38, human lymphocyte antigen (HLA)-DR or CD45RO (Part II). The results are shown as the mean percentage±SEM for 10 HIV-1-negative and 10 HIV-1-positive Centers for Disease Control and Prevention stage II individuals. †Percentage increases in all markers were noted within the CD3+ gate in HIV-1-infected individuals (see Results). ND, not determined because of the paucity of cells of this phenotype.

negative blood 55% of CD28– T cells were CD8+. As expected, in HIV-1 infection the vast majority of CD3+CD28 lymphocytes were CD8+ (89.8±2.1; Table 2; Fig. 2b).

When CD57+ lymphocytes were investigated, CD57 and CD28 were found in separate lymphocyte populations in both HIV-1-negative and HIV-1-positive blood, and only few (<5%) double-positive cells were seen (Fig. 2c and d). The percentages of CD3+CD57+ cells increased in HIV-1 infection (control, 6.9±1.5; HIV-1-positive CDC stage II, 26.7±5.0; $P<0.001$) and most CD57+ cells were CD3+CD28–, representing a significant proportion of the CD3+CD28– population (control, 65.4±4.9; HIV-1-positive, 54.2±7.1). Importantly, a particularly large proportion of CD3+CD57–CD28– cells remained in HIV-1 infection (0.35±0.04, 0.42±0.087 and 0.37±0.53×10^9/l in the CDC stage II, III and IV groups, respectively. These cells were rare in the control blood (<0.05×10^9/l).

Previous studies have indicated that the various isoforms of the CD45 antigen discriminate between resting CD45RA+ and recently activated CD45RO+ cells. In HIV-1-negative individuals, among the few CD3+CD28– cells, 52.6% were CD45RO+ (Fig. 2i), representing less than 10% of the total number of CD3+CD45RO+ cells. In HIV-1 infection, large proportions of CD8+ lymphocytes express CD45RO, and the absolute numbers of CD3+CD28–CD45RO+ cells were greatly increased (control, 0.05±0.01; HIV-1, 0.30±0.02; $P<0.001$). Within the increased CD3+CD28– subset 57.7% were CD45RO+. The CD28– component comprised up to 60% of the entire CD3+CD45RO+ population.

In HIV-1 infection CD3+ lymphocytes expressing activation markers such as CD38 and HLA-DR also increase. In seronegative donors, low levels of CD38 (<5×10^3 molecules/cell) were detected on a proportion (21.1±3.9%) of CD3+ T cells; these were predominantly CD28+ (>80%; Fig. 2e). The increase in CD3+CD38+ lymphocytes in HIV-1 infection was, however, caused by an expansion of the CD3+CD38+CD28– population (Fig. 2f). These CD38+ cells constituted 45.7±2.0% of the CD3+CD28– lymphocytes. Using a triple combination for CD3, CD28 and HLA-DR it was found that although both CD3+CD28+ and CD3+CD28– subsets could express HLA-DR (Fig. 2h) a greater proportion of these (65.4±8.3%) were CD28-negative.

TIA-1 and perforin expression

It is known that cells with cytotoxic capacity, such as polymorphs, NK cells and activated CD8+ effector T cells express the granule-associated TIA-1 molecule and, in a smaller proportion of cells, the perforin molecule [34,35]. These are cytoplasmic markers that were analysed in cytocentrifuge preparations. During HIV-1 infection both TIA-1 and perforin-positive cells accumulated within the CD8+ population. In HIV-1-negative individuals, after removal of CD16+ NK cells, 37% (range, 29–49%; n=10) of CD8+ lymphocytes expressed TIA-1 and 9.4% (range, 2–63%) contained perforin. This was increased in HIV-1-positive individuals (n=10) to 56% (range, 38–82%) and 21% (range, 2–52%) for TIA-1 and perforin, respectively. The expression of TIA-1 and perforin was also examined in purified CD3+CD28– cells from HIV-1-negative and HIV-1-positive patients. As CD57+ cells have been shown previously to have potent cytolytic activity [37] the TIA-1 and perforin content of the CD3+CD28–CD57+ population was determined by dual-colour immunofluorescence (Fig. 3). Within the CD3+CD28– population 90–100% of CD57+ lymphocytes from HIV-1-negative and HIV-1-positive donors expressed both TIA-1 and perforin (Fig. 3). Although perforin expression was restricted largely to the CD57+ population, TIA-1 was observed in CD57–CD28– cells (40–60%)

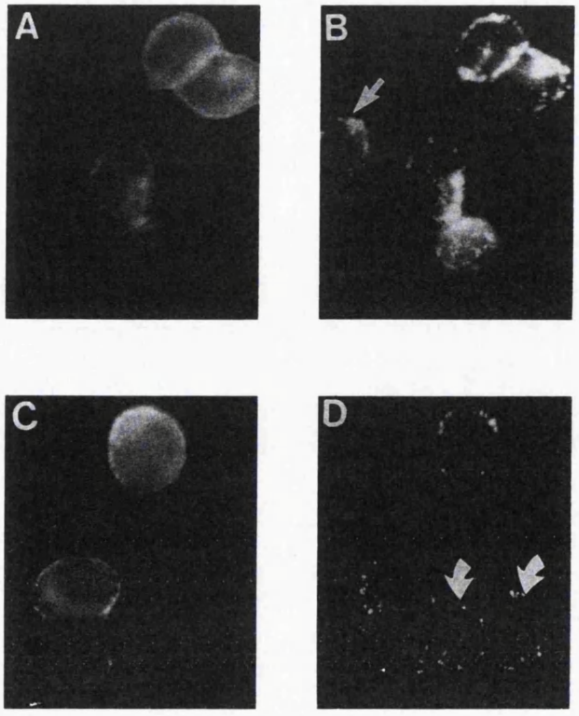

Fig. 3. The presence of cytotoxic granules in CD28– lymphocytes. A purified CD28– population was prepared from E+, CD16-depleted cells by negative selection with CD28. The remaining CD3+CD28– cells were double-stained for the membrane marker CD57 (a,c) and one of the cytotoxic granule-associated proteins; TIA-1 (b) or perforin (d). Note that all of the CD57+ cells express both TIA-1 and perforin. The arrows indicate the presence of CD57– cells that also contain the cytotoxic granules.

The proliferative defects of CD28– lymphocytes in HIV-1 infection

To determine whether the defects in lymphocyte stimulation in HIV-1 infection were related to the presence of CD3+CD28– lymphocytes, PBMC from HIV-1-positive individuals and controls were stimulated with PHA plus rIL-2. The number of lymphoblasts recovered on day 3 were used as a measure of lymphocyte activation and this method also provided useful information regarding the phenotype of responding lymphoblasts. In 56 HIV-1-positive individuals investigated, there was a highly significant positive correlation between the number of CD28+ cells present at the start of the culture and the number of lymphoblasts recovered after 3 days activation ($P<0.001$; Fig. 4). This correlation was further tested on CD28– cells following the removal of CD28+ T lymphocytes. Less than 10% of this population from HIV-1-positive individuals were able to transform into lymphoblasts after activation with PHA despite the addition of rIL-2. Conversely, the removal of CD57+ (CD28–) lymphocytes proportionally increased the number of lymphoblasts recovered, indicating that in this assay the residual CD28+ cells from HIV-1-positive donors are not handicapped and can respond to mitogens in the presence of rIL-2.

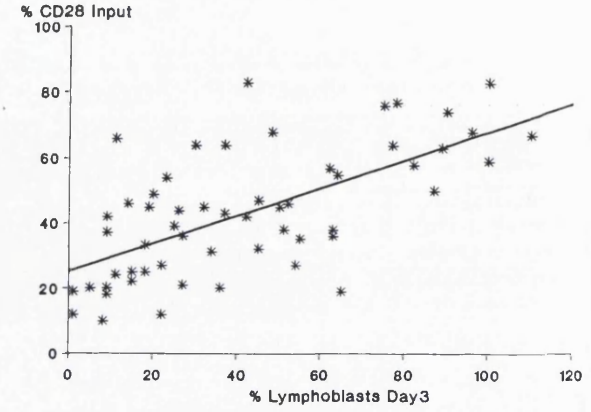

Fig. 4. The correlation between CD28 expression and lymphocyte activation responses. Peripheral blood mononuclear cells (PBMC) from 56 HIV-1-positive individuals were assayed for their expression of CD28 before activation with phytohaemagglutinin. The percentage of lymphoblasts developing in the cultures compared with the total cell input (x-axis), is plotted against the percentage of CD28+ cells present in the culture prior to activation (y-axis). Regression analysis confirmed the highly significant relationship between these two variables. The 56 HIV-1-positive individuals had a mean CD4+ lymphocyte count $0.301 \pm 0.034 \times 10^9$/l. The proportions of CD4+ and CD8+ lymphocytes in the PBMC were $16.2 \pm 1.1\%$ and $58.7 \pm 1.5\%$, respectively. $r = 0.66$; $P < 0.001$.

To demonstrate further that CD28– T cells, but not CD28+, have a defective proliferative response, we investigated the phenotypic features of cells surviving after 3 days activation (Fig. 5). The majority ($95 \pm 2.0\%$) of lymphoblasts recovered from the HIV-1 control cultures were CD28+. Among the fewer remaining blasts detected in the cultures from HIV-1-positive donors, the CD28 positivity was high ($89 \pm 3.70\%$); the absolute blast count at day 3 corresponded to the original CD28+ input at day 0. Thus the CD28+ cells selectively responded but the CD28– cells remained unresponsive. In addition, very few CD57+ blasts were seen in both the control and HIV-1-positive samples (control, 2.6 ± 0.36; HIV-1-positive 5.5 ± 0.63) and the rare surviving CD57+ blasts coexpressed low levels of both CD57 and CD28, indicating that the expression of CD28 might have contributed to their survival. Some other CD57–CD28– lymphoblasts constituted <10% of the total blast population but these were B cells.

When investigated for CD25 (IL-2Rα chain) expression after 2 days in culture, the majority of lymphocytes from HIV-1-negative or HIV-1-positive individuals expressed IL-2R (Fig. 6a). These CD25+ cells included the partially overlapping CD28– and

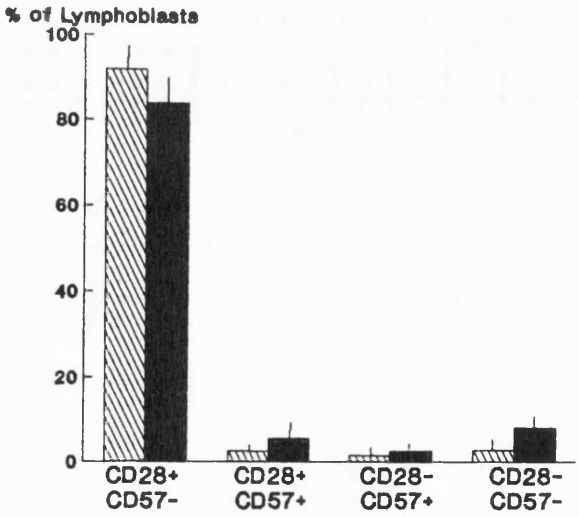

Fig. 5. Phenotypic study of the lymphoblasts developing in phytohaemagglutinin (PHA) stimulated cultures after 3 days. The phenotype of the lymphoblasts recovered after activation with PHA was determined with respect to CD57 and CD28. During data acquisition a gate was set up around the large lymphoblasts based on their forward and side scatter profiles. The results are shown as the percentage of total lymphoblasts from five HIV-1-negative controls (▨) and 10 HIV-1-positive individuals (■).

CD57+ populations but remained small lymphoid cells that failed to transform into lymphoblasts as measured by their cell size and BrdU incorporation (Fig. 6b). By day 3 of culture, however, these CD25+CD28–CD57+ cells had died.

The effect of costimulation via CD28 on activation responses

It has been reported that costimulation via CD28 restores the defective proliferative response of lymphocytes from HIV-1-infected individuals [38]. To evaluate this in relation to CD28 expression, cells were stimulated with combinations of anti-CD2 antibodies. These were used in suboptimal concentrations because mitogenic activity is significantly boosted by adding anti-CD28. PHA-stimulated cells were used as positive controls. Lymphocyte activation was measured by both a bulk assay of ^{3}H-thymidine uptake (c.p.m.; Fig. 7a and b) and by determining lymphoblast counts at day 3 in relation to the original T-cell input (Fig. 7c and d). In all cases the magnitude of response correlated closely with the expression of CD28 (c.p.m.: $r = 0.797$, $P = 0.006$ and lymphoblast percentage: $r = 0.834$, $P = 0.003$).

Individuals responded to each of the mitogens tested as shown in Fig. 7. In addition, when anti-CD28 was added as a costimulus to CD2 there was a significant increase in thymidine uptake. In the lymphocyte cultures from both HIV-1-negative donors and HIV-1-positive individuals the enhancement was similar (220–240%), indicating that this pathway of lymphocyte activation is still intact in CD28+ cells. However, the addition of anti-CD28

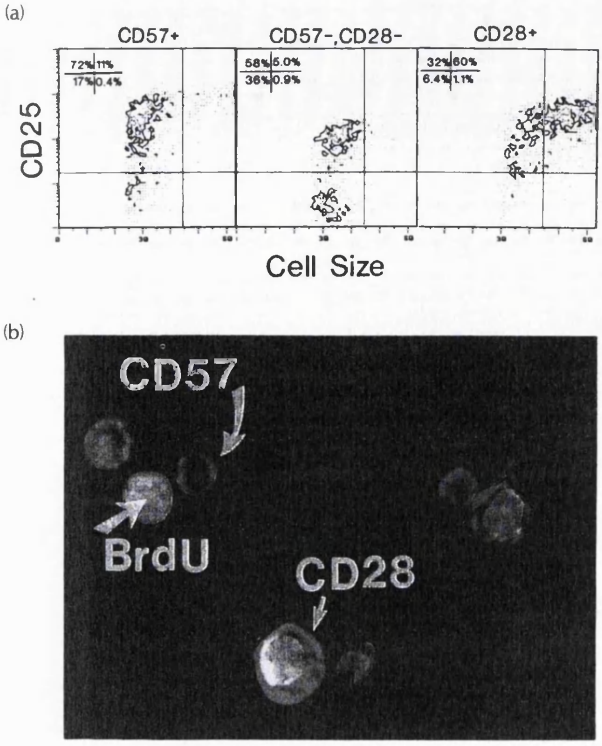

Fig. 6. The defective activation response of CD28– lymphocytes. The early upregulation of CD25 was investigated on CD57+CD57–CD28– and CD28+ lymphocytes after 2 days in culture with phytohaemagglutinin (PHA) (6a). During data acquisition the cells were gated according to their CD57 and CD28 phenotypes and the cell size (x-axis) in relation to CD25 expression (y-axis) plotted. The result from the representative patients shown indicate that although CD28 lymphocytes can upregulate CD25 they fail to transform into lymphoblasts. This was confirmed in cells incubated with bromodeoxyuridine (BrdU) (6b). Lymphocytes stimulated with PHA for 2 days were pulsed with BrdU and the phenotype of the BrdU+ blast cells investigated by three-colour immunofluorescence using confocal microscopy. The membrane stain for CD57 appears on small BrdU-lymphocytes while cells positive for membrane CD28 have transformed into large lymphoblasts with nuclear staining for BrdU.

to cultures of lymphocytes from HIV-1-positive individuals did not reconstitute the number of lymphoblasts observed at day 3 to the higher levels seen in cultures from HIV-1-negative donors. Neither the thymidine uptake nor the recoverable blast cell counts were fully normalized (Fig. 7b and d). These results indicate that the defective activation of CD28– T cells in HIV-1-positive individuals cannot be corrected by costimulation via anti-CD28.

Discussion

We have demonstrated that in asymptomatic individuals with HIV-1 infection CD3+ T cells accumulate that fail to express CD28. Such cells form only a minor population in HIV-1-negative individ-

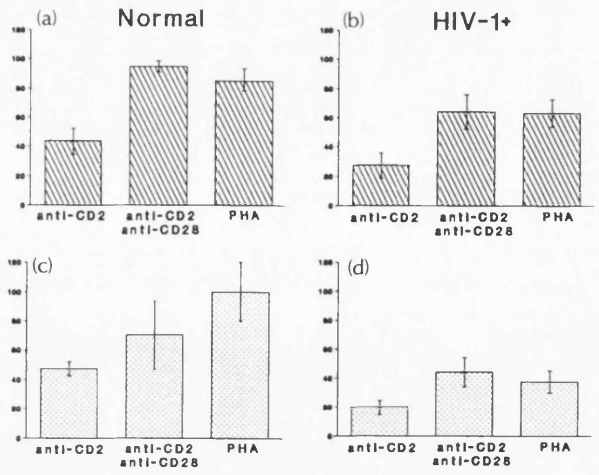

Fig. 7. The effect of costimulation via anti-CD28 on the activation responses of lymphocytes from HIV-1-negative and HIV-1-positive individuals. Peripheral blood mononuclear cells from five HIV-1 controls (a,c) and nine HIV-1-positive individuals (b,d) were activated with three different stimuli; combinations of anti-CD2, anti-CD2 plus anti-CD28, and phytohaemagglutinin. The activation response was measured by ^{3}HTdR incorporation (a,b) and lymphoblast recovery (c,d). ▨, c.p.m. $\times 10^3$; □, lymphoblast percentage.

uals where virtually all CD28– cells are members of the NK-cell family and do not express CD3. This enriched CD3+CD28 population in HIV-1 infection is essentially composed of CD8+ T cells. This cohort is responsible for the CD8 lymphocytosis seen throughout the whole course of disease. The data on CD28–CD8+ cells adds to the findings of Gruters *et al.* [24] who, in a study of five HIV-1-positive individuals, demonstrated a shift within the CD8+ population to a CD28– phenotype without documenting these cells' CD3 positivity. Although absolute numbers of CD4+CD28– cells do not increase in HIV-1 infection, this subset becomes proportionally more dominant with disease progression. These CD4+CD28– cells were found to coexpress CD57 (unpublished data), a marker that in CD4+ T cells is associated with a population unresponsive to mitogenic stimulation and that fails to release IL-2 [39]. Thus, the proportional increase in CD4+CD28–CD57+ cells in HIV-1 infection might add to the functional deficiencies of the CD4+ subset.

The CD28 antigen is the most important costimulatory molecule described to date on normal T cells because it transmits a second signal obligatory for T-cell activation [40]. We confirm that the expression of CD28 is linked to the ability of T lymphocytes to undergo blastogenesis in response to PHA, anti-CD3 and -CD2 *in vitro*, and also extend the relevance of these findings to HIV-1 disease. The CD3+CD28– populations in the blood of individuals with HIV-1 infection are deficient in responding to the strongest mitogens including PHA, anti-CD3, combinations of anti-CD2 and anti-CD28 stimuli even in the presence of rIL-2. We also observed that both the CD3+ and CD3– cohorts of CD28– T and NK cells transiently express CD25 but then perish in culture despite the addition of rIL-2. It has been reported previously that CD57+, presumably CD28–, lymphocytes from HIV-1-negative individuals do not respond to mitogenic stimuli as measured by BrdU uptake. A proportion of these cells remained lymphocytic in appearance after short-term activation with PHA [36] but could not be maintained *in vitro* for more than 48 h [41], suggesting an inherent defect in these cells.

We found that the increase in cells expressing the previously reported activation-associated markers CD38 and HLA-DR [8–10] occur predominantly in the CD3+CD28– population. Furthermore, the observations of CD45 isotypes reveal that these particular cells exhibit the features of primed T cells expressing CD45RO, while in HIV-1-negative blood the majority of CD28– lymphocytes are CD16+ NK-like cells which retain CD45RA+, a characteristic feature of unprimed lymphocytes [23]. The observations above also reveal that the primed CD8+CD28– T cells contain both TIA-1 and perforin, molecules associated with cytotoxic granules [34,35].

The functional commitments of these CD8+CD28– cells are still ambiguous. Studies using purified CD8+CD28+ and CD8+CD28– lymphocytes showed that only the CD28+ fractions generated cytotoxic cells during culture with allogeneic cells [42], while CD8+CD28– lymphocytes suppressed the mixed lymphocyte reaction response. These cells, as well as the same population which express CD11b, have also been shown to have suppressor activity in other systems [43–45] and in HIV-1-positive individuals CD8+CD57+ cells were found to secrete suppressor factors [46]. However, other studies reveal that CD3+CD8+CD28– cells exhibit cytotoxic activity but only when freshly isolated from blood because these cells do not respond well to activation *in vitro* [47], as documented in our analysis. The CD57+ cells in the peripheral blood of individuals with HIV-1 infection also included some cells with cytotoxic T lymphocyte activity [37]. These sets of data are not contradictory because CD28– T cells are likely to be a population of terminally differentiated effector cells that are cytotoxic only in short-term cultures while secreting suppressor moieties.

It has been demonstrated that activation of T cells via ligation of TCR in the absence of a costimulatory signal induces a state of anergy [48]. A powerful cosignal required for stimulatory T-cell activation is given through an interaction between CD28 and its ligand B7/BB-1 [17], which is expressed on antigen-presenting cells such as activated B cells [49] and dendritic cells [20]. While anergy is generally taken to be a transient state of unresponsiveness it has been suggested recently that there are vari-

ous levels of tolerance including anergy, and that anergic cells remain susceptible to further tolerogenic signals that eventually lead to deletion [50]. For example, Liu and Janeway [51] have shown that in mice, activation of T helpler$_1$ (TH_1) clones in the absence of accessory cells causes cell death by an as yet undefined mechanism. The lack of expression of CD28 on T cells from HIV-1-positive individuals may therefore explain the activation-induced cell death observed by the absence of a second signal [5].

Apoptosis is typically an active signal-dependent process with characteristic morphological and biochemical changes including nuclear degradation whilst membrane integrity is maintained [52]. Based upon these criteria, we believe that the activation-induced cell death described as a result of second signal defects may not be a typical apoptosis. In these stimulated cultures of blood taken from HIV-1-positive individuals only rare cells show intact membranes and fragmented nuclei. Furthermore, the proportion of DNA broken into oligosomal bands is less than that seen in suspensions of bcl-2–CD8+ cells undergoing typical apoptosis during acute infection with various viruses such as Epstein–Barr virus and varicella-zoster virus [53].

Costimulation with anti-CD28 has been reported to return proliferative responses to normal levels when measured by ^{3}H-TdR [39]. These results have been interpreted to show that anti-CD28 is able to save some cells from death and/or apoptosis. Nevertheless, anti-CD28 accelerates the activation cycle of CD28+ cells but is unlikely to act upon the CD28– populations. In our study, we confirmed that costimulation via anti-CD28 increased ^{3}H-TdR incorporation but this enhanced uptake was not due to the activation of CD28– cells that remained unresponsive. The quantitative results from the Cytoron absolute showed that the numbers of transforming lymphoblasts on day 3 were low despite adding anti-CD28, thus, among mixed CD28+ and CD28– T-cell populations costimulation via CD28 did not induce CD28– cells to regain their proliferative potential.

The process that leads to the development of CD28–CD8+ T cells during HIV-1 infection remains obscure. Studies on the JA3 cell line have shown that stimulation with anti-CD28 induces rapid modulation of CD28 [54]. Similarly, stimulation of PHA-activated lymphocytes with anti-CD28 results in a temporary decrease in both CD28 mRNA and surface expression of the protein [55]. The interaction of CD28 with its ligand might therefore result in the loss of the antigen from the cell surface. To determine whether CD28 had been modulated from the cell surface *in vivo* but was still detectable inside the cells, lymphocytes from HIV-1-positive individuals were investigated for the presence of cytoplasmic CD28; these were, however, undetectable (unpublished data). Furthermore, stimulation of PBL via anti-CD3 in short-term cultures does not comodulate CD28, indicating that these receptors are not physically linked [54]. In fact, activation for 7 days in culture has been shown to increase CD28 expression [56]. However, long-term cultures of T cells in medium supplemented with rIL-2 results in a progressive loss of CD28 [57]. It is therefore likely that CD28 reduction may require 2–3 weeks of continuous activation to occur, in agreement with the phenotypic characteristics suggesting the differentiated features of CD8+CD28– cells (see above).

The appearance of CD28– T lymphocytes in the peripheral blood, as shown in this study, provides one mechanism for severe functional immunodeficiency in asymptomatic HIV-1-infected individuals. These CD28–CD8+ T cells are primarily a blood-borne population, absent from the CD8+ infiltrates in lymph nodes taken from either HIV-1-negative or HIV-1-infected individuals (unpublished data). Similarly, CD16+ and CD57+ NK cells are also restricted to the blood and absent from the normal lymphoid tissue [58]. Conversely in HIV-1-infected lymph nodes the dominant CD8+ T cells express CD28 and show decreased levels of bcl-2 [53], a protein that would normally save the cells from apoptosis [59]. Thus, different CD8+ T lymphocytes show differential tissue distribution during HIV-1 infection.

A corollary of these observations is that at least two mechanisms may contribute to CD8+ T cells dysfunction in HIV-1 infections. In the blood, CD28– cells lack a costimulatory signal and remain anergic when challenged by antigens. In the lymph nodes the dominant CD8+ T cells may be short-lived and primed for apoptosis [53]. The regulation disorders driven by chronic viral load in HIV disease that lead to these divergent changes should be the subject of further investigations.

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Flow cytometric analysis of the stimulatory response of T cell subsets from normal and HIV-1$^+$ individuals to various mitogenic stimuli *in vitro*

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SUMMARY

A novel technique is described which allows the study of the responses of T cell subpopulations stimulated in bulk cultures without interfering with cell–cell interactions. The number and phenotype of lymphoblasts developing following stimulation with phytohaemagglutinin (PHA), anti-CD3, staphylococcal protein A (SPA) and pokeweed mitogen (PWM) was determined in HIV-1$^-$ and HIV-1$^+$ patients using a new five-parameter flow cytometric method. We found that normal T cells responded faster to PHA than to any of the other mitogens tested. The peak of the PHA response occurred on day 3, followed by anti-CD3 and SPA on day 4 and PWM mitogen on day 5. Although PHA and anti-CD3 stimulated up to 95% and 80% of lymphocytes, respectively, SPA and PWM stimulated only 40% and 30% of cells, respectively. A defective T cell response was observed in lymphocytes cultured from asymptomatic HIV-1$^-$ patients compared with negative controls. This loss of response was related to a selective mortality of T cells following mitogenic stimulation, referred to as activation-associated lymphocyte death (AALD). The results showed that stronger mitogens (PHA and anti-CD3) induced AALD in a larger proportion (50–60%) of T cells than weaker mitogens such as SPA and PWM (30–40%), and that AALD affected different lymphocyte subsets to different extents. AALD occurred more frequently in total CD8$^-$ and CD45RO$^-$ T cells compared with CD4$^-$ and CD45RA$^-$ T cells, but memory CD4$^-$ T cells were the population most severely affected in samples from HIV-1$^-$ donors.

Keywords Flow cytometry T cell subsets mitogenic stimulation HIV-1 infection

INTRODUCTION

Lymphocyte stimulation by mitogens *in vitro* provides a model for the study of T cell activation *in vivo* [1]. The conventional way of assessing the responsiveness of T lymphocytes to mitogenic stimulation is based on the incorporation of ^{3}H-thymidine into the DNA of proliferating cells in bulk cultures [2]. This method, however, is not fully quantitative in terms of the numbers of responding cells, and there are difficulties in determining which cell populations respond to a given stimulus. An alternative method is to analyse single cells using activation markers such as the IL-2 receptor (IL-2Ra; CD25) in combination with subset markers, such as CD4, by flow cytometry [3,4]. In the present study we have further developed this concept and describe a simple quantitative method for the enumeration of mitogen-stimulated T lymphoblasts using a recently developed absolute cell counter, the Cytoron Absolute. This flow cytometer can count the number of lymphoblasts and define their phenotype. Thus the mitogenic responses of activated blasts can be established at a single-cell level.

Previously, to assess the stimulatory response of lymphocyte subsets required their isolation from heterogeneous peripheral blood mononuclear preparations. Such isolation procedures may change the response pattern of cells to mitogens and antigens by depriving them of helper effects which develop in the culture *in situ* [5,6]. With the method described here, individual cell populations can be investigated when stimulated in mixed cultures, and the number of cells responding related to the original input.

Poor proliferative responses or death after lymphocyte stimulation have been reported in HIV-1 infection [7]. The question we have investigated using this new technique is whether the blast transformation of CD4$^+$ or CD8$^+$ cells is handicapped during the asymptomatic phase of HIV-1 infection in unseparated populations. Recently, the phenotypic features of unprimed (CD45RA$^+$) and primed (CD45RO$^+$) T lymphocytes have been described, and we have also enquired

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whether either of these populations is particularly handicapped in HIV-1 infection.

The aims of this study are therefore: (i) the standardization of the Cytoron Absolute to identify lymphoblasts of different phenotypes developing in response to various commonly used mitogens in normal and disease conditions without disturbing cell–cell interactions; (ii) to analyse the time course of blast transformation and proliferation of T cells responding to phytohaemagglutinin (PHA), soluble anti-CD3, staphylococcal protein A (SPA) and pokeweed mitogen (PWM) in non-infected normal T lymphocytes; (iii) to delineate the most severely handicapped cell types in early asymptomatic stages of HIV-1 infection using this quantitative technique.

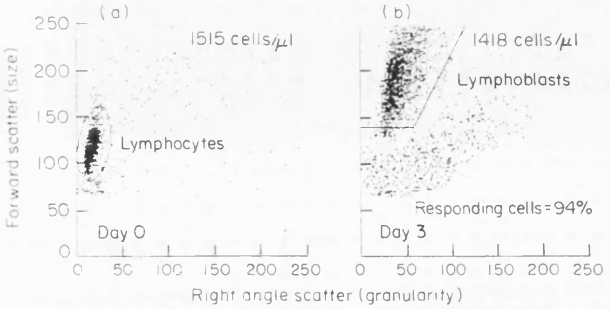

Fig. 1. Acquisition gates for lymphocytes (a) and lymphoblasts (b) which developed after 3 days stimulation with phytohaemagglutinin (PHA), using forward (size) and right angle (granularity) scatter.

SUBJECTS AND METHODS

Subjects

Fifteen HIV-1$^+$ asymptomatic carriers and 10 with AIDS defined according to the CDC classification system [8] attending the out-patients clinics at the Royal Free Hospital, London, UK, were investigated. The asymptomatic subjects had a CD4$^+$ lymphocyte count of $>200/mm^3$. The HIV-1$^-$ group ($n = 10$) was taken from volunteer donors attending an Open Access Clinic.

Lymphocyte isolation and culture

Mononuclear cells were isolated from heparinized venous blood by Ficoll–Hypaque density gradient centrifugation and resuspended to 2×10^6/ml in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 1 ng/ml of rIL-2 (Sandoz, Basel, Switzerland) and antibiotics. The omission of rIL-2 did not substantially vary our results (data not shown), but it was added routinely to overcome the lack of IL-2 production reported in HIV-1 infection.

Peripheral blood lymphocytes (PBL) were cultured at 1×10^6/ml of CD3$^+$ T cells in triplicates of 200 μl in 96-well microplates. The stimuli used were PHA [9] at 1 μg/ml (cat. no. HA16; Wellcome Reagents Ltd, Beckenham, UK), Protein A (SPA) [10] at 20 μg/ml (code no. 17-0770-01; Pharmacia, Uppsala, Sweden), PWM [11] at 10 μg/ml (cat. no. 670-5360AC; Gibco Labs, Grand Island, NY), and anti-CD3 MoAb [12] at 0·1 μg/ml (OKT3; deposit no. 8133 from ATCC, Rockville, MD). Cells were also cultured without mitogens as a control. These mitogens were pre-titrated to give optimal stimulation.

Measurement of blast transformation using ^{3}H-thymidine incorporation

The incorporation of ^{3}H-thymidine was measured after 72 h, using 2 Ci/mmol ^{3}H-thymidine (^{3}H-TdR; cat. no. 7777; Amersham Plc, Aylesbury, UK). The incubation was performed at 37°C, at 100% relative humidity and at a CO_2 of 5%. After a 16-h pulse, the cells were collected on glassfibre filters using an automatic cell harvester (Skatron, Lierbyen, Norway) and the incorporation of the radiolabel measured on a β-counter (LKB Ltd., Croydon, UK). The results are given as medians of triplicate determinations.

Measurement of blast transformation using the Cytoron Absolute

PBL were cultured with either PHA, SPA, PWM or anti-CD3 MoAb for 5 days. The cells were collected daily and resuspended in 2 ml of 1% paraformaldehyde in PBS. The number of unstimulated cells and the number of lymphoblasts developing in the cultures was determined using a Cytoron Absolute (ORTHO Diagnosis, High Wycombe, UK). The Cytoron Absolute is a flow cytometer that not only allows a five parameter analysis but in addition gives the total number of cells present in a fixed volume. The staining of the sample does not require a washing step, and therefore allows the quantification of specific populations labelled after culture. These facilities were utilized to measure accurately the recovery of small resting lymphocytes and lymphoblasts after culture. The lymphocyte population was selected using the forward (FSC) and side scatter (SSC) gates as shown in Fig. 1a,b. This lymphocyte gate excludes cells with high granularity (including monocytes), as well as clusters of dead cells and contaminating erythrocytes. The results obtained are expressed as the percentage of blasts per number of CD3$^+$ T cells placed into culture at time 0 h (blasts/input T cells). More than 92% of blast cells harvested at 72–96 h had a CD3$^+$, T cell phenotype (see also below). Natural killer (NK) type CD57$^+$ cells did not proliferate in culture.

Phenotype of proliferating cells

The phenotypic analysis was performed by double and triple labelling using antibodies conjugated to FITC, PE and biotin together with TANDEM (streptavidin R-PE/TXRD; cat. no.7100-10; Southern Biotechnology Ass., Birmingham, AL) or streptavidin-Tricolor (CALTAG; cat. no. SA 1006; Bradsure Biological Ltd, Market Harborough, UK). The antibodies used were: CD3-FITC (clone OKT3), CD8-FITC (clone RFT8), CD4-PE and CD4-FITC (clone RFT4), CD45RA-PE and CD45RA-FITC (clone SN130), CD45RO-biotin (clone UCHL1), and CD25-FITC (clone RFT5). The analysis of the developing lymphoblasts within the different cell populations was carried out using a combination of absolute counting and three-colour IF using the Cytoron Absolute or the Paint-a-gate program on the FACScan (Becton Dickinson, Oxford, UK).

Statistical analysis

The Wilcoxon Mann–Whitney test and paired t-test were used to compare the groups; $P < 0{\cdot}05$ was considered significant. Linear regression was used to assess the correlation between variables.

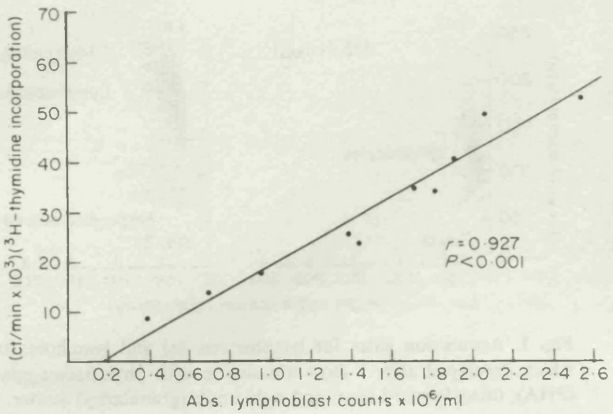

Fig. 2. Correlation between lymphoblasts developed in culture expressed as absolute lymphoblast counts and ^{3}H-thymidine incorporation expressed as ct/min after 3 days stimulation with phytohaemagglutinin (PHA).

RESULTS

Reliability of Cytoron Absolute counts versus *^{3}H-thymidine uptake*

We compared the amount of blasts detected by flow cytometric and ^{3}H-TdR incorporation methods in 10 samples taken from HIV-1$^-$ and HIV-1$^+$ donors after activation for 3 days with PHA (Fig. 2). To determine if the number of lymphoblasts developing after activation correlated with DNA synthesis, the percentage of lymphoblasts measured by flow cytometry was plotted against the same patients' ^{3}H-TdR counts. The results displayed in Fig. 2 show a highly significant correlation between the two sets of data ($r = 0{\cdot}927$, $P < 0{\cdot}001$).

Investigation of the number of T cells responding to various mitogens

The kinetics of cell responses to the different mitogens were assessed over 5 days (Fig. 3). The number of lymphoblasts developing in cultures stimulated with PHA peaked on day 3 ($126 \pm 5{\cdot}0$). After this time, the numbers declined as the culture medium became exhausted (Fig. 3a). The maximum number of lymphoblasts in anti-CD3- ($198 \pm 9{\cdot}0$) and SPA- ($119 \pm 8{\cdot}1$) stimulated cultures appeared on day 4 (Fig. 3b,c, respectively). Stimulation with PWM was slower, and the peak response ($100 \pm 5{\cdot}0$) was seen between days 5 and 6 (Fig. 3d). The kinetics of the PHA and anti-CD3 responses were similar, although PHA was a slightly stronger mitogen. A proportion of small resting lymphocytes were seen even after 5 days' activation with SPA and PWM, indicating that these two mitogens stimulate only a subpopulation of lymphoid cells.

The immunofluorescence profiles of IL-2 receptor (CD25) expression after 96 h activation confirmed the different behaviour of cells responding to PHA, anti-CD3, SPA and PWM (Fig. 4). The percentages of CD25$^+$ cells were determined within the lymphoblast gate. Although in cultures stimulated with PHA almost all blasts were positive for CD25, at early time points a negative population was identified among the SPA, PWM and anti-CD3 activated cells. The majority of lymphoblasts from PHA- ($97 \pm 0{\cdot}5\%$) and anti-CD3- ($87 \pm 1{\cdot}2\%$) stimulated cells expressed CD25 after 3 days (Fig. 4a,b). However, this was reduced in cultures stimulated with either SPA ($80 \pm 0{\cdot}6\%$) or PWM ($53 \pm 1{\cdot}0\%$) (Fig. 4c,d).

Quantification of blast transformation in HIV-1 infection

The number of cells recovered from PHA, anti-CD3, SPA or PWM stimulated cultures was determined in 10 normal and 15 HIV-1$^+$ asymptomatic individuals. A proportion of activated lymphocytes from HIV-1-infected patients died prematurely when compared with the negative control, irrespective of the mitogen used (Fig. 5). After 3 days, the percentage of cells recovered from HIV-1$^+$ cultures compared with input was $60 \pm 7{\cdot}5$, $55 \pm 7{\cdot}7$, $76 \pm 5{\cdot}3$, $71 \pm 3{\cdot}5\%$ in PHA-, anti-CD3-, SPA- and PWM-stimulated cultures, respectively. Under the same conditions the number of lymphoblasts in cultures from HIV-1$^-$ donors was more than 100% ($126 \pm 5{\cdot}0$, $125 \pm 7{\cdot}1$, $119 \pm 4{\cdot}7$, $103 \pm 3{\cdot}1\%$). These data suggest that activation with the strong mitogens PHA and anti-CD3 causes the loss of an increased number of lymphocytes in HIV-1$^+$ samples compared with the weaker mitogens SPA and PWM. We have previously shown that the poor lymphoblast recovery in HIV-1$^+$ samples does not result from a failure of lymphocytes to respond to mitogenic stimulation, but from the death of cells after activation through a process of activation-associated lymphocyte death (AALD) [7]. The earliest discrimination between HIV-1$^-$ and HIV-1$^+$ samples appeared in cultures stimulated with PHA after 2 days ($P < 0{\cdot}001$).

Poor survival of CD4$^+$ and CD8$^+$ T cells from HIV-1$^+$ patients after mitogenic activation

To determine the T cell population most affected by AALD in HIV-1$^+$ patients, 15 samples from asymptomatic HIV-1$^+$ individuals were stimulated with PHA, anti-CD3, SPA or PWM, and analysed after 3 days. The results in Table 1 reveal a poor blast transformation in every lymphocyte population tested compared with the negative control. The mean percentage of CD4$^+$ blast cells recovered from cultures stimulated with PHA and anti-CD3 was significantly lower in HIV-1$^+$ samples compared with negative controls ($P < 0{\cdot}001$). The differences found in SPA- and PWM-stimulated cultures were, however, not statistically significant.

The analysis of the number of CD8$^+$ blasts recovered from HIV-1$^+$ patients compared with the control group showed significant differences in PHA ($53 \pm 8{\cdot}7$, $165 \pm 4{\cdot}3$) and anti-CD3 ($48 \pm 10{\cdot}3$, $192 \pm 22{\cdot}9$) as well as in SPA-stimulated cells ($37 \pm 5{\cdot}7$, $71 \pm 3{\cdot}7$). No significant differences were observed in PWM-stimulated cultures ($33 \pm 3{\cdot}3$, $41 \pm 4{\cdot}6$).

Taken together, these results indicate that although both CD4$^+$ and CD8$^+$ lymphocytes from HIV-1$^+$ patients were affected by AALD, the CD8$^+$ T cells were more damaged than the CD4$^+$ lymphocytes. Thus, the proliferative defects in the CD8$^+$ subset were more marked when mitogens which preferentially activate CD8$^+$ T cells (i.e. PHA and anti-CD3) were used.

Memory cells are more vulnerable to AALD

In support of our previous finding [7], following mitogenic stimulation, the mean percentages of CD45RO$^+$ lymphoblasts seen after culture were significantly lower in HIV-1-infected samples compared with the control group (Table 1). A significant decrease in the proliferative response of CD45RA$^+$

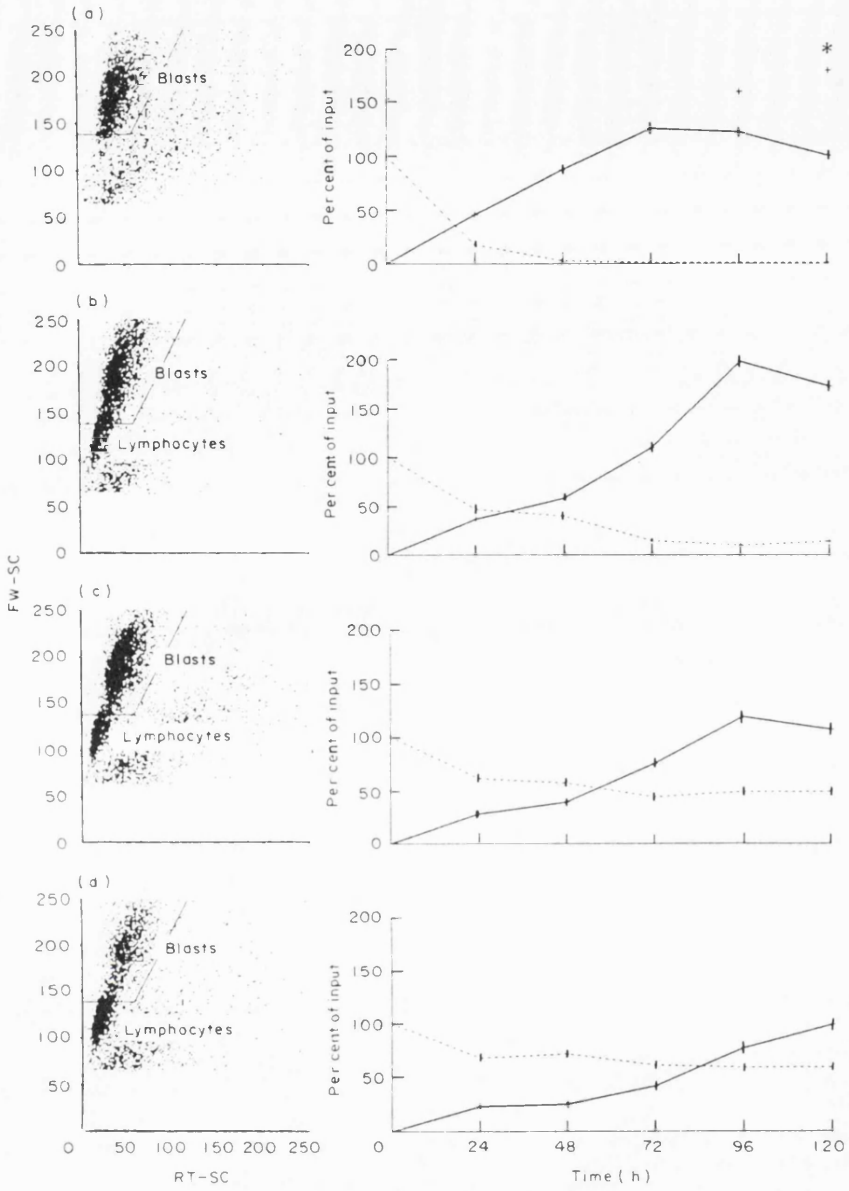

Fig. 3. Flow cytometric analysis of proliferating T cells. The acquisition gates for analysing lymphoblasts (left) and a time course of 5 days stimulation (right) with phytohaemagglutinin (PHA) (a), soluble anti-CD3 (b), staphylococcal protein A (SPA) (c) and pokeweed mitogen (PWM) (d). The percentage of blast cells (——) is shown as well as the percentage of unstimulated lymphocytes (- - - -). Each point represents the mean and s.e.m. of 10 samples. Some of the cultures (*) were fed with fresh culture medium after 72 h stimulation.

lymphocytes from HIV-1 donors was observed only in PHA- and anti-CD3-stimulated cultures compared with healthy donors.

We next studied the proliferative response of the $CD45RA^+$ and $CD45RO^+$ populations among the $CD4^+$ and $CD8^+$ lymphocytes from $HIV\text{-}1^+$ patients. A slightly decreased survival was found in the unprimed $CD4^+$ T cells compared with $HIV\text{-}1^-$ controls. In contrast, the capacity of mitogens to induce blast transformation in the primed $CD4^+$ T cells was greatly reduced in $HIV\text{-}1^+$ samples in relation to the values seen in normal controls ($32 \pm 5{\cdot}5$, $82 \pm 10{\cdot}5$ for PHA; $20 \pm 5{\cdot}5$, $70 \pm 14{\cdot}7$ for anti-CD3; $39 \pm 5{\cdot}5$, $63 \pm 5{\cdot}9$ for SPA; and $15 \pm 2{\cdot}0$, $32 \pm 2{\cdot}6$ for PWM).

We also examined the proliferation of unprimed *versus* primed $CD8^+$ lymphocytes from $HIV\text{-}1^+$ patients after mitogenic stimulation. Significantly lower responses were observed in unprimed $CD8^+$ T cells taken from $HIV\text{-}1^+$ patients when stimulated with PHA ($66 \pm 9{\cdot}8$, $136 \pm 10{\cdot}3$) or anti-CD3 ($55 \pm 7{\cdot}9$, $148 \pm 16{\cdot}8$), but the differences found between $HIV\text{-}1^+$ and $HIV\text{-}1^-$ samples activated with SPA ($38 \pm 5{\cdot}4$, $52 \pm 3{\cdot}6$) or PWM ($34 \pm 4{\cdot}3$, $34 \pm 4{\cdot}1$) were not significant. These results therefore show that the response of primed $CD8^+$

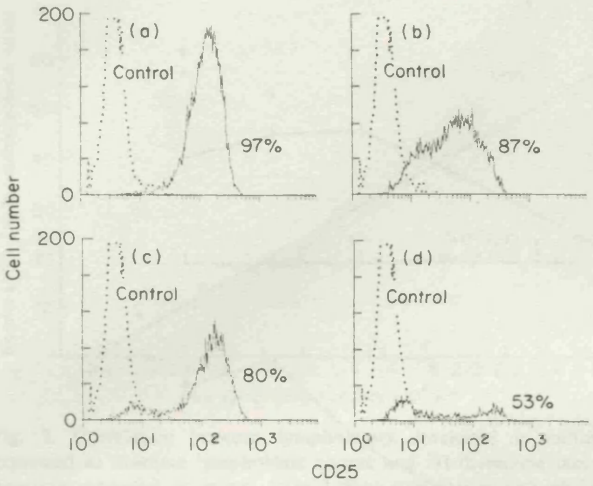

Fig. 4. Immunofluorescence analysis of IL-2 receptor (CD25) expression on blast cells from phytohaemagglutinin (PHA) (a), anti-CD3 (b), staphylococcal protein A (SPA) (c) and pokeweed mitogen (PWM) (d) stimulated cultures. The percentage of $CD25^+$ cells was determined within the lymphoblast region on day 4 of culture. The dotted line represent the negative control. Values on *x* and *y* axes indicate relative fluorescence intensity (log_{10}) and percentage of cells, respectively.

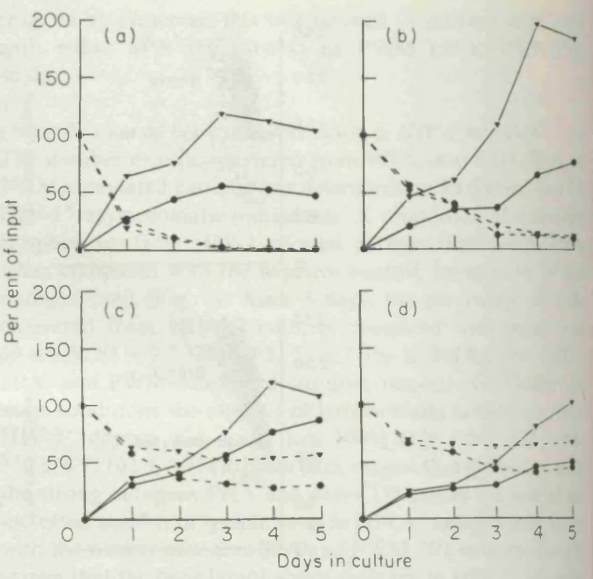

Fig. 5. Percentage of lymphocytes (----) and lymphoblasts (——) recovered from phytohaemagglutinin (PHA) (a), anti-CD3 (b), staphylococcal protein A (SPA) (c) and pokeweed mitogen (PWM) (d) stimulated cultures over 5 days from 10 HIV-1$^-$ (▼) and 15 HIV-1$^+$ (●) donors.

T cells from HIV-1$^+$ donors after stimulation *in vitro* with the strong mitogens is particularly poor.

DISCUSSION

The method used to measure lymphocyte proliferation in most of these studies was the incorporation of ^{3}H-TdR [2]. Albeit easy to perform, this method has severe drawbacks: the counts obtained after stimulation do not directly correlate with the number of stimulated cells, and the method does not distinguish between unresponsiveness and activation-induced cell death. More importantly, no information about the nature of responding cells can be obtained in such a bulk assay unless subpopulations are separated first. Such a separation process can, however, interfere with lymphocyte responses. Different T cell populations produce various lymphokines after stimulation, and may even synergize with each other [13]. For instance, $CD4^+$, $CD45RA^+$ cells are efficient IL-2 producers but make little IL-4, while $CD4^+$, $CD45RO^+$ cells produce higher levels of IL-4 and relatively low amounts of IL-2. These populations are dependent on each other for optimal proliferation [13]. Consequently, the proliferation of an isolated population might not mirror what happens in unseparated culture.

To avoid these problems some investigators have used features of lymphocyte activation which can be quantified on individual cells. One example is the expression of CD25 on recently activated blasts [4]. Others have measured bromodeoxyuridine (BrdU) incorporation or analysed mitosis in

Table 1. Lymphocyte subpopulation responses to phytohaemagglutinin (PHA), anti-CD3, staphylococcal protein A (SPA) and pokeweed mitogen (PWM) activation

	PHA		Anti-CD3		SPA		PWM	
	HIV$^-$	HIV$^+$	HIV$^-$	HIV$^+$	HIV$^-$	HIV$^+$	HIV$^-$	HIV$^+$
$CD4^+$	110 ± 4·3*	50 ± 8·5	64 ± 8·1	31 ± 10·5	75 ± 3·6‡	66 ± 9·6†	38 ± 2·7‡	27 ± 5·5†
$CD8^+$	165 ± 11·9	53 ± 8·7	192 ± 22·9	48 ± 10·3	71 ± 3·7	37 ± 5·7	41 ± 4·6‡	33 ± 3·3†
$CD45RA^+$	127 ± 5·1	72 ± 9·1	96 ± 9·2	53 ± 9·0	70 ± 4·7‡	54 ± 8·8†	37 ± 3·1‡	35 ± 4·7†
$CD45RO^+$	129 ± 7·0	42 ± 5·4	146 ± 30·7	41 ± 8·0	88 ± 10·0	45 ± 7·5	42 ± 3·4	23 ± 2·3
$CD4^+/CD45RA^+$	119 ± 4·5	78 ± 10·3	59 ± 2·8‡	52 ± 12·2†	81 ± 7·9‡	89 ± 14·7†	43 ± 4·5‡	35 ± 8·8†
$CD4^+/CD45RO^+$	82 ± 10·5	32 ± 5·5	70 ± 14·7	20 ± 5·5	63 ± 5·9	39 ± 5·5	32 ± 2·6	15 ± 2·0
$CD8^+/CD45RA^+$	136 ± 10·3	66 ± 9·8	148 ± 16·8	55 ± 7·9	52 ± 3·6‡	38 ± 5·4†	34 ± 4·1‡	34 ± 4·3†
$CD8^+/CD45RO^+$	160 ± 21·4	60 ± 8·7	180 ± 28·9	51 ± 8·3	80 ± 17·7	49 ± 10·6	60 ± 12·5	34 ± 5·2

* Mean ± s.e.m. of absolute lymphoblasts count × $10^3/\mu l$.
†, ‡ are not statistically different.

cytospin preparations [14]. These assays can be combined with phenotypic analysis, but are difficult to perform.

In this study we have introduced a simple practical method for analysing the blast transformation of lymphocytes in combination with two- and three-colour immunofluorescence in order to determine the number of transforming cells in the various T cell subpopulations. The total number of lymphocytes placed into the cultures at the start of incubation and the lymphoblasts seen after varying periods of time have been determined using the forward and side scatter profiles. The absolute numbers of $CD45RA^+$ and $CD45RO^+$ cells among the $CD4^+$ and $CD8^+$ population of peripheral blood mononuclear cultures taken from HIV-1^- and HIV-1^+ individuals were analysed following stimulation with PHA, anti-CD3, SPA and PWM.

Our studies have confirmed, as expected, that T cells respond faster to PHA than to the other mitogens. In this study we have made no attempt to prolong further optimal mitogenicity by subcloning the cultures of proliferating T cell blasts. Thus the different time courses of activation by the various mitogens simply confirm that PHA and anti-CD3 are stronger mitogens than SPA and PWM. This may be due to a stronger signal delivered by PHA which interacts with several glycoproteins on the surface of T cells [15,16]. The lower proportion of cells which respond to SPA and PWM could be explained by the observations that these stimulants act as superantigens and are recognized by fewer T cells. Thus, White *et al.* [17] and Janeway *et al.* [18] reported that SPA stimulates human T cells by interacting with the portion of the TCR encoded by certain Vβ segments. Somewhat surprisingly, differences in mitogenic strength are also reflected in the rate of expression of IL-2Rα chain, identified by the CD25 antibody on activated cells during the period of culture. The population of $CD25^-$ lymphoblasts observed in culture stimulated with SPA and PWM might represent an early stage of activation which is only readily detectable when this process is relatively slow. It is therefore important to emphasize that the low ^{3}H-TdR uptake in these cultures is due not only to the fewer responding cells, but is also a reflection of the slower kinetics of activation.

As mentioned above, not all the cells responded to weak mitogenic stimuli. This lack of responsiveness did not reflect a fully selective stimulation of one or another subset. We have observed that all the populations investigated responded to all mitogens tested, although to a different degree. In our hands during lymphocyte culture in the presence of added recombinant IL-2 (rIL-2), PHA effectively activated all T cell subsets.

The poor response of T cells from HIV-1-infected individuals to recall antigens, alloantigens, PHA, anti-CD3 and PWM has been widely documented [19–22], but this is the first detailed investigation about the phenotypic features of mitogen-responsive cells. This is an issue of practical importance, because these mitogens have been utilized in various systems to establish prognostic factors and predict responsiveness to antiviral therapy [19–21,23]. In these studies PWM, and more recently anti-CD3, were found to discriminate between HIV-1^+ patients with good and poor prognosis [21,24], but the method used was bulk ^{3}H-TdR uptake with only a limited analytical capacity.

We have previously shown [7] that T cells from HIV-1^+ individuals can in fact respond to PHA and anti-CD3 to a variable degree, but that many T cells died after 24–48 h. This AALD was not due to typical apoptosis. We, and others, have described that $CD8^+$ lymphocytes were more affected than $CD4^+$ and, again, that $CD45RO^+$ cells were more vulnerable than $CD45RA^+$ populations [7,25–27].

In this study we now confirm that following activation with strong mitogens the flow cytometric method shows a greater sensitivity in measuring cellular immunodeficiency, by quantifying poorer blast transformation and AALD in HIV-1^+ lymphocytes, than ^{3}H-TdR uptake. It is also clear that some cells can initiate DNA synthesis and incorporate BrdU but die afterwards, appearing as $BrdU^+$ dead cells in cytospin preparations (data not shown). These dead cells may contribute to erroneously high ^{3}H-TdR counts. Few ($< 10\%$) apoptotic bodies were seen in these cultures, indicating that AALD is different from the underlying, low levels of apoptosis seen in unstimulated mononuclear cells from patients with HIV-1 and other viral infections [27]. It is important to point out that when smaller proportions to T cells are activated, e.g. by antigens, ^{3}H-TdR incorporation remains the method of choice to quantify proliferative responses, as the few blasts present may be 'swamped' by residual unstimulated cells during a flow cytometric analysis.

The relevance of the observations above in respect of the analysis of HIV-1-related defects is as follows. We have now extended our previous studies to show that all four mitogens tested induced cellular deficiency in T cells even in patients at an early asymptomatic stage of disease with >200–400 $CD4^+$ cells/mm^3. Nevertheless, the numbers of T cells undergoing AALD in our culture system were dependent upon the stimuli used. Thus powerful mitogens such as PHA and anti-CD3 induced much more lymphoid cells (50–60%) to undergo AALD than the weaker mitogens such as SPA and PWM (30–40%). This was clearly shown by the fact that higher numbers of small resting lymphocytes remained viable in PWM- and SPA-activated cultures.

The strength of the stimuli not only increased the number of cells undergoing AALD but, although to different degrees, affected different subpopulations. With all mitogens tested in HIV-1^+ compared with HIV-1^- individuals, particularly low numbers of $CD4^+$, $CD45RO^+$ and $CD8^+$, $CD45RO^+$ cells were activated successfully. In fact, when PWM and SPA were used as the stimuli these were the only significant differences between the HIV-1^- and HIV-1^+ groups. These findings confirm previous studies about the preferential handicap of memory cell responses in HIV-1 infection [7,26–28]. With stronger mitogens, such as anti-CD3 and PHA, all T cell subsets were affected when samples from HIV-1^+ patients were analysed but, again, the magnitude of defect was higher among the $CD45RO^+$ T cells.

Finally, it is important to point out that the method standardized in our study using the Cytoron Absolute counter to measure blast transformation within the various T cell subsets is the first practical and convenient method to quantify the defects in both $CD4^+$ and $CD8^+$ cells with a $CD45RO^+$ phenotype in HIV-1 infection. In fact, in many individuals the functional damage in these two populations varied independently. The clinical significance of these findings is under investigation.

The mechanisms by which $CD4^+CD45RO^+$ and $CD8^+CD45RO^+$ populations are damaged are unknown, but

may not be the same. The viral load in HIV-1$^+$ individuals has previously been grossly underestimated, as the more recent studies of HIV virus detected in the patients' plasma by competitive polymerase chain reaction (PCR) indicate that an active productive infection is present at all stages of the disease [29]. A more severe viral load in CD4$^+$, CD45RO$^+$ cells has also been reported [28]. It is nevertheless unlikely that HIV-1 infection is the only mechanism of CD4$^+$ cells' destruction, and apoptotic damage within CD4$^+$ cells has also been suggested [22].

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