DEFECTIVE CYTOTOXIC T LYMPHOCYTE FUNCTION IN HIV INFECTION

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DEFECTIVE CYTOTOXIC T LYMPHOCYTE FUNCTION IN HIV INFECTION

By

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ABSTRACT

The primary objective of this thesis is to determine whether cytotoxic T lymphocytes (CTL) detectable against $CD4^+$ lymphocytes *in vitro* act *in vivo* to promote $CD4^+$ depletion and thereby contributing to immune dysfunction and disease progression in HIV-1 infected individuals. During this study, the relationship between the level of CTL activity against CD4 lymphocytes and disease progression was assessed by carrying out a series of *in vitro* experiments in a HIV-positive cohort of ~70 individuals.

It is well established that CTL use clonotypic T cell receptors (TCR) associated with the invariant CD3 signalling complex, to recognize antigenic peptides bound to major histocompatibility complex (MHC) molecules on the target cells. Since P815 cells express an FcR and Fas antigen. IgG anti-CD3 antibodies can trigger non-specific killing of P815 cells by a variety of effector cells. Comparable inhibition of cellular cytotoxicity against P815 cells by Jo-2 or by cycloheximide, a protein synthesis inhibitor preventing Fas ligand induction, confirmed that the different levels of killing of Jo-2 treated and untreated P815s reflected the extent that perforin and Fas ligand, respectively, were utilized in target cell killing.

Abnormally high numbers of T cells from HIV-infected individuals undergo spontaneous and activation-induced cell death (AICD), and also are especially sensitive to Fas-mediated apoptosis, suggesting that Fas/Fas ligand (FasL) interactions might contribute to AICD in HIV infection. We used treatment with PMA and ionomycin to investigate the possible role of Fas/FasL interactions in AICD in HIV infection. PMA/ionomycin-induced AICD measured using Cr release, DNA analysis and electron microscopy, demonstrated that PMA and ionomycin acted synergistically to induce up to 70% release of incorporated Cr from fresh PBMC of HIV-infected individuals compared with up to 26% release by healthy volunteers. Cell death required cell-cell contact and extracellular calcium, while it did not involve Fas/FasL interactions or

DNA fragmentation, but showed plasma membrane disruption with intact nuclear membranes of damaged cell. We describe a novel form of AICD in T lymphocytes from HIV-infected individuals.

The presence, number and proportion of activated $CD8^+$ T lymphocytes in the peripheral blood of HIV-infected individuals correlates with disease progression. We examined the associations between autoreactive CTL in the peripheral blood of HIV-infected individuals and disease progression. A significant percentage of HIV-seropositive persons (>50%) in our study cohort, in contrast to healthy individuls showed cytolysis of PHA-activated uninfected lymphocytes. These autoreactive CTL were found to be $CD28^-$ $CD8^+$ T cells which expanded with disease progression. A high proportion of $CD28^-$ $CD8^+$ T cells was seen in all HIV-infected individuals with demonstrable levels of circulating CTL.

We have shown direct association between the autoreactivity and other markers of disease progression such as plasma viral load, $CD4^+$ T-cell count, $CD8^+$ T cell count, and plasma levels of β_2 microglobulin . The data is in agreement with the proposed hypothesis that these CTL actually contribute to immunodeficiency and clinical progression to AIDS. Based on our data, CTL appear to be a major contributor to disease progression. Further studies based on longitudinal follow-up of these patients may help uncover the functional significance of autoreactive CTL.

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ABBREVIATIONS

ADCC:	Antibody Dependent Cellular Cytotoxicity
ADE:	Antibody Dependent Enhancement
AICD:	Activation-induced Cell Death
AIDS:	Acquired Immunodeficiency Syndrome
APC:	Antigen Presenting Cell
ARC:	AIDS Related Complex
ARV:	AIDS associated retrovirus
Aspase	Aspartase
ASMase	acidic sphingomyelinase
ATP:	Adenosine Triphosphate
CAD	caspase-activated DNase
CAT:	Chloramphenicol Acetyl Transferase
CCR5	Cemokine Receptor 5:
CD:	Cluster Designation
CDC:	Centre for Disease Control
CDR:	Complementarity Determining Region
CL:	Cytolytic Lymphocyte
CMV:	Cytomegalovirus
Con A:	Concanavalin A
CsA:	Cyclosporin A
CTL:	Cytotoxic T Lymphocyte
CXCR4:	Chemokine receptor 4

DD:	Death Domain
DED:	Death Effector Domain
DISC:	Death-Inducing Signaling Complex
DN:	Dominant Negative
DNA:	Deoxyribonucleic acid
EBV:	Epstein Barr Virus
EDTA:	Ethylene diamine tetra acetic acid
FADD:	Fas-associated death domain
FAP-1:	Fas-associated phosphatase-1
FasL:	Fas ligand
FCS:	Fetal Calf serum
FITC:	Fluorescein Isothiocyanate
gld:	Generalized lymphoproliferative disease
HCl:	Hydrochloric acid
HHV-6:	Human Herpes Virus-6
HIV:	Human Immunodeficiency Virus
HLA:	Human Leukocyte Antigen
HTLV:	Human T Lymphotropic Virus
ICE:	Interleukin-1β Converting Enzyme
IEL:	Intra Epithelial Lymphocyte
IFN-γ:	Interferon-Gamma
IgE:	Immunoglobulin E
IgG:	Immunoglobulin G
IgM:	Immunoglobulin M
IL-1:	Interleukin-1

IL-2:	Interleukin-2
IL-2R:	Interleukin-2 receptor
IL-3:	Interleukin-3
IL-4:	Interleukin-4
IL-5:	Interleukin-5
IL-6:	Interleukin-6
IL-7:	Interleukin-7
IL-8:	Interleukin-8
IL-9:	Interleukin-9
IL-10:	Interleukin-10
IL-11:	Interleukin-11
IL-12:	Interleukin-12
IL-13:	Interleukin-13
IL-14:	Interleukin-14
IL-15:	Interleukin-15
IL-16:	Interleukin-16
LAK:	Lymphokine Activated Killer
LAS:	Lymphadenopathy Syndrome
LAV:	Lymphadenopathy Associated Virus
LCMV:	Lymphocytic Choriomeningitis Virus
LFA-1:	Lymphocyte function Antigen-1
lpr:	Lymphoproliferation
LTR:	Long terminal repeats
MA:	Matrix
mAb:	Monoclonal Antibodies

MHC:	Major Histocompatibility Complex
MIP-1α:	Macrophage inflammatory protein-1α
MIP-1β:	Macrophage inflammatory protein-1β
NC:	Nucleocapsid
Nef	Negative factor
NGF:	Nerve Growth Factor
NK:	Natural Killer
NSI:	Non-syncytium Inducing
PBL:	Peripheral Blood Leukocyte
PBMC:	Peripheral Blood Mononuclear Cells
PHA:	Phytohaemagglutinin
PI:	Propidium Iodide
PKC:	Protein Kinase C
PMA:	Phorbol myristic acetate
RANTES:	Regulated upon activation normal T cell expressed and secreted
RBC:	Red Blood Cell
RBL:	Red Blood Leukemia
Rev	Regulator of viral protein expression
RNA:	Ribonucleic acid
Rnase H:	Ribonuclease H
RT:	Reverse transcriptase
sCD4:	Soluble CD4
SCID:	Severe Combined Immunodeficiency
SCID-hu	Severe Combined Immunodeficiency-human
SCID-hu/PBL:	Severe Combined Immunodeficiency-human lymphocytes

SI:	Syncytium Inducing
SIV_{agm}:	Simian Immunodeficiency virus-African Green Monkey
SIV:	Simian Immunodeficiency Virus
SLE:	Systemic Lupus Erythematosus
TAT	Transactivating protein
TCR:	T Cell Receptor
TH1:	T Helper 1
TH2:	T Helper 2
TM:	Transmembrane
TNF-α:	Tumor Necrosis Factor-Alpha
TNF-R:	TumorNecrosisFactor- receptor
TRAIL:	TNF-related apoptosis-inducing ligand
TRAMP:	TNF-receptor-related apoptosis-mediating protein
v-FLIP:	viral FLICE-inhibitory protein

CHAPTER I.

INTRODUCTION

I. 1.0 INTRODUCTION

This chapter broadly outlines current concepts of the immunopathogenesis of human immunodeficiency virus (HIV) infection. This is followed by a more specific discussion of cytotoxic T lymphocytes (CTL) and their possible roles in the progression of HIV infection to the invariably fatal immunodeficient state or acquired immune deficiency syndrome (AIDS).

I. 1.1 DISCOVERY OF THE VIRUS CAUSING AIDS

HIV is a lentivirus that has only recently been recognised as the causative agent of AIDS. The first indication that AIDS could be caused by a retrovirus came in 1983, when Barre-Sinoussi et al. (1983) at the Pasteur Institute recovered a reverse transcriptase containing virus from the lymph node of a man with persistent lymphadenopathy syndrome (LAS). A concomitant publication by Gallo et al. (1983) reported the isolation of human T-cell leukemia virus (HTLV-III) from individuals with AIDS and argued that the causative virus was this previously recognised human retrovirus. Further studies by Montagnier and coworkers clarified these issues regarding the LAS agent, indicating that AIDS-associated human retrovirus was distinct from HTLV. Their virus, later called lymphadenopathy associated virus (LAV), grew to high titer in $CD4^+$ cells and killed these cellular targets (Montagnier et al.1984). These observations on LAV supported the potential etiological role of a retrovirus in AIDS. Levy et al. (1984) also reported the identification of a retrovirus, they named the AIDS-associated

retrovirus (ARV). All these viruses were recovered from AIDS patients from different known risk groups, as well as from other symptomatic and asymptomatic people. HIV isolates were subsequently recovered from the blood of many patients with AIDS, AIDS related complex (ARC), and neurological syndromes, as well as from the peripheral blood mononuclear cells (PBMC) of several clinically healthy individuals (Levy et al., 1985a; Salahuddin et al., 1985). Soon after the discovery of HIV-1, a separate virus, HIV-2, was identified in Western Africa (Clavel et al, 1986). It is now established that both viruses can lead to AIDS, although the pathogenic course with HIV-2 appears to be longer.

I. 1.2 HIV VIRION

By electron microscopy, HIV-1 and HIV-2 have cone shaped cores which are biochemically constituted by the viral p25 Gag protein. Inside the capsid, are two identical RNA strands with which the viral RNA dependent DNA polymerase (Pol or reverse transcriptase) and the nucleocapsid (NC) proteins are closely associated. The inner portion of the viral membrane is surrounded by a myristoylated p17 core (Gag) protein that provides the matrix (MA) for the viral structure and is vital for the integrity of the virion (Gelderblom et al., 1988; Gelderblom et al., 1989). Recent studies have suggested that MA is required for incorporation of Env proteins into mature virions (Yu et al., 1992).

The viral envelope (env) is characteristically made up of trimers or tetramers of glycoproteins (Earl et al., 1990; Gelderblom et al., 1988; Ozel et al., 1988; Pinter et al., 1989; Weiss et al., 1990). The mature Env proteins are derived from a 160,000 D precursor, which is cleaved inside the cell into a glycoprotein (gp) 120 external surface (SU) envelope protein and a gp41 transmembrane (TM) protein (McCune et al., 1988). These proteins are transported to the cell surface, where parts of the central and N terminal portions of gp41 are also expressed on the outside of the virion. The central region of the TM protein binds to the external viral gp120 in a noncovalent manner, most probably in the

hydrophobic regions in the amino and carboxy termini of gp120 (Helseth et al., 1991). Generally, the virion has about 100 times more p25 Gag protein than envelope gp120 (Layne et al., 1992; Moore et al., 1991) and 10 times more p25 than the polymerase protein (Layne et al., 1992).

The gp120 situated on the virus surface contains the binding site for cellular receptor (s) and the major neutralizing domains. Nevertheless, the external portion of gp41 and perhaps part of p17 have also been reported to be sensitive to neutralizing antibodies (Chanh et al., 1986; Dalgleish et al., 1988; Naylor et al., 1987; Sarin et al., 1986).

I. 1.3 TRANSMISSION OF HIV

There are essentially three modes of transmission of the virus from an infected individual to another person: exposure to blood or blood products, sexual transmission and vertical transmission.

I. 1.3.1 TRANSMISSION BY BLOOD AND BLOOD PRODUCTS.

The potential risk of infection of transfusion recipients depends on the virus load in the contaminated blood used for transfusion, which increases as an infected individual (as donor) advances to disease (Perkins et al., 1987). In hemophiliacs, infection could only occur through transmission of free virus and was associated with receipt of many vials of unheated coagulation factors (Evatt et al., 1984; Eyster et al., 1987; Goedert et al., 1989; Koerper et al., 1989).

I. 1.3.2 SEXUAL TRANSMISSION

AIDS was first identified as a sexually transmitted disease in homosexual men. However subsequent studies demonstrated heterosexual spread of HIV, which accounts for the large majority of infections worldwide (Nkowane et al., 1991; Stoneburner et al., 1990). Transmission of HIV in genital

fluids most probably occurs through virus-infected cells since they can be present in large numbers in the body fluids. Presence of other concomitant sexually transmitted diseases can increase levels of HIV in genital fluids and thus make transmission more likely (Cameron et al., 1989; Plummer et al., 1991). Infection through anal intercourse could occur following interaction of virus with cellular receptors, especially those on the bowel mucosa (Yahi et al., 1992) or the attachment of virus-antibody complexes to Fc receptors on the mucosal cells (Hussain et al., 1991). Another possible means of HIV entry could be via intestinal M cells present in the bowel epithelium (Amerongen et al., 1991).

In the case of vaginal intercourse, the columnar and squamous cell epithelium of the vagina can be a barrier to virus infection, so that ulcerations caused by venereal diseases might be required for infection at this site.

The insertive partner in sexual contact carries a relatively lower risk of infection, although (Winkelstein et al., 1987) transmission could occur through infection of macrophages or lymphocytes in the foreskin or the urethral canal. Finally oral-genital contact could also potentially lead to infection of either partner, albeit at a lower frequency (Winkelstein et al., 1987). Non traumatic oral exposure to cell-free SIV was shown to infect adult macaques (Baba et al., 1996). These infected macaques later developed full blown AIDS indicating the possibility of an oral transmission of the virus and subsequent systemic disease in humans also (Baba et al., 1996). In all routes of sexual contact, an increased number of virus infected cells in the genital fluids and or lesions on the mucosal surfaces (eg., resulting from venereal disease) would increase the risk of transmission of the virus.

I. 1.3.3 TRANSMISSION FROM MOTHER TO CHILD

The transmission of HIV from mother to child appears to occur in 11 to 60% of children born to HIV^+ mothers (Ades et al., 1991; Blanche et al., 1989). It appears that transmission

can occur either *in utero* during or after delivery (Rossi, 1992). Support for intrauterine transmission comes from the detection of HIV in placentas and fetuses by *in situ* hybridization, polymerase chain reaction (PCR) and immunohistochemistry (Courgnaud et al., 1991). HIV has also been isolated from cord blood amniotic fluid, and placental and fetal tissues (Chandwani et al., 1991).

The factors involved in transfer of HIV from mother to child could be studied in relation to the SIV infection. Transmission of this primate virus appears to occur primarily when the animals are sexually active and not during birth (McClure et al., 1992). SIV transmission to one of the three offsprings was demonstrated in pigtailed macaques (*Macaca nemestrina*) (Ochs et al., 1991). The consistency of this result, which resembles transmission in humans, needs further study. However this observation could suggest an HIV transmission model, since pigtailed macaques have been found to be sensitive to HIV infection (Agy et al., 1992). If the factors influencing maternal transmission of HIV can be well defined, antiviral approaches could be better targeted.

I. 1.4 CLINICAL SYNDROME OF ACUTE HIV INFECTION

A recently infected individual can present within 1 to 3 weeks with signs of acute virus infection. Symptoms include headaches, retroorbital pain, muscle aches, sore throats, low grade or high grade fever, and swollen lymph nodes, as well as a non pruritic maculopapular erythematous rash involving the trunk and later the extremities (Cooper et al., 1985). In acutely infected individuals, pneumonitis, diarrhea, and other gastrointestinal complaints have also been reported (Tindall and Cooper,1991). These symptoms usually last for 1 to 3 weeks, although lymphadenopathy, lethargy, and malaise can persist for many months. In general, primary HIV infection is followed by an asymptomatic period of many months to years.

In a group of 23 persons at risk of HIV infection who were followed every six months and who became infected, 87% had symptomless acute infection and 95% of these patients sought medical evaluation (Schacker et al., 1997). But only one in four patients in this study received the appropriate diagnosis of acute HIV infection at the first clinical visit, even though there should have been a high level of suspicion. Laboratory studies performed during the initial infection may show lymphopenia and thrombocytopenia, but atypical lymphocytes are infrequent (Quinn, 1997).

Infected individuals are often lymphopenic and thrombocytopenic during the first week following HIV infection. In the second week, the total number of lymphocytes increases, primarily because of expansion of $CD8^+$ cells. $CD4^+$ cells are reduced in number. Thus, during this period, the $CD4^+/CD8^+$ cell ratio is inverted. Moreover, atypical lymphocytes can appear in the blood (Cooper et al., 1988) but usually in smaller numbers in primary HIV infection than in EBV, CMV or other infections that elicit a similar response (Cooper et al., 1988; Gaines et al., 1988). Over the following months, number of the $CD8^+$ cells remains greater than that of the $CD4^+$ cells, which increases to a smaller extent and so the ratio remains inverted.

During acute HIV infection, the infected individual demonstrates antigenemia and viremia with high levels of infectious virus in the peripheral blood (Clark et al., 1991; Daar et al., 1991). Seroconversion can occur days after infection, but antibodies to HIV generally appear after 1 to 4 weeks. In studies performed by Cooper et al., (1985) and Gaines et al., (1988), IgM antibodies were first detected in some patients as early as 6 days post infection. IgG levels could usually be demonstrated by an indirect-immunofluorescent assay within two weeks.

Some studies have reported HIV specific helper T cell responses shortly after acute infection before seroconversion (Clerici et al., 1991). Conceivably other cellular immune responses are evident

before humoral immunity is evident (Clerici et al., 1992a). Mackewicz and Levy, (1992) have reported $CD8^+$ cell anti-HIV activity in one individual before seroconversion.

I. 1.5 HIV INFECTION OF CELLS

HIV infection of human cells involves a series of steps.

I. 1.5.1 CD4 MOLECULE: VIRUS RECEPTOR ON CELLS

An early breakthrough in the study of HIV was the discovery of its major cellular receptor, the CD4 molecule. Preferential growth of HIV in $CD4^+$ lymphocytes was then explained by its attachment to the CD4 protein on the cell surface (Dalgleish et al., 1984; Klatzmann et al., 1984; Klatzmann et al., 1984a).

With the crystal structure of CD4 now known (Ryu et al., 1990; Wang et al., 1990) the gp120 binding site has been located on a protuberant ridge along one face of the V1 domain. Recently using viral mutants and high resolution CD4 atomic structure, Moebius et al., (1992) have delineated the viral attachment site further. These studies indicate that the class II MHC binding site appears to include the same CD4 region as the gp120-binding site. (Moebius et al., 1992). Thus, this overlap might affect the use of inhibitors of the CD4-gp120 interaction.

Further sites on CD4 could still be involved in HIV binding and /or fusion such as the CDR3 domain of the V1 region (Autiero et al., 1991; Corbeau et al., 1993). It was suggested that this region play a role because CDR3-related peptides block the CD4-gp120 interaction (Lifson et al., 1991) and mutations in this region decrease fusogenic activity of HIV (Camerini and Seed, 1990).

Recent findings by Deng et al. (1996) and Dragic et al. (1996) have indicated that in order to infect a cell, not only does HIV have to bind to the CD4 receptor on the cell surface, it must also

enrol the help of a second receptor known as CC-CKR-5. This co-receptor is a binding site for the attractant molecules RANTES, MIP-1α, and MIP-1β. These studies show that RANTES, MIP-1α, and MIP-1β all inhibit HIV-1 infection by blocking viral fusion and entry, and that expression of CC-CKR-5 chemokine-receptor gene together with that for CD4 renders the cells susceptible to infection by primary non-syncytium forming (NSI) strains of HIV-1. It is not yet clear whether the respective chemokines competitively block their receptors against HIV entry, or whether the chemokine binding results in down-regulation of cell-surface expression of the receptor.

I. 1.5.1.1 CORECEPTORS IN HIV INFECTION

The first identified coreceptor, CXCR-4, is a receptor for the α (CXC) subclass of chemokines and mediates entry/fusion of T-cell tropic strains of HIV (Feng et al., 1996). Another receptor for the members of the β (CC) chemokine subclass, CCR-5, mediates entry/fusion of macrophage-tropic isolates of HIV (Alkhatib et al., 1996). This molecule serves as a receptor for RANTES, MIP-1α, and MIP-1β and thus provides the basis for chemokine-mediated suppression of HIV. Later, a CXC chemokine called SDF-1, a ligand for CXCR-4 was shown to suppress replication of T-cell tropic HIV isolates (Bleul et al., 1996).

The selectivity for the specific coreceptor is governed by the HIV envelope glycoprotein gp120. The third hypervariable region (V3) of the molecule appears to be the major determinant (Cochi et al., 1996). Paradoxically the V3 loop is also the least conserved region of gp120 and contains highly strain specific neutralizing epitopes. However, certain highly conserved residues are present, and they may contribute to a conserved structural motif that broadly facilitates chemokine receptor interactions. Chemokines suppress HIV infection by blocking the viral entry process supported by chemokine

receptors. Following the binding of oligomeric viral gp120 to CD4 on the host cell surface, the resulting CD4-gp120 complex binds a coreceptor (Wu et al., 1996) which in turn, exposes the N-terminal fusogenic sequence of gp41 (Chan et al., 1997). Chemokines disrupt this process by inducing coreceptor internalization (Chan et al. 1997) into endosomes (Chan et al., 1997) which effectively prevents the formation of gp120/CD4/corecptor tricomplex. Most of the evidence indicates that this inhibition does not require coreceptor activation. First, treatment of cells with pertussis toxin, which is a selective inhibitor of G proteins involved mainly in chemokine-induced intracellular signalling, fails to reduce chemokine-mediated suppression of HIV replication. Doranz et al., 1996). Second, mutant chemokine receptors are impaired in their ability to transduce intracellular signal-exhibited coreceptor activity (Atchison et al., 1996). Third, the chemokines modified at their N-termini act as antagonists of their wild-type counterparts, but do not trigger receptor activation, and are active as anti-HIV (Simmons et al., 1996). Finally, some monoclonal antibodies that possess anti-HIV activity bind to bind to CCR-5 or CXCR-4 without triggering intracellular signalling (Endres et al., 1996).

Another enigmatic feature of chemokine-mediated HIV suppression is that a ligand specific for one coreceptor can be effective against some viral strains even when other usable coreceptor species are present. Brain derived cell cultures expressing both CCR-3 and CCR-5 were protected from infection by reported pseudovirions containing NSI envelopes by treatment with either eotaxin or MIP-1β alone (Bron et al., 1997). This suggests that certain chemoreceptors might be downregulated from the cell surface in a co-ordinated manner or that there may be cooperative use of different coreceptors by at least some HIV isolates.

I. 1.5.1.2 ROLE OF CHEMOKINES IN HIV PATHOGENESIS

At the coreceptor level, it has been established that specific alleles in coreceptor genes modulate expression and profoundly influence HIV infection. The most studied is a CCR-5 allele (Δ32) encoding a 32 base pair deletion. This gene produces a truncated and non-functional receptor form that is not transported to the cell surface. Homozygosity of this gene confers strong resistance to HIV infection although a few cases of HIV-infected Δ32 homozygous individuals have been reported (Michael et al, 1997). Heterozygosity for the mutated allele is associated with a slower course of disease progression (Endres et al. 1996). The effect in heterozygotes appears to be a transdominant suppression of wild-type CCR-5 coreceptor function due to an intracellular association of defective and normal gene products (de Roda Husman et al., 1997) leading to retention in the endoplasmic reticulum. The net result is decreased surface CCR-5 expression relative to wild-type homozygotes. However, the presence of Δ32 CCR-5 is not associated with any known pathology in either the homozygote or the heterozygote.

In addition to the control at the genetic level, coreceptor expression is a function of receptor-ligand interactions that mediate surface down-regulation. Given the significant effects of reduced coreceptor expression on HIV infection, it is entirely plausible to expect that chemokine levels themselves correlate with disease progression. It is also possible that certain genetic alleles that reduce coreceptor expression work in concert with chemokines to modulate infection. Several groups have attempted to determine whether there is an inverse correlation with chemokine levels and disease progression. One approach was to measure plasma or serum levels of RANTES, MIP-1α and MIP-1β in several cohorts. Overall, these studies have failed to show an inverse correlation between chemokine levels and disease status (Zanussi et al., 1996). A much more succesful approach has been to measure

chemokine release by primary PBMCs activated *in vitro*. In early studies, two groups failed to detect a correlation between production of chemokines and disease progression (Luster et al., 1995; Blazevic et al., 1996). However, other groups subsequently found a significant correlation between RANTES production and resistance to infection and decreased MIP-1α production in symptomatic HIV$^+$ patients (Mackewicz et al. 1996). The disparities between various studies likely arise from subtle differences in sample acquisition, storage and manipulation.

Taken together, these studies present an emerging pictue of HIV pathogenesis in which the production of suppressive chemokines controls disease progression. The results also suggest that in response to HIV antigens, CD4$^+$ effector T cells release antiviral chemokines at the site of viral production. This will not only protects local target cells, but will also protect the activated effector cells by inducing autocrine down-regulation of CCR-5. The induction of this response produces an asymptomatic state for some period of time in all individuals, but a broader and more robust response leads to non-progression or in rare cases, protection from infection.

I. 1.5.1.3 SUPPRESSIVE CHEMOKINES SECRETED BY CD8$^+$ T CELLS

It is known that CD8+ T cells from HIV seropositive individuals produce a soluble non-cytolytic activity that suppresses infection by HIV *in vitro* (Walker et al. 1986). The production of suppressive activity was shown to correlate with immune status and to steadily decline in parallel with disease progression (Walker et al. 1986), indicating that the responsible factors may control infection. Because of these properties, the identification of factors responsible for HIV-1 suppression has been a major objective in HIV-1 research.

Although SDF-1 is an obvious possiblity for the balance of soluble activity effective with SI isolates, recent studies have shown that this cytokine is not involved (Moriuchi et al., 1996). Therefore,

an alternate possibilty is that other chemokines make up the balance of soluble suppressive activity. When HTLV-I immortalized $CD8^+$ T cells from HIV-1 infected individuals were screened for HIV-SF, using an acute infectivity assay with activated $CD8^+$ T cell-depleted PBMCs and virus HIV_{IIIB}, a cell line exhibited suppressive activity against HIV_{IIIB} as well as primary T-tropic and M-tropic isolates (Lacey et al., 1997). The molecule was later identified as a β chemokine, macrophage-derived chemokine (MDC) (Pal et al. 1997). The purified chemokine suppressed a variety of T-tropic and M-tropic primary isolates and thus demonstrated a broader pattern of suppression. It has been reported that MDC mRNA and protein are also expressed by activated $CD4^+$ and $CD8^+$ T cells from healthy individuals (Pal et al., 1997; Godiska et al., 1997). The production by $CD8^+$ T cells further implicates MDC as the soluble suppressive factor.

I. 1.5.1.4 CHEMOKINE-BASED THERAPEUTIC APPROACHES

Given the features of coreceptor functions and novel insights into the pathogenesis of HIV infection, there are at least two different ways one can exploit the knowledge for therapeutic implications. First, molecules can be designed that can prevent HIV binding to the coreceptors, without triggering intracellular signalling. Second, molecules can also be designed that down-regulate receptor exposure on the cell surface.

An approach described recently uses a novel concept to induce receptor down-regulation. Chemokine receptors are coupled with an endoplasmic reticulum retention signal, thus causing the chemokine to be retained intracellularly in the endoplasmic reticulum ("intrakine"). Receptor expression on the membrane is down-regulated in cells producine intrakines, as the receptor binds to its ligand intracellularly and therefore its exposure is prevented (Doranz et al., 1997; Donzella et al.,

1998). As a result HIV infection is inhibited in cells expressing intrakines. This approach could be useful as a gene therapy protocol to protect cells from infection.

Another recent study showed that viral pseudotypes containing CD4 and appropriate chemokine receptors were able to target HIV- and SIV-infected cell lines and primary macrophages. The targeting was shown to be related to the specificity of the viral envelope for a given CD4-chemokine receptor complex. This novel approach might contribute a new strategy to target specifically HIV-infected cells, thus providing an important tool for gene therapy approaches (Chen et al. 1997).

I. 1.5.2 POST BINDING EVENTS IN VIRUS INFECTION

The post binding events of HIV entry are also made up of a set of sequential steps.

I. 1.5.2.1 ENVELOPE SHEDDING

The HIV envelope proteins can be involved in steps other than binding to cell surface receptor, during virus infection of a cell. Some reports have suggested that after attachment to the CD4 molecule, the gp120 is displaced, uncovering the domains of gp41, which is needed for virus-cell fusion (Sattentau and Moore, 1991). Recent analyses suggest that this displacement results from the dissociation of a knob and socket like structure involving the carboxy terminal region of gp120 and the central portion of gp41 (Schultz et al., 1992). Shedding does not appear to occur (Dimitrov et al., 1992) or to be necessary as long as the fusion domain on gp41 is exposed (Sattentau and Moore, 1991). In this regard, the soluble CD4 (sCD4)-induced shedding of gp120 from viruses, observed *in vitro*, has not correlated well with virus entry or the viral envelope syncytial properties (Berger et al., 1992).

I. 1.5.2.2 ENVELOPE CLEAVAGE

Another event involving the HIV envelope that influences HIV entry into cells is the intracellular cleavage of gp120. Certain studies of gp120 have revealed sites within the V3 loop that could be sensitive to selected cellular proteases (Hattori et al., 1989). The proposed concept was that these enzymes, when present in the cell, cleave gp120 in the V3 region after binding. This in turn facilitates a conformational change in the envelope so that a viral region like gp41 can subsequently fuse with the cell membrane (Sattentau and Moore, 1991). Thus, the $CD4^+$ cells that cannot be infected by certain HIV strains might lack the necessary proteolytic enzymes recognizing a cleavage site of that particular viral V3 region.

The hypothesis of envelope cleavage has gained some support from evidence demonstrating gp120 digestion by proteases (Clements et al., 1991). Recent studies have shown that exposure of HIV to sCD4 leads to cleavage of gp120 by proteolytic enzymes (Moore et al., 1991). This phenomenon can be blocked by monoclonal antibodies (mAbs) to certain regions of the V3 loop (McKeating et al., 1992). However the importance of this event has to be further investigated.

I. 1.5.3 VIRUS CELL FUSION

Enveloped viruses such as HIV enter cells following fusion with the cell membrane. The mechanism for this process in HIV invasion is not yet known. The fusion step could follow a conformational change in the CD4 molecule as well as, dissociation of the envelope gp120 or exposure of its V3 loop to proteolytic cleavage.

The kinetics of this fusion reaction suggests continued attachment of virus to membrane CD4 while the fusion takes place (Dimitrov et al., 1992). Thus, complete gp120 shedding does not occur

although some displacement, might be involved. The V3 loop as well as gp41 could be important in this membrane fusion event (Berger et al., 1992). Infectivity presumably results from the virus-cell fusion (Bergeron et al., 1992).

Some observations suggest that the CD4 molecule, in addition to binding could be required for the fusion of the viral envelope with the cell surface. Elimination of the proximal region of CD4 through molecular techniques reduces viral infection. It also eliminates the ability of cells to fuse to the infected cells (Poulin et al., 1991). Nevertheless, whether virus-cell fusion and cell-cell fusion involve the same processes is still unknown. Finally, the nature of putative cellular fusion receptor (s) is unknown although a glycolipid that mediates HIV infection has been identified on $CD4^-$ brain-derived and bowel-derived cells (Harouse et al., 1991).

I. 1.5.4 DOWN MODULATION OF THE CD4 PROTEIN

Another early event seen with HIV infection in human T cells is the disappearance of the CD4 protein from the cell surface (Hoxie et al., 1986). The extent and timing of the down-regulation depend on the level of virus production in the infected cells (Stevenson et al., 1987; Zagury et al., 1986).

In vitro, loss of CD4 expression generally occurs several days after HIV infection of cells when sufficient progeny virions are produced. Thus, a reduction in chronic HIV production of a T-cell line with a Tat antagonist, restored CD4 expression (Shahabuddin et al., 1992). The mechanism for altered expression of this cell surface receptor is still not clear. By using interviral recombinants, this CD4 down-modulation has been linked to the envelope region (York-Higgins et al., 1990). The CD4 receptor does not internalize with HIV during infection, and CD4-related signal transduction events are not involved in virus entry (Orloff et al., 1991a & b).

Some reports indicate that down-modulation involves an arrest of CD4 mRNA transcription (Salmon et al., 1988), while others demonstrate complexing of CD4 with the envelope gp160 within the cell (Crise et al., 1990). Some studies have also suggested masking of CD4 by the envelope gp120 attached to the cell surface (McDougal et al., 1986; Sattentau et al., 1986). Finally, some researchers suggest that the CD4 molecule is removed by budding virions (Meerloo et al., 1992). The relative effect of each of these processes on CD4 expression again most probably depends on the particular virus and the cell infected. The relevance to pathogenesis is unclear since some viruses do not modulate the expression of CD4, however the removal of this HIV binding site does prevent superinfection of the cells with other HIV strains.

I. 1.5.5 POSSIBILITY OF ANOTHER CELLULAR RECEPTOR: INFECTION OF CD4$^-$ CELLS

HIV can infect many types of CD4- cells. These include human skin fibroblasts, (Tateno et al.,1989) muscle and bone derived fibroblastoid cell lines (Clapham et al., 1989), human trophoblast cells, follicular dendritic cells, brain derived glial cells (Cheng Meyer et al., 1987), brain capillary endothelial cells fetal adrenal cells (Barboza et al., 1992) and human liver carcinoma cell lines (Cao et al., 1990). Evidence for another cellular recptor in virus entry into these cells comes from studies with Mabs to CD4, incubation of virus with sCD4, and lack of detectable CD4 mRNA in the virus-targetted cells.

The rate of viral replication is generally low in the CD4$^-$ cells (Tateno et al., 1989), and the limited virus production is a consequence of inefficient viral entry, since usually fewer than 1% of CD4$^-$ cells become infected (Mellert et al., 1990). To detect HIV production in these CD4$^-$ cells, co-cultivation of the cells with other sensitive targets, such as PBMC, has been required (Tateno et al.,

1989). Recent studies suggest that cytokines produced by the PBMC can enhance HIV production in $CD4^-$ cells, in particular those of brain origin (Swingler et al., 1992).

The nature of the cell surface molecule (s) responsible for viral entry into the $CD4^-$ cells is not known, but entry conceivably could involve a fusion receptor (Tateno et al., 1989). This route of entry, however, as noted above, is quite limited when compared to the CD4-mediated process. One conclusion could be that for $CD4^+$ cells, the attachment to CD4 enhances the interaction of the viral envelope with the cell surface fusion receptor. Cells lacking the CD4 molecule would use the same mode of entry but it would be much less efficient.

Finally, work demonstrating the lymphocyte function associated antigen-1 (LFA-1) adhesion molecule as a participant in HIV infection offers alternative mechanism for viral entry, although its role appears primarily to be in cell-cell fusion (Hansen et al., 1989).

I. 1.5.6 CELL-TO-CELL TRANSFER OF VIRUS

Besides entering a cell as a free infectious particle, HIV might be passed during cell to cell contact. Evidence has been presented that HIV can spread rapidly from one cell to another without forming mature virions (Sato et al., 1992). Transfer of nucleocapsids is probably involved, with subsequent *de novo* reverse transcription (Li et al., 1992). Moreover, HIV can be transmitted from monocytes or lymphocytes to epithelial cells during such close contact that neutralizing antibodies do not block the transfer (Philips et al., 1992). Thus, HIV spread in the host could result from cell-to cell transfer (via cores or virions) as well as from circulating free virus. During cell-to-cell contact, neutralizing antibodies might not prevent this type of infection.

In summary, the leading concept on early events in HIV infection is that attachment of HIV to the CD4 molecule most probably leads to some conformational changes in virus gp120 and perhaps

CD4 molecule. The initial attachment appears to be at one site on CD4 (the complementarity determining region (CDR) 2 domain). Subsequent displacement of gp120 or cleavage of the envelope protein by cellular enzymes most likely causes changes in the viral envelope, permitting the interaction of gp41 with the target cell membrane. This could possibly involve another cell surface receptor and subsequently, virus-cell fusion occurs (Sattentau and Moore, 1991). Without CD4 expression, fusion of viral gp41 with the cell membrane might take place but the efficiency of this process might be greatly limited. Finally, the spread of the HIV in the host results from production of infectious progeny and also, most probably, by cell-to-cell transfer of immature virions.

1.6 OTHER MECHANISMS OF HIV ENTRY INTO CELLS

HIV can infect cells by mechanisms other than the interaction of its envelope proteins with surface receptors. Antibody-dependent enhancement (ADE) of HIV infection involves binding of the Fab portion of non-neutralizing antibodies to the surface of the virion and transfer of the virus into cells through complement or Fc receptors (Homsy et al., 1989; Horvat et al., 1989). Fc-mediated infection by HIV highlights a potential role of herpes viruses as cofactors in HIV infection. Since these viruses can induce Fc receptors on the surface of infected cells (Keller et al., 1976; Bauke and Spear, 1979) they can then serve as potential target cells for HIV. Further serological studies of individuals infected with HIV are needed to answer the question of the clinical relevance of ADE.

Another mechanism for HIV entry into cells is phenotypic mixing (Boettiger, 1979). By this process, a viral genome can be enclosed within the envelope of a different virus and have the host range of that virus. Moreover, HIV pseudo-types have been produced *in vitro* with herpes virus and rhabdoviruses (e.g., vesicular stomatitis virus) (Weiss et al., 1986). Viruses that can undergo phenotypic mixing with HIV-1 are HIV-2, HTLV-1, vesicular stomatitis virus, herpes virus and murine

xenotropic, amphotropic, and polytropic type C retroviruses. Whether formation of pseudo-type virus particles occurs in nature is unknown. If this is the case, HIV-infected individuals co-infected with herpes viruses or with HTLV-1 would have virus populations representing phenotypic mixtures with these two agents (Landau et al., 1991).

The term 'superinfection' is also used to denote infection of an individual by more than one HIV strain. In this case, individuals carrying both HIV-1 and HIV-2 are documented (Evans et al., 1988; Rayfield et al., 1988), although this event is most probably uncommon. Moreover, chimpanzees can be simultaneously infected by more than one HIV-1 strain (Fultz et al., 1989). In none of these cases, has recovery of two distinct viral strains from the same cell been documented.

I. 1.7 HIV CYTOPATHOLOGY

Another important biologic feature of HIV infection is formation of multinucleated cells in culture (syncytium), resulting from the fusion of infected cells with uninfected $CD4^+$ cells (Lifson et al., 1986; Lifson et al., 1988). Syncytium formation is often the first sign of HIV infection in culture and can appear within 2-3 days. Ballooning of the cells accompanies this cytopathic effect most probably resulting from membrane permeability changes. This cell-cell fusion does not require DNA, RNA or protein synthesis (Hansen et al., 1989; Tang and Levy,1990). Whether this process is directly related to virus-cell fusion is not clear, but the cell fusion process certainly involves the CD4 molecule and both the HIV gp120 and gp41 envelope proteins (Sodroski et al., 1986).

The role of cellular membrane proteins, such as the adherin LFA-1, in cell-cell fusion has recently been emphasized (Hildreth and Orentas, 1989). Monoclonal antibodies (Mab) to this cell surface protein block cell aggregation and syncytium formation, but not virus infection (Pantaleo et al.,

1991). The process of cell fusion has been linked to viral cytotoxicity and cell death (Lifson et al., 1986).

Cytopathology and cell death during acute HIV infection in *vitro* is often associated with accumulation of viral DNA in the cytoplasm of the infected cells (Levy et al., 1986; Shaw et al., 1984). However, it is not known whether this process occurs during virus infection *in vivo*. Similar observations have been made with infected T cells arrested in division (Tang et al, 1992). These observations support the conclusion that high levels of intracellular viral DNA can be toxic to the cell and, in the early events of infection, contribute to cell killing (Levy et al., 1986). Nevertheless, single cell killing is not associated with accumulation of viral DNA in the cytoplasm (Bergeron and Sodroski 1992). A variety of processes can be involved in virus-mediated cell death, reflecting toxicity of viral proteins.

I. 1.8 CONTROL OF HIV REPLICATION

Once the virus enters cells as a ribonucleocapsid, several intracellular events take place that lead to the integration of a proviral form into the cell chromosome. The viral RNA, still associated with core proteins, undergoes reverse transcription, using its RNA dependent DNA polymerase and Rnase H activities and eventually forms doubled stranded DNA (reviewed in Greene, 1991). These DNA copies of viral RNA then migrate into the nucleus, where they integrate randomly to the cell chromosome. Integration of the provirus appears to be random and is essential for the cells to produce progeny virions. Recent observations on the early events of viral infection have revealed noteworthy features of HIV replication. In T cells arrested in division, virus infection is abortive, whereas in nondividing macrophages and epithelial cells, progeny production takes place. In permissive activated T cells, HIV undergoes integration and replication within 24 h (Kim et al., 1990). In macrophages, the

process is similar but progeny production appears to require 48 h (Munis et al., 1992). The earliest mRNA species made in the infected cell have low molecular weights, representing the viral long terminal repeats (LTR) and the regulatory genes, particularly *tat, rev* and *nef* (Greene, 1991). It appears that Tat is made first and up regulates the production of Rev. Presumably, the predominance of any one of these gene products can determine whether HIV infection will lead to a productive or latent state. The presence of Tat at high levels will stimulate substantial virus production (Dayton et al., 1986). In the late stages of the virus replicative cycle, Rev would down-regulate its own production and cause decreased progeny formation and perhaps latency. In cells not fully permissive to HIV replication, the relative expression of these regulatory proteins can differ, leading to abortive infection, persistence of virus traces, or a latent state (Pomerantz et al., 1990).

Cellular proteins that could influence virus replication are those reported to increase Tat binding to TAR (Alonso et al., 1992). Recent experiments have suggested that two related cellular Tat-binding proteins might compete to up regulate (e.g., MSSI) or down-regulate (e.g., Tat binding protein 1) Tat activity and thereby affect HIV production (Nelbock et al., 1990; Shibuya et al., 1992). Their mechanisms of action need further elucidation. The potential for using these observations with Tat to develop more effective antiviral therapy merits further evaluation.

The processes involved in T cell activation are not fully defined but are known to affect HIV replication via the interaction of intracellular regulatory factors with regions in the viral LTR (reviewed in Gaynor, 1992). This activation is part of a signal transduction process by which the binding of antigens or mitogens to the surface TCR and CD28 molecules affects gene expression within the cell. The activation is reflected by an increase in the concentration of intracellular free calcium and depends on the subsequent activation of calcium-dependent protein kinase C (PKC) and other phosphorylation events (Kinter et al., 1990). Following the T cell stimulation, cellular transcription factors are released

from intracellular inhibitors (e.g., NF-κB from its inhibitor IκB) (Nolan et al., 1991), via phosphorylation by PKC. They enter the nucleus, where binding to other cellular proteins and, in the case of HIV, attachment to viral DNA sequences take place. The interaction of these transcriptional factors with viral LTR regions can up regulate viral replication. Identification of the cellular transcription factors involved is still ongoing, but many have been recognised (reviewed in Gaynor, 1992). Certain cytokines, as well as transactivating proteins encoded by other viruses, can also increase HIV production via these intracellular events (Kinter et al., 1990).

Cytokines and other external stimuli often effect HIV expression via interaction with the viral LTR, through their influence on intracellular factors, like NFκB (Matsuyama et al., 1991). For example, tumor necrosis factor (TNF-α) increases HIV production. Some viruses such as CMV and herpes simplex virus can enhance HIV production through activation of the viral LTR by viral proteins.

Finally, a *tat* gene product, after interacting with cellular factors binds to the TAR of the viral LTR in conjunction with cellular RNA binding proteins (Wu et al., 1991) that may be phosphorylated (Han et al., 1992) and up-regulation of viral expression occurs. The induction of TNF-α production in T cells by HIV Tat could also be involved in increased virus production (Buonagouro et al., 1992). These observations reflect how both viral and cellular factors interact to affect HIV replication.

Many viruses also enhance HIV production by induction of cytokines. Co-infection of cells with viruses like herpes virus, papovavirus, hepatitis viruses, and retroviruses can enhance the production of HIV-1. Generally, the transactivating factors produced by the infecting virus, usually early gene products, interact directly or indirectly via intracellular factors with the HIV-LTR, usually at the responsive κB region. For example, CMV, human herpes virus 6 (HHV-6) and EBV activate the HIV-LTR as measured in cell culture by the chloramphenicol acetyl transferase (CAT) assay (reviewed

in Nelson et al., 1990). Some biological assays involving HTLV-1 and other animal viruses have demonstrated an increased production of HIV after coinfection (Canivet et al., 1990). Similarly, several B cell lines already transformed by EBV appear to replicate the virus best, perhaps resulting from a postinfection process (Dahl et al., 1987; Monroe et al., 1988; Montagnier et al., 1984). The mechanism (s) for this enhanced expression of HIV in coinfected cells is unknown, but again could reflect a cross-reaction of the HIV LTR.

I. 1.9 MECHANISMS OF HIV INDUCED KILLING

Some understanding of the pathogenesis of HIV infection has come from studying the direct toxic effect of the virus or its proteins on individual cells. Certain strains of HIV-1, particularly those recovered from individuals with advanced disease, have a greater capacity for killing infected cells.

Several observations associate cell death with direct toxicity of the virus or viral proteins. The relative quantities of viral envelope protein produced by the cell can determine cytopathicity (Sodroski et al., 1986). Moreover, the cell fusion that often leads to cell death has been associated with gp120 (Lifson et al., 1988). In one study, doubling the production of gp120 produced cytopathic effects and cell death following HIV infection (Stevenson et al., 1988). Moreover, addition of gp120 to PBMC or cultured brain cells caused killing in a dose dependent manner (Dreyer et al., 1990). The *vif* gene has been linked to cytopathic effects, probably by increasing infectious virus replication (Sakai et al., 1991).

The mechanism of this induction of cell death by the viral envelope protein is not clear at the moment. Disturbances in the membrane permeability could be involved, as reflected by the balloon degeneration of cells observed *in vitro*. HIV binding to and entry into these cells produces membrane discontinuities and pores in association with ballooning (Fermin and Garry, 1992). Hence, cells

infected by and producing cytopathic HIV demonstrate an inability to control the influx of monovalent and divalent cations that accumulate in the cell along with water (Cloyd and Lynn, 1991). The resulting loss in intracellular ionic strength not only leads to cell death, but also at relatively non-cytopathic levels, could change the electric potential of the cell so that normal cell function is compromised.

I. 1.9.1 HIV INDUCED APOPTOSIS

Recently, apoptosis was put forward as a cause of $CD4^+$ cell loss in HIV infection (Groux et al., 1992; Laurent-Crawford et al., 1991). This process has also been observed in T cells during other viral infections like EBV (Uehara et al., 1992). This phenomenon involves the reemergence of a programmed T cell death that is a normal physiological response during thymocyte maturation (Coffin, 1992; Kerr et al., 1972). The process requires cell activation, protein synthesis, and the action of a calcium dependent endogenous endonuclease that produce fragmentation of cellular DNA. Apparently $CD4^+$ cells do not undergo apoptosis in HIV-infected chimpanzees (DeRienzi et al., 1992). This may help to explain the lack of disease in these animals. Cell proliferation induced by phytohemagglutinin (PHA) alone, does not lead to apoptosis. However, stimulation with MHC-restricted class II recall antigens (e.g., tetanus toxin) or with the pokeweed mitogen can cause death of up to 40% in the $CD4^+$ cells from asymptomatic HIV-infected individuals in two days (Groux et al., 1992). Although most studies focus on $CD4^+$ cells undergoing apoptosis in HIV-infected individuals, some indicate that many $CD8^+$ cells also die by this process (Meyaard et al., 1992). However, the role of $CD8^+$ T cell apoptosis in the pathogenesis of HIV infection requires further investigation.

There is considerable speculation as to whether apoptosis results from direct effects of HIV or its viral proteins, antibodies to CD4, gp120 antibody complexes, variations in cytokine production, or finally superantigens from other infecting pathogens (e.g., staphylococci, streptococci, or mycoplasma).

Some results suggest that gp120 or virus-antibody complexes can elicit apoptosis (Terai et al., 1991). Recently, cross linking of the gp120 bound to human $CD4^+$ lymphocytes followed by T cell activation by anti CD3 antibodies was shown to induce apoptosis (Banda et al., 1992). Some cytokines like IL-4 can also increase apoptosis in macrophages by countering the protective effects of other cytokines (TNF-α and interferon-γ) on these cells (Mangan et al., 1992). These types of interactions could be taking place in HIV infection.

Since unstimulated $CD4^+$ cells removed from the infected individual do not undergo apoptosis, whether this phenomenon occurs to a substantial extent *in vivo* is not clear. However, recent reports suggesting enhanced cell death from this process even in PBMC taken directly from the blood of infected individuals (Groux et al., 1992) are especially relevant.

I. 1.9.2 INFLUENCE OF SUPERANTIGENS

HIV may have a peptide that acts like a superantigen by attaching to $CD4^+$ lymphocytes by one portion of the T cell receptor and triggering cell death by apoptosis (Coffin, 1992). Support for this concept comes from the observation that the individuals with AIDS show a disproportionate loss of T cells with a certain TCR β chain V regions (Imberti et al., 1991). Moreover, superantigens are responsible for the loss of T cells in other retrovirus infections, such as the murine mammary tumour virus and the murine model of AIDS (Woodland et al., 1991). If this process occurs in HIV infection the antigen involved has yet to be characterised. Conceivably, this mechanism for the elimination of $CD4^+$ cells may be caused by other organisms or antigens present during HIV infection.

I. 1.9.3 OTHER CAUSES OF HIV-INDUCED CELL DEATH

A number of other events in the viral cycle have also been believed to be involved with cell death. The accumulation of unintegrated viral DNA appears to be toxic, and the viral Tat protein can kill brain cells (Sabatier et al., 1991). Moreover, interactions of certain cytokines, like TNF-α, with HIV-infected fragile cells might bring about additional damage (Matsuyama et al., 1991). Finally, anti cellular responses of immune cells also could be involved.

I. 1.10 HIV INDUCED IMMUNE DEFICIENCY

The mechanism by which HIV causes a loss of immune responsiveness is a major mystery in AIDS research. Numerous studies have confirmed that immune abnormalities can be observed in T cells, B cells, and macrophages early in the infection well before loss of $CD4^+$ cells begins (Clerici et al., 1992b).

I. 1.10.1 DIRECT CYTOPATHICITY OF THE VIRUS

The prominent immunologic disorder recognised in-patients with AIDS is a loss of $CD4^+$ T lymphocytes (Mildvan et al., 1982). Whether this cell loss reflects direct cell destruction by the virus or its proteins or a secondary effect of immune dysfunction is unclear.

Many features of direct HIV-1 infection may contribute to the reduction in $CD4^+$ cells and their function. First, despite the inability to detect HIV-1 in a large number of $CD4^+$ cells, even in healthy individuals, HIV could be present in a latent or silent state and affect the function, long term viability, and proliferation of these cells (Bagasra et al., 1992). Second, the virus could infect or suppress the production of the early precursors of the $CD4^+$ cells and reduce the quantity of fresh lymphocytes added regularly from the bone marrow to the peripheral blood (Folks et al., 1988). A loss of memory T cells

has been reported in asymptomatic HIV individuals (van Noesel et al., 1990). Third. the HIV *tat* gene expressed in infected cells might reduce the responses of $CD4^+$ cells to recall antigens (Viscidi et al., 1989) and contribute to immunodeficiency. Finally, even if HIV does not replicate at high levels, it might alter the membrane integrity of $CD4^+$ cells sufficiently to affect not only normal function but also increase their overall sensitivity to cellular factors.

I. 1.10.2 SIGNAL TRANSDUCTION ABNORMALITIES

Besides the direct effects of HIV on $CD4^+$ lymphocytes and macrophages, infection of these cells by HIV could interfere with the normal events in signal transduction. This may involve activation by an extracellular signal that subsequently affects the activity of sequence specific transcription factors. This process occurs when natural ligands bind to CD4 or interact with other membrane surface proteins activating T cells and elliciting immune response *in vivo* (reviewed in Greene, 1991). The HIV-1 gp120 has been found to form an intracellular complex with CD4 and $p56^{lck}$ in the endoplasmic reticulum (Crise and Rose, 1992). The retention of this tyrosine kinase in the cytoplasm could be toxic to the cell or affects its function. Furthermore, a possible gp120-receptor interaction with cellular proteins on CD4 negative brain cells with subsequent activation of tyrosine phosphorylation of certain cellular proteins might be involved in the pathogenesis of neurological manifestations of HIV infection (Schneider-Schaulies et al., 1992).

I. 1.10.3 BYSTANDER EFFECT

Another possible mechanism of $CD4^+$ cell loss is absorption of soluble gp120 by uninfected cells carrying the CD4 molecule. These cells can be then recognized as virus infected cells by NK effector cells or CTLs (Lanzavechia et al. 1988) and destroyed, even though they are not infected by

the virus. This hypothesis requires the detection of circulating gp120 in the blood of individuals or on uninfected cells. Although some gp120 released from cells has been found by *in vivo* studies (Gilbert et al., 1991), this feature has not been well documented *in vivo*.

I. 1.10.4 IMMUNE COMPLEXES OF VIRAL PROTEINS

Many investigators have showed that viral envelope proteins have immunosuppressive effects on the mitogenic responses of T lymphocytes (Chanh et al., 1988) or NK cell activity (Cauda et al., 1988). In the case of B cell function, gp120 could interfere with normal T cell help via a block in contact-dependent interactions (Chirmule et al., 1992). Finally, the formation of anti-viral antigen complexes (Morrow et al., 1986) could tie up the reticulo-endothelial system, affect cytokine production and influence immune function.

I. 1.10.5 CYTOTOXIC T CELLS AND $CD8^+$ SUPPRESSOR CELL DERIVED FACTORS

Studies using lymphocytes from infected individuals have suggested that cytotoxic $CD8^-$ cells may kill normal $CD4^+$ cells as well as those infected with HIV (Pantaleo et al 1990; Zarling et al., 1990). Some have found cytotoxic $CD4^+$ T cells against infected $CD4^+$ cells (Orentas et al., 1990). Production of immunosuppressive factors by $CD8^+$ cells has been described (Laurence, 1990) and recently a factor produced by $CD8^+$ cells was found to reduce the response of $CD4^+$ cells to certain recall antigens (Clerici et al., 1992). Production of this factor could explain the early abnormalities seen in helper T cell function (Shearer and Clerici, 1992).

I. 1.10.6 ANTI-LYMPHOCYTE ANTIBODIES

Autoantibodies to lymphocytes could also play a role in immunodeficiency. In early studies, antibodies to both helper and suppressor T lymphocytes were detected, and their presence has since been confirmed (Ardman et al., 1990). Some of these antibodies may result from anti-MHC responses induced by HIV proteins. Moreover, autoantibodies to CD4 protein itself have been detected in HIV infected individuals (Thiriat et al. 1988) and might contribute to $CD4^-$ lymphocyte death.

I. 1.10.7 ROLE OF CYTOKINES

Cytokines are produced by a variety of immune cells during infection and inflammation. Many of these can affect HIV replication *in vitro* (reviewed in Matsuyama et al., 1991) and in some instances promote cell death. Some studies suggest that on stimulation, HIV-infected macrophages release diminished amounts of cytokines or show no change in production of these cellular factors upon stimulation (Roy and Wainberg, 1988). Thus, the relative extent of cytokine expression during HIV infection is not clear, and whether these cellular products act as cofactors to influence the $CD4^-$ cell destruction or compromise their function needs further evaluation.

I. 1.11 HUMORAL IMMUNE RESPONSES TO HIV INFECTION

In this section, the host humoral immune responses that could influence HIV-induced disease are discussed.

I. 1.11. 1 NEUTRALIZING ANTIBODIES

A conventional response of the host to a viral infection is the production of antibodies that attach to the virus and neutralize it. The HIV envelope is the major target for the humoral antibody responses. The viral proteins believed to be primarily involved in antibody neutralization have been

localized to the envelope gp120 and the external portion of gp41 (Broliden et al., 1992). Moreover, as the disease progresses neutralizing antibodies can be replaced by enhancing antibodies (Homsy et al., 1990). In general, sera from HIV-1 infected individuals can neutralize HIV-1 but not HIV-2 strains. In contrast, sera from HIV-2 infected individuals have been reported to cross react with and neutralize some HIV-1 strains (Weiss et al., 1988). This cross reactivity could be governed by antibodies to the CD4 binding site, particularly conformational epitopes (Steimer et al., 1991).

The principal neutralizing domain of gp120, called the V3 loop, is found in the central portion of the third variable region, located in the N -terminal portion of gp120 (Broliden et al , 1992). The V3 loop contains both neutralizing and nonneutralizing epitopes, since sera with high titer antibody to V3 peptides do not always neutralize the homologous HIV strain (Warren et al , 1992).

The neutralizing antibodies detected against gp41 have received little attention. Nevertheless, immunization of animals with the N-terminal portion of this envelope protein (Chanh et al. , 1986) has elicited antibodies to homologous and heterologous strains.

The clinical relevance of these neutralizing antibodies remains uncertain. Whether levels of neutralizing activity correlate directly with the clinical state is still controversial (Alesi et al., 1989). Patients including those with AIDS can have substantial titers of neutralizing antibodies against laboratory strains (Robert -Guroff et al., 1985). In most cases, however, their anti viral response to a homotypic strain, which would be of great importance clinically, was not demonstrated. Moreover, the virus mutates under immunologic pressure to escape neutralization (Nara et al., 1990). Thus, the induction of neutralizing antibodies would appear to be most beneficial early in the course of HIV infection and to have less influence at later stages.

I. 1.11.2 ANTIBODY DEPENDENT CELL CYTOTOXICITY

Antibodies to both gp120 and gp41 envelope proteins induce antibody dependent cellular cytotoxicity (ADCC) (Koup et al., 1989). Here, the antibody coated cells are recognized by effector cells, like NK cells, bearing the Fc receptors or by monocytes and killed by a cytotoxic mechanism, most probably cytokine mediated (reviewed in Yagita et al., 1992). Whether ADCC is relevant clinically in HIV infection is not known.

I. 1.11.3 ANTIBODY ENHANCEMENT

In HIV infected individuals, the presence of antibodies, that can enhance viral infection either via complement or Fc receptors, has been demonstrated (Robinson et al., 1988). Whether the CD4 molecule play a role in this process is still controversial. If CD4 is involved in ADE, many investigators prefer to conclude that the enhancement occurs because the virus-antibody complexes are brought closer to the CD4 molecule after attachment to the Fc or complement receptors. Alternatively, if CD4 is not involved, perhaps HIV is brought to the cell via Fcγ-receptor binding and then the virus fuses directly with the cell membrane. The clinical significance of ADE in HIV infection is not known but its association with disease suggests that it plays a role in the pathogenesis (Homsy et al., 1990).

In summary, it can be concluded that neutralizable HIV strains can mutate to become resistant to or enhanced by the same antibody species. Immunization of individuals, with a particular virus strain, might induce neutralizing antibodies to the immunizing strain, but enhancement of a different strain, particularly one from another part of the world. Defining envelope regions that will induce only neutralizing and not enhancing antibody responses could be very difficult. Some studies have suggested that a very small change at the critical region, perhaps in one amino acid, might determine the sensitivity of a virus to antibody neutralization or enhancement.

I. 1.12. CELL MEDIATED IMMUNE RESPONSES TO HIV

In the next few pages, the cellular immune responses that are directed against HIV through specific recognition of the virus or virus infected cell are reviewed. In most viral infections, the cell-mediated immune response plays a critical role in arresting or eliminating the infectious agent (Doherty et al., 1984).

I. 1.12.1 CYTOTOXIC NATURAL KILLER CELLS

A major component of cellular immunity is the NK cell, which recognizes and kills virus infected cells in a non-MHC dependent manner. In HIV infection, this cell type has been found to decrease function, particularly as infected individuals progress to disease (Cai et al., 1990). This finding appears to reflect a reduction in NK cytotoxic factor production (Bonavida et al., 1986) and polarization of cytolytic machinery upon binding to target cells does not occur (Sirianni et al., 1988). Recently, the reduced NK-cell activity noted *in vitro* was countered by the addition of a B cell cytokine IL-12 to the assay (Chehimi et al., 1992). Since IL-12 also restored the responses *in vitro*, the potential of IL-12 for therapy has to be considered.

I. 1.12.2 $CD4^+$ CELL RESPONSES

$CD4^+$ T cell responses can also be decreased early in HIV infection. Recent observations indicate that, like in the murine system, human $CD4^+$ cells can be separated into two functional subsets, TH1 and TH2. TH1 cells secrete IL-2, and IFN-γ; while TH2 cells produce IL-4, IL-6 and IL-10. From studies of HIV infected individuals, a hypothesis was put forward that levels of TH1 and TH2 cytokines play an immunoregulatory role in HIV infection and they can affect progression to AIDS (Shearer and

Clerici, 1991; Sher et al., 1992). It is noteworthy that TH1 responses are found primarily in healthy asymptomatic individuals and high-risk individuals without evidence of HIV infection (Clerici et al., 1992b; Shearer and Clerici, 1991). Several investigators have suggested that this type of cell mediated immune response could protect individuals from HIV infection (Sher et al., 1992). A subsequent TH2 response would lead to B cell activation and hypergammaglobulinemia, most probably secondary to IL-4 and IL-6 production by the TH2 cells. In this regard, the balance appears to favour TH2 cells, in AIDS patients. Moreover the secretion of high levels of IL-10 by TH2 cells can suppress the TH1 response (Shearer and Clerici, 1991; Sher et al., 1992).

Since TH1 cells produce IL-2 and other cytokines that enhance the generation and activity of $CD8^+$ cells, this subset could also be very important in the cellular immunologic control of HIV infection and prevention of AIDS. Some studies with human T cell clones demonstrate that certain $CD4^+$ lymphocytes, although sensitive to infection by HIV, can also show cytotoxicity against HIV-infected targets (Orentas et al., 1990).

Virus-specific CD4 T lymphocytes are particularly undetectable in human immunodeficiency syndrome infection. In individuals who control the infection without the antiviral therapy, polyclonal antiviral CD4 responses are present and they persist (Rosenberg et al., 1997). HIV-1 specific proliferative responses were also demonstrable after treatment of acute HIV infection (Rosenberg et al., 1997).

I. 1.12.3 CYTOTOXIC $CD8^+$ CELLS

Cytotoxic T lymphocytes (CTL) use clonotypic T cell receptors (TCR) associated with the invariant CD3 signaling complex, to recognize antigenic peptides bound to major histocompatibility complex (MHC) molecules on the target cell. It has long been realized that more than one mechanism

of cytolysis is used by cytotoxic lymphocytes (CL). Even when redirecting human peripheral blood T lymphocyte subsets to lyse antigen coated red blood cells (RBC) or nucleated target cells, it was apparent that effector T cells were using distinct mechanisms depending upon the target cell, the presence of Ca^{2+}, the need for *de novo* protein synthesis, and effector granule exocytosis (Smyth and Ortaldo, 1993). More recently, through the development of gene knockout mice and identification of membrane-bound mediators of target cell apoptosis, it has become evident that two major forms of cytotoxicity are used by CTL.

I. 1.12.3.1 THE GRANULE EXOCYTOSIS MECHANISM

Although the mechanisms of recognition of target cells by CTL and NK cells are very different, evidence indicates that the lethal hit delivered by both cell types involves components of their characteristic electron dense cytoplasmic granules (Henkart, 1994). In the presence of Ca^{2+}, CTL cytotoxic granules are vectorially secreted into the intercellular space formed during conjugation of the CTL and the target cell (Henkart, 1985) and lysis is often associated with membrane lesions on the target cell (Podack and Dennart, 1983). The granules of CTL contain a number of proteins including a pore forming protein termed perforin, and a family of serine proteases collectively called granzymes. Perforin causes osmotic damage through its binding of phosphoryl choline headgroups, polymerization and subsequent pore formation in the lipid bilayer of the target cell. These pores formed in the presence of Ca^{2+} have been shown to allow efflux of large proteins and ions, and it was thought that this damage was lethal to the target cell. These observations along with the purification and subsequent cloning of perforin, led some to believe that the mechanism of cell mediated cytolysis was ultimately solved (Lichtenheld et al., 1988). However, CTL-mediated target cell death generally involves changes such as chromatin condensation, extensive membrane blebbing and ultimately,

nuclear DNA fragmentation (apoptosis) (Duke et al., 1983). These events clearly occur some time before appreciable perforin mediated cell lysis, and purified perforin alone is incapable of causing DNA fragmentation (Duke et al., 1989). The recent development of perforin gene knockout mice has allowed the cytotoxic function of perforin *in vivo* to be definitively addressed (Kagi et al., 1994a & b). Experiments in perforin (-/-) homozygous gene knock out mice indicated that perforin is critical for: (1) effective CTL clearance of lymphocytic choriomeningitis virus (LCMV) i.e. anti viral activity; (2) CTL lysis of allogeneic fibroblasts and tumor cells; (3) clearance of *Listeria monocytogenes* infection (Kagi et al., 1994a & b) (i.e. intracellular bacterial infection and (4) the cytotoxicity of peritoneal exudate, lymphokine activated killer cells (LAK) and NK effector cells.

I. 1.12.3.2 GRANZYME-PERFORIN SYNERGY

Considerable *in vitro* and *in vivo* experimental evidence suggests a supplementary role for granzymes in target cell killing. The enzyme activities of various granzymes have been designated according to their hydrolysis of synthetic thio benzyl ester substrates, such as tryptase (cleavage after arg or lys) aspase (cleavage after asp or glu), chymase (cleavage after aromatic amino acids) and met-ase (cleavage after met) (Odake et al., 1991). Three enzyme activities that have been definitely matched with certain granzymes are tryptase (granzyme-A and Tryptase-2) (Sayers et al., 1994), Aspase (granzyme B); (Poe et al ., 1991),and Met-ase (Met-ase -1); (Smyth et al., 1992b). A role for granzymes in cellular cytotoxicity had been postulated for several years, principally on the basis that cytotoxicity could be completely abrogated in some cases by a variety of protease inhibitors (Shi et al., 1992). Granzymes by themselves do not have cytolytic activity, but the ability to induce DNA fragmentation has been described for many granzymes (Shi et al., 1992). Granzymes are able to fragment the DNA from many target cells of diverse lineages and the actions of different granzymes can be synergistic.

Irreversible inhibitors like aspartate (Shi et al., 1992) can effectively block DNA fragmentation induced by granzymes. Transfection studies have demonstrated that expression of perforin by a granulated noncytolytic rat basophil leukemia (RBL) enables this cell line to kill non-nucleated target cells, such as immunoglobulin E coated erythrocytes provided the cells are cross linked using RBL Fcε receptor (Shiver and Henkart, 1991). However, nucleated target cells could not be killed in this manner unless the RBL transfectants also expressed granzyme A or B. Co-transfected perforin and granzymes are perfectly targeted to the granules of RBL, and these transfectants were several fold less cytolytically active than CTL.

Gene knockout mice with a homozygous null mutation of granzyme A (Ebnet et al., 1995) or granzyme B (Heusel et al., 1994) genes develop normally and have normal hematopoesis, lymphopoiesis, and CL granule formation. *In vitro*, CTL, NK, and LAK derived from the granzyme B knockout mice are unable to induce rapid DNA fragmentation in allogenic target cells. The defect is kinetic in nature and can be rescued with longer incubation periods, implying that other granule proteins may also play an important supplementary role. In addition, ^{51}Cr release due to low concentrations of perforin can be augmented in a dose dependent manner by the addition of granzyme B. The granzyme A (-/-) mice recover from primary listeria monocytogenes infection and eradicate syngeneic tumors with kinetics similar to those of the wild type littermates. Also, the absence of granzyme A or B results in delayed clearance of LCMV from spleen and liver.

To summarise these *in vivo* observations, it can be concluded that while granzymes may not play a primary role in CL effector responses to foreign or infected target cells, their peculiar function may be in host CL eradication of viral infection.

I. 1.12.3.3 PERFORIN-GRANZYME COLLABORATION

Detergents and other pore forming agents (as a substitute for perforin) cannot synergise with granzymes to cause DNA fragmentation. Furthermore, microinjection of granzyme B into the target cell cytoplasm induced plasma membrane blebbing, but only limited nuclear damage and chromatin condensation (Greenberg, 1996). These data suggest that perforin does more than merely enable other cytotoxic granule contents to enter the cell. More recently, immunoelectron microscopy and other studies have indicated that granzyme B can enter the cells in the absence of perforin, but without measurable cytotoxic effect (Greenberg, 1996; Froelich et al., 1996). Hence perforin's major role following pore formation in target cells may be help to trigger an internal disintegration pathway in the cell.

I. 1.12.3.4 EFFECTS ON CELL CYCLE CONTROL

It remains to be established what downstream events are triggered immediately after perforin binds to the target cell membrane and granzyme B enters the cytoplasm, but clearly both sets of events coincide in a death signal. Shi et al, (1992) have been able to demonstrate that cdc2, the mitosis-regulating cyclin dependent kinase is required for perforin/granzyme induced apoptosis. When added with perforin to target cells, granzyme B induces premature activation and tyrosine dephosphorylation of cdc2 and apoptosis is induced at all stages of cell cycle. This contrasts with the dogma that quiescent cells are refractory to DNA fragmentation and that G_0 cells appear to be relatively resistant to CL-induced apoptosis (Nishioka and Welsh , 1994). Normally, throughout the cell cycle and until the cell is prepared to enter mitosis, a nuclear kinase, Wee-1, which maintains mitotic timing negatively regulates cdc2 kinase activity by phosphorylation of a residue within its ATP binding domain. Wee-1 can rescue a target cell from granzyme B-induced apoptosis by preventing cdc2 dephosphorylation

(Chen et al., 1995). CL must activate a mechanism for which all the necessary molecules are already present in the target cell, as DNA fragmentation induced by granzyme B/perforin does not depend on new protein synthesis in the target cell. It makes evolutionary sense for this type of defense system to operate independently of the host cell protein synthesis, since many viruses shut off host cell protein synthesis early in infection.

I. 1.12.3.5 SPECIFIC SITE OF GRANZYME ACTION

The fact that granzyme B can enter target cells independently of perforin suggests that receptors for granzymes must exist in the plasma membrane. The first report of a novel serine protease proteolytic mechanism of receptor activation is drawn from the isolation and subsequent cloning of a thrombin receptor (Vu et al., 1991). A similar subfamily of G protein coupled receptors have been suggested to be possible candidate granzyme receptors, given that granzyme A can activate the thrombin receptor itself. These receptors do not internalize their ligands and therefore this would not explain the uptake of granzyme B into target cells. Many have drawn parallels between granzyme B and a family of intracellular cysteine proteases [such as the interleukin- 1β converting enzyme (ICE)] based upon their shared Aspase-induced apoptotic activity (Vaux et al., 1994). These proteases probably have common or similar intracellular target substrates.

I. 1.12.3.6 A SECOND MECHANISM - THE FAS / FAS LIGAND SYSTEM

Previous observations that target cell death can also occur in the absence of Ca^{2+}, granule exocytosis or perforin suggested the existence of an alternative pathway of CL-mediated cytotoxicity. Rouvier et al. (1993) demonstrated that this Ca^{2+}-independent killing involves CTL-mediated crosslinking of the target cell Fas receptor. This induced death process occurs within a few hours, in

the absence of new protein synthesis or extracellular calcium and can be triggered in target cells by monoclonal antibodies against Fas (Trauth et al., 1989). Structurally, Fas belongs to the tumor necrosis factor (TNF) and nerve growth factor (NGF) receptor families (Itoh et al., 1991). Mutational analyses of the cytoplasmic domains of these receptors have identified a conserved region that is necessary for transduction of the apoptotic signal (Tartaglia et al., 1993). Fas ligand (FasL) is a CL surface receptor of the TNF family (Suda and Nagata., 1994). FasL expression appears to be constitutive in NK cells (Arase et al., 1995) and can be rapidly induced in T cells by activation with phorbol esters or by TCR engagement (Anel et al., 1994).

I. 1.12.3.7 THE NATURE OF THE FAS DEATH SIGNAL

Signaling via the Fas receptor can trigger apoptosis, with characteristic cytoplasmic and nuclear condensation and DNA fragmentation (Trauth et al., 1989; Itoh et al, 1991). Triggering of this pathway generally requires cross-linking of Fas and, like TNF, the soluble form of FasL has a trimeric structure. The Fas triggered pathway to death is independent of extracellular Ca^{2+} and macro molecular synthesis (Rouvier et al., 1993; Itoh et al., 1991). As for most death pathways, the cellular environment plays an essential role in the interpretation of the Fas-originating signal and thus cell sensitivity involves other factors than just the level of Fas expression. Conflicting evidence exists concerning the sensitivity of Fas-transduced cell death to bcl-2 expression as some groups claim no effect (Chiu et al., 1995), while others observed partial inhibition (Itoh et al., 1991) or complete inhibition, by co-expression of bcl-2 and its binding protein BAG-1 (Takayama et al., 1995). It is unknown whether molecules like cdc2 kinase play a role in Fas-dependent cell death, however, cytosolic molecules have been identified that can associate with other members of the TNF/NGF receptor family (Rothe et al., 1994). Furthermore, thymocytes from ICE (-/-) deficient mice were resistant to apoptosis induced by

anti-Fas mAb, suggesting that this cysteine protease normally plays a role in the Fas death pathway (Kuida et al., 1995). In addition, the complex lipid, ceramide, a breakdown product of sphingomyelin (a sphingosine-fatty acid-phosphoryl choline molecule found in the plasma membrane and the cytoplasm), can specifically activate protein kinases that have been implicated in Fas -mediated cell death signalling (Cifone et al., 1994).

The death receptors that have been defined are CD95, TNFR1, TRAMP (TNF-receptor-related apoptosis-mediating protein), TRAIL (TNF-related apoptosis-inducing ligand) and TRAIL-R2 (reviewed in Peter et al., 1998). All ligands form trimers and trimerize their receptors upon binding. The pathway that was reported to be involved in CD95 and TNF-R1 signaling was the activation of acidic sphingomyelinase (aSMase) and generation of ceramide, a putative mediator of apoptosis. The aSMase was also reported to be significant for the production of another mediator of cell death, the GD3 ganglioside (De Maria et al., 1997). The role of ceramide has been challenged recently by many investigators. Firstly, C2-ceramide, at low concentrations, induces apoptosis by upregulation of CD95L and not by direct engagement of an intracellular apoptosis inducer (Herr et al. 1997). Secondly, production of ceramide has been noted by many people to be independent of activation of caspases that are essential for apoptosis (Watts et al., 1997). Thirdly, in aSMase$^{-/-}$ mice, the role of activation of aSMase in CD95-mediated apoptosis could be tested. The aSMase-knockout mice have a partial defect in radiation-induced apoptosis, even though no defect in death receptor signaling has so far been reported in these mice (Santana et al., 1996).

Oligomerization of CD95 creates a conformational change of the death domain (DD), which, attracts the adapter Fas-associated death domain (FADD) through its DD. FADD also possesses an amino terminal death effector domain (DED), through which it attracts, procaspase-8a-b and CAP3. Procaspase-8a/b is then cleaved at the death-inducing signaling complex (DISC) leading to the

formation of the active caspase-8 (Medema et al. 1997). The prodomain of the caspase-8 remains at the DISC while active caspase-8 dissociates from the DISC to initiate the cascade of caspases leading to the execution of apoptosis.

Several transgenic mice and knockout mice have recently been generated in experiments that underscore the central role of the DISC-associated molecules FADD and caspase-8 in signaling via the the death receptors. In $FADD^{-/-}$ chimeric mice, CD95-mediated apoptosis was completely blocked in the thymocytes. In addition, fibroblasts from these mice showed no defect in TRAIL-R1-mediated apoptosis, whereas those signals through TNF-R1 and TRAMP were impaired (Yeh et al., 1998). In these T- cells, activation-induced proliferation was severely impaired in spite of normal IL-2 secretion (Zhang et al., 1998). These data suggest that death receptors that use $FADD^{-/-}$ as a signaling adapter may mediate apoptosis and proliferation.

So far, ten caspases have been described in humans (Alnemri et al., 1996). Caspases are classified into three groups that may have redundant functions. Firstly, Caspase-1, 4 and 5, then Caspase 3, 7 and 2 and lastly caspase 6, 8 and 9 (Thornberry et al., 1997). In CD95-mediated apoptosis, caspase-8 plays an important role. It is the apical caspase and is activated at the DISC (Medema et al., 1997). Activated caspases finally cleave a multitude of cellular substrates that yield the morphological picture of apoptosis and oligosomal DNA damage (Martin and Green 1995).

In many forms of apoptosis, one of the first events that is noticed is a drop in mitichondrial transmembrane potential ($\Delta\Psi_m$) which may be in part due to the opening of permeability transition (PT) pores, multiprotein complexes built up at the contact site between the inner and outer membrane (Susin et al. 1997). Mitochondria also release cytochrome c into the cytoplasm resulting in the activation of caspase-9, which in turn activates caspase-3 (Zou et al., 1997).

Recent work has thrown some light into the function of Bcl-2 in blocking apoptosis (Scaffidi et al., 1998). There have been two different apoptosis-signaling cell types (type I and type II) described so far. In type I cells, caspase-8 is activated at the DISC in large quantities resulting in processing of caspase-3. This step is independent of mitochondrial activation and cannot be blocked by Bcl-2. In type II cells, the amount of active caspase-8 generated at the DISC is very small. Apoptosis in type II cells depends on mitochondrial activation and large quantities of caspase-3 are activated. In these cells the overexpression of Bcl-2 and Bcl-x completely blocks activation of caspases (Scaffidi et al, 1998). Release of cytochrome c is believed to be essential for the activation of caspases downstream of mitochondria (Zou et al., 1997).

I. 1.12.3.8 IMMUNOREGULATION BY FAS/FASL

The mouse spontaneous mutants *lpr* (lymphoproliferation) and *gld* (generalized lymphoproliferative disease) carry autosomal recessive mutations. *Lpr/lpr* and *gld/gld* mice develop lymphadenopathy and splenomegaly and produce large quantities of immunoglobulin G and M antibodies, including anti-DNA and rheumatoid factor (Cohen and Eisenberg, 1991). They develop nephritis and arthritis and usually die around five months of age. Many studies indicate that the *lpr* mutation is a loss of function mutation in the Fas gene and the *gld* represents a point mutation in the FasL gene, abolishing the ability of FasL to bind Fas. The abnormal accumulation of lymphocytes the *lpr* and *gld* mice suggest that Fas and Fas L are involved in normal lymphocyte regulation. Positive and negative selection in the thymus is apparently normal in the *lpr* mice (Sidman et al., 1992), indicating that a Fas mediated regulatory mechanism is not critical for the thymic selection processes. However, peripheral clonal deletion and elimination of activated T cells are impaired in *lpr* and *gld* mice (Singer and Abbas, 1994), suggesting that Fas and FasL are normally involved both in the clonal deletion of

autoreactive T cells in peripheral lymphoid organs and in the elimination of activated T cells following recognition of foreign antigens. Among the classical $CD4^+$ T helper populations, TH1 cells can express FasL and lyse target cells in a Fas dependent manner more readily than TH2 cells (Ju et al., 1994). In contrast, $CD8^+$ T cells and NK cells, the professional CTL, can usually utilize both the Fas based and perforin based mechanisms (Arase et al. , 1995; Ju et al., 1994).

Fas and FasL may interact on the same cell (Russel et al., 1993) or on different cells or alternatively, FasL may be secreted and activate Fas in solution (Dhein et al., 1995). In each case, the cytotoxicity is not directed against non-self, or modified self, but against activated self. However, in the physiological context it is unclear whether Fas induced elimination of mature T cells is strictly suicidal, or FasL is effectively provided by neighbouring activated T cells, or possibly even antigen presenting cells. Although T cells upregulate Fas expression within 24 hours of TCR stimulation, they only become sensitive to Fas-mediated cell death several days later. Therefore, an antiviral $CD8^+$ T cell may normally have only a narrow time-span in which to carry out its immune function, prior to its Fas-mediated 'suicide' or 'murder'. In addition, it is not yet clear how NK cells that constitutively express FasL fit into various proposed models of Fas-medited immunoregulation (Crsipe, 1994). There is considerable collective data (Kagi et al., 1994a & b) to argue that a combination of granule exocytosis and Fas pathways account for all cytolysis measured *in vitro*, but it remains to be established whether there is cross-talk between these mechanisms of cytotoxicity.

I. 1.12.3.9 FAS /FASL IN PATHOLOGY

The Fas system may play several different roles in human pathology. First, several patients have been described with a phenotype similar to that of *lpr* mice (Sneller et al., 1992). Furthermore, Cheng et al., (1994), have suggested that soluble Fas may cause the systemic lupus erythematosus

(SLE) phenotype. This second category of Fas-related diseases may be caused by excessive act of the Fas system. There is circumstantial evidence that Fas might be involved in the death of $CD4^+$ T cells during the course of an HIV infection (Debatin et al., 1994; Ameison et al., 1995), and the death of hepatocytes during acute fulminant hepatitis B (Ando et al., 1993). Katsikis et al. (1995) have shown that peripheral blood $CD4^+$ and $CD8^+$ T lymphocytes from HIV-infected individuals undergo apoptosis *in vitro* in response to antibody stimulation of Fas at a much higher frequency than from uninfected controls. Clearly, more work is required to establish the possible pathophysiological roles of Fas/FasL interactions and the results should contribute to a better understanding of the basic mechanism of many human diseases.

I. 1.12.3.10 IMPLICATIONS OF FAS -MEDIATED CELL DEATH IN HIV

Freshly isolated T cells from many HIV^+ individuals spontaneously undergo apoptosis *in vitro* (Ameison and Capron, 1991). These cells were increasingly susceptible to apoptosis when stimulated with mitogens (Groux et al., 1992). This led some researchers to propose that depletion of $CD4^+$ T cells in HIV infected individuals occurred via apoptosis (Ameison and Capron, 1991). Freshly isolated T cells from healthy volunteers do not undergo apoptosis to the same extent as the freshly isolated T cells from HIV^+ individuals. Hence the hypothesis that inappropriate apoptosis may be responsible for the $CD4^+$ T cell loss in these individuals.

Cross-linking of CD4 on murine T cells primes these cells to undergo apoptosis (Newell et al. 1990). Oyaizu et al. (1993) observed similar results using human T cells when CD4 was crosslinked using gp120/anti-gp120 immune complexes. $CD4^+$ cells from normal mice, but not from *lpr/lpr* mice, show selective depletion *in vivo* after treatment with the anti-CD4 antibody (Wang et al., 1994). It was also shown that T cells from mice expressing a CD4 transgene were susceptible to apoptosis when

treated with gp120/anti-gp120 immune complexes (Wang et al., 1994a). The crosslinking of CD4 molecules on the surface of T cells appears to induce the expression of Fas on the cell causing these cells to become susceptible to Fas-mediated cell death. It has been shown that CD4 crosslinking can upregulate the expression of Fas on human T cells (Oyaizu et al., 1994). In addition, anti-Fas antibodies can induce a markedly higher rate of apoptosis in T lymphocytes from HIV-infected individuals than in the controls (Katsikis et al., 1995). Therefore, this hypothesis of Fas-mediated activation-induced cell death (AICD) is one of the mechanisms for CD4 depletion in HIV holds a lot of promise and has to be explored in greater detail.

Interestingly, TH1 cells can be induced to express FasL whereas TH2 cells show little or no FasL expression upon stimulation. The circulating immune complexes composed of gp120 and antibody in HIV^{+} individuals can stimulate both TH1 and TH2 cells of which only TH1 cells express FasL. Thus, the activated TH1 cells express FasL and might be interacting lethally with other TH1 cells. Moreover, the stimulation of TH1 cells would lead to clonal proliferation of TH1 cells that are susceptible to Fas-mediated apoptosis. This situation would be expected to lead to the preferential loss of TH1 cells resulting in a relative increase in the proportion of TH2 cells. Many people have associated an increased frequency of TH2 like cells with progression of disease in HIV-infected persons. This could be a second mechanism by which the Fas /FasL regulatory system influences the course of HIV infection (Clerici et al., 1994).

In summary, CTL primarily utilize a potent pore-forming toxin, perforin, in conjunction with a series of serine proteases capable of inducing fragmentation of target cell DNA. FasL, a cell surface molecule belonging to the TNF family, binds to its receptor Fas and induces apoptosis of Fas bearing cells. Description of the exact nature of these mechanisms may lead to a better understanding of all

forms of cell death and the pathophysiology of many diseases. This may ultimately lead to the introduction of novel therapeutic reagents in the management of a variety of pathological conditions.

I. 1.12.3.11 ROLE OF CTL IN VIRAL INFECTIONS

Another cell type, besides NK, that commonly react with virus infected cells is the cytotoxic T lymphocyte (CTL). Classically, this response is human leukocyte histocompatibility antigen (HLA) dependent and requires cell to cell contact. These cells are very important in the control of certain viral infections (Doherty et al 1984) and probably in the control of HIV. Specific cellular cytotoxicity can be demonstrated with high numbers of unstimulated $CD8^+$ cells, typically at a CD8 to target ratio of 25:1, 50:1, to 100:1, is measured in a 4h ^{51}Cr release assay. Moreover, as expected, the cytotoxicity is observed only with viral protein expressing target cells with the same MHC class I phenotype as the $CD8^+$ cells.

All these studies have shown that $CD8^+$ cells from HIV-infected individuals can kill cells expressing several different HIV proteins, including reverse transcriptase (RT), envelope, core, and some accessory proteins (Autran et al., 1991; Clerici et al., 1991; Hoffenbach et al. , 1989; Kundu and Merigan, 1992; Langlade-Demoyen et al., 1988; Nixon and McMichael, 1991; Plata, 1989). The cytotoxic $CD8^+$ cells can be found in relatively large numbers during the asymptomatic period, but then, they appear to decline, at least in anti-HIV activity in some individuals, with disease progression (Autran et al., 1992; Gotch et al., 1992; Hoffenbach et al., 1989). Incubating the effector cells with anti CD8 or anti-CD3 antibodies blocks target cell killing. A surprising observation is the finding of high levels of anti-HIV CTL precursors in normal uninfected individuals (Hoffenbach et al., 1989).

Although this kind of antiviral activity has prevented virus spread in some animal model systems (Byrne et al., 1984), the role of CTLs in HIV infection is not clear. Despite some correlation

of CTL activity with a healthy clinical state (Autran et al., 1991; Gotch et al., 1992), progression of disease with increasing levels of HIV infected cells occurs in the presence of these CTLs. Recently, a cellular factor that blocks the CTL response was identified in symptomatic individuals (Autran et al., 1991). Finally, several studies suggest that HIV can escape the CTL response (Philips et al., 1991) which could be a means for progression towards the disease. Recently, Wolinsky et al. (1996) demonstrated amino acid changes within the appropriate epitopes of HLA-restricted CTL during the natural course of HIV infection. Thus, evolutionary dynamics exhibited by the HIV virus under natural selection might play a role in progression of disease.

In addition to cytotoxic activity, $CD8^+$ cells can suppress HIV replication in $CD4^+$ cells. Initially, this cellular antiviral activity was identified in infected asymptomatic individuals whose cultured PBMC did not yield HIV. When their $CD8^+$ cells were removed from the blood sample by panning, high levels of virus were released from the $CD4^+$ cells remaining in the culture (Walker et al., 1986). The replacement of $CD8^+$ cells in this culture at levels far below those used to demonstrate cytotoxicity, led to complete suppression of virus replication. Subsequent removal of $CD8^+$ cells again revealed virus-releasing cells.

Several other studies have indicated that the $CD8^+$ cells could suppress virus production without affecting activation markers on $CD4^+$ cells or by killing the virus infected cells (Mackewicz and Levy, 1992). This was confirmed in a large number of studies in which the number of virus infected cells before and after mixing with $CD8^+$ cells remained essentially the same or even increased (Walker et al., 1991; Wiviot et al., 1990). This noncytotoxic antiviral response can be measured with naturally infected $CD4^+$ cells obtained from infected individuals, as well as with normal $CD4^+$ cells obtained from seronegative individuals acutely infected in culture with HIV (Mackewicz and Levy, 1992)

The extent of $CD8^+$ cell suppression varies among subjects (Walker et al., 1989) and decreases in patients with disease (Landay et al., 1993). In many asymptomatic individuals, a ratio of $CD8^+$ cells to $CD4^+$ cells as low as 1:20 in the cell culture assay suppresses endogenous virus replication; but in AIDS patients the ratio often changes to 2:1. Healthy infected individuals monitored over time show a reduction of this $CD8^+$ cell response concomitant with the onset of symptoms (Landay et al., 1993). The significance of this loss of antiviral activity is of special interest as the absolute number of $CD8^+$ cells does not correlate with this antiviral activity (Landay et al., 1993). The $CD8^+$ cell-suppressing activity has also been demonstrated in the SIV system (Kannagi et al.,1988) and in HIV infected chimpanzees (Castro et al., 1992). Moreover, human $CD8^+$ cells show similar effectiveness against different isolates of HIV-1, HIV-2, and SIV (Walker et al., 1991). $CD8^+$ cells from several uninfected individuals also demonstrate this response. However, their reactivity occurs only with naturally infected individuals, not acutely infected cells and generally only at a $CD8^+$:$CD4^+$ cell ratio of 0.5:1 or higher.

It has been shown that a soluble factor produced by $CD8^+$ cells is involved in part in this $CD8^+$ antiviral response (Walker and Levy, 1989) even though cell to cell contact is the most effective method of suppressing HIV replication. The presence of the factor can be shown by adding supernatant from $CD8^+$ cell cultures directly to infected $CD4^+$ cells (Walker et al., 1991). Virus replication is substantially reduced without any effect on cell viability or replication. The level of the factor produced is associated with the clinical state (Walker and Levy, 1989) as the highest levels are derived from $CD8^+$ cells from healthy HIV -infected individuals with high $CD4^+$ counts.

The mechanism by which the $CD8^+$ cells inhibit HIV replication has been an interesting area of study. Suppression appears to occur at or before RNA transcription (Levy et al., 1991). Naturally infected $CD4^+$ cells mixed with autologous $CD8^+$ cells have a marked reduction in viral RNA and protein synthesis. However, at the time of suppression, almost equal number of infected $CD4^+$ cells in

the culture as those present in control cultures can be detected. Thus, no infected cells are lost, and a reduction in virus expression occurs. Recently, Cochi et al. (1995) described three chemokines, RANTES, MIP-1 alpha, and MIP-1 beta as HIV suppressive factors. Recombinant RANTES, MIP-1 alpha, and MIP-1beta induced a dose-dependent inhibition of different strains of HIV-1. HIV-2, and SIV (Cochi et al., 1995). These findings may have relevance for the prevention and treatment of AIDS.

The literature also relates $CD8^+$ cell activity to clinical outcome through the resistance shown by PBMCs from asymptomatic individuals to superinfection by other strains of HIV-1. Despite the known presence of many uninfected $CD4^+$ cells in these cultured PBMC, no acute infection takes place unless $CD8^+$ cells are first removed. Apparently, the uninfected $CD4^+$ cells are protected from infection, mostly by the $CD8^+$ cells, the factor (s) they secrete or both. The study on protection against disease progression in HIV infection has always focussed on the suppressive effects of $CD8^+$ cells on virus replication by cytolytic and non-cytotlytic mechanisms (Walker et al., 1987). Van Kuyk and his colleagues (1994) have also showed that anti-HIV CTL can protect SCID/Hu mice from HIV infection. Hence, it was proposed that a gradual decline in these CD8 cell responses might allow for progression of the disease. There have been a lot of discussions regarding the causes for the ultimate failure of an anti-HIV CTL response. Selective emergence of viral escape mutants (Philips et al., 1991), clonal exhaustion theory (Moscophidis et al., 1993), anergy of specific CTL (Pantaleo et al., 1990), inappropriate T cell help (Shearer et al., 1991), and T-cell mediated suppression (Joly et al., 1989) have all been put forth to explain the defect in CTL activity in AIDS patients. A wide variety of experimental strategies for enhancing the CTL function in HIV patients have been attempted. However, it will take time to determine whether these novel therapeutic modalities are actually beneficial or even detrimental for the patients (Ho et al., 1993). Rapid significant activation of CD8 cells including those that are capable of lysing uninfected lymphocytes occurs during HIV infection (Zarling et al. 1990;

Grant et al., 1993; Grant et al., 1994). It has also been reported that many HIV-infected individuals with very low CD4 counts have high levels of circulating anti-HIV CTL (Grant et al., 1992). Most of the patients with AIDS retain their anti-HIV CTL activity, at least *in vitro* (Grant et al., 1992). In summary it can be concluded that increased numbers of CD8 cells bearing markers associated with anti-HIV CTL activity signifies a poor clinical prognosis (Giorgi et al., 1993; Ho et al., 1993a). Alternatively, the relative dynamics of the stimulation and expansion of anti-HIV CTL and viral replication and spread determines the role for these CTL in terms of protection or damage (Odermatt et al., 1991).

I. 1. 13 CTL IN HIV INFECTION: ARE THEY AUTOREACTIVE?

The initial hematological data from HIV individuals indicating reciprocal changes in $CD4^+$ and $CD8^+$ lymphocyte populations led to the idea that the $CD8^+$ cells could be autoaggressive (Ziegler and Stites, 1988). This argument looked attractive as progressive histopathological observations of lymphoid sections from HIV patients showed CD8 cell hyperplasia followed by a lymphocyte depletion (Ziegler and Stites, 1988). Based on the previous conclusions regarding the immunopathology of lymphocytic chorio-meningitis virus (LCMV) infection in mice and human hepatitis B, a proposal was put forward that HIV infection produces a similar CTL-mediated immunopathology (Zinkernagel and Hengartner, 1994). These studies suggested that anti-HIV CTL-mediated lysis of CD4 cells and other antigen presenting cells (APC) infected with HIV cause CD4 cell depletion and subsequent immunodeficiency.

I. 1.13.1 EPIDEMIOLOGICAL EVIDENCE

Polk et al. (1987), in a comprehensive epidemiological study of factors contributing to disease progression in HIV infection, found that individuals with CD8 counts above 600/μL peripheral blood

had increased relative risks (from 2.01 to 3.69) of developing AIDS within the next 10-18 months. Many recent epidemiological studies also corroborate an association between the elevated CD8 cell counts and proximity of AIDS (Anderson et al. 1991). It was shown by Giorgi et al., (1994), that non-progressors (those with persistent CD4 counts >500/μL), exhibit a slower rate and lesser absolute increases in $CD8^+$ cell counts following HIV infection than rapid progressors.

I. 1.13.2 PHENOTYPE OF CD8

The percentage of particular subsets of $CD8^+$ cells may be more relevant to disease progression than the total CD8 cell number. Multiparametric flow cytometric studies have indicated that an increase in $CD8^+$ T cells that are T cell receptor (TCR) $\gamma\delta^+$, HLA-DR^+, $CD38^+$, interleukin-2 receptor IL-$2R^+$, or $CD57^+$, precedes disease progression (Kestans et al., 1992; Autran et al., 1989). Class II HLA and IL-2R are markers expressed on activated T cells, while CD38 is expressed on immature and activated T cells. The expansion of $CD38^+$ $CD8^+$ T cells was recently shown to indicate a poor prognosis for HIV-infected individuals even though the majority of anti-HIV CTL is $CD38^+$ and HLA DR^+ (Ho et al., 1993). CD57 may distinguish T cells with a particular function or specificity and γδ T cells constitute a distinct T lymphocyte lineage with an unclear function.

Although a causal relationship between the proliferation of $CD8^+$ T cells and progression to AIDS cannot be implied at this time, disease progression and changes in the $CD8^+$ cell repertoire are closely linked. It is possible that activation of $CD8^+$ cells reflects increased replication of HIV or other opportunistic pathogens. The inevitable course of HIV infection indicates that the CD8 cell response is at best temporarily protective. Alternatively, it could be suggested that a strong anti-viral $CD8^+$ T cell response could turn pathological. In any case, the presumption that viral replication is the primary

factor responsible for the activation of $CD8^+$ T cells and that these cells are in turn protective in HIV infection is unsubstantiated.

However, several experimental (Grant et al., 1993; Grant et al., 1994), epidemiological (Polk et al., 1987), and clinical (Devergne et al., 1991) studies suggest that some of the CD8s activated in HIV infection may contribute to disease progression. CD8 cells rise early following HIV infection and remain elevated during the stages when opportunistic infections are rare and HIV levels are low. An aberrant CD8 repertoire is a consistent and early observation in HIV infection during the clinically asymptomatic period. $CD8^+$ T cell infiltration can cause immunopathology of many organs and tissues including the skin (Ringler et al.,. 1992), lungs (Autran et al., 1988), salivary glands (Itescu et al., 1990), central nervous system (Jassoy et al., 1992), lymph nodes (Devergne et al., 1991), and blood vessels (Calabrese et al., 1989) of HIV- infected individuals.

Many cytotoxic drugs block T lymphocyte activation *in vivo* and are used clinically to prevent diseases or conditions with an underlying immune pathology. Many HIV-infected individuals classified according to the Center for Disease Control (CDC) stages 2 and 3, who received cyclosporin A (CsA), an immunosuppressive drug, showed a dramatic reversal of the abnormal CD4 to CD8 ratio as well as showed reduced lymphadenopathy (Andrieu et al., 1988). Upon discontinuation of the drug, the previous abnormalities were restored. These positive results were not reproduced when CsA was administered to AIDS patients (Philips et al.,1989). The response to this immunosuppressive drug, at least in the early stages of disease, could be the result of an inhibition of T cell activation leading to reduced viral replication rather than a direct effect on the virus. In a recent study, HIV-infected individuals were immunized with their own peripheral blood lymphocytes (PBL), or given an intravenous injection of S6F1, an antibody against an activation-induced conformational determinant of LFA-1 on CTL (Allen et al., 1993). The level of CD8s fell and that of CD4s increased. The CD4

increases were sustainable with repeated immunizations with PBLs, but it appeared that the percentage of S6F1$^+$ PBL in the vaccine dictated the initial outcome (Allen et al., 1993). This suggests the autologous lymphocyte vaccines induced an immune response that down-regulated the CTL levels when the proportion of CTL in the vaccine preparation was above a certain threshold level. Individuals treated with S6F1 also showed reversion of cutaneous anergy to recall antigens, suggesting an inverse relationship between the CD8 cell count and immune responsiveness (Allen et al., 1995). Since treatment with an antibody against CTL is not expected to reduce viral replication, the increases in the CD4 cell levels appear more directly linked to diminished CD8 cell activity.

In the human-PBL-SCID mouse model of HIV infection, CD4 cell depletion occurred more rapidly when scid mice reconstituted with human PBL were infected with non-cytopathic variants of HIV (Mosier et al., 1993). These non-cytopathic viruses also induced the greatest proliferation and activation of CD8$^+$ lymphocytes (Mosier et al., 1993). Enhanced CD8 cell activation in this system is not protective and since anti- HIV CTL were not detected among the CD8 cells activated by HIV, it does not necessarily reflect an adaptive response to greater replication of HIV (Mosier et al., 1993).

Speculations that CD8 cells might contribute to CD4 cell depletion and disease progression are supported by experimental observations like CD8-mediated killing of HIV-infected CD4 cells (Siliciano et al., 1988). More recently, it was shown that CTL from HIV patients directly kill uninfected CD4$^+$ cells (Grant et al., 1993; Zarling et al., 1990). Zarling et al. (1990) also detected CTL, which killed uninfected CD4$^+$ cells in 11 out of 13 HIV$^+$ individuals. They also showed that HIV-infected chimpanzees in contrast to humans, do not have CTL that kill uninfected CD4$^+$ cells (Zarling et al. 1990). Even when infected with an HIV variant highly cytopathic to chimpanzee lymphocytes *in vitro*, chimpanzees do not suffer CD4 depletion and hence are not susceptible to AIDS (Watanabe et al., 1991). Although they become chronically infected and mount a vigorous anti-HIV response, HIV-

infected chimpanzees show none of the CD8 cell subset derangements or immune dysfunctions characteristic of human HIV infection (Ferrari et al., 1993).

Investigators have also found that CTL that can kill uninfected CD4s can be isolated from the cultured PBL of HIV-infected hemophiliacs and homosexuals (Grant et al., 1993; Lederman et al., 1996). CTL activity against uninfected CD4s detectable following *in vitro* stimulation and is selectively present in stimulated PBL from HIV-infected individuals (Grant et al., 1993). An important consideration is that these CTL may act locally *in vivo* within the spleen or lymph nodes and the activity detectable in the peripheral blood is a weak reflection of *in vivo* events.

In murine model systems, such as LCMV infection, otherwise innocuous viruses can induce CTL responses that kill the host (Zinkernagel et al., 1985: Odermatt et al., 1991). The pathogenesis of HIV infection could be exacerbated by the unusually vigorous CD8 cell response, without being an all or none system, like LCMV. This has to be investigated further especially in light of recent reports by Pantaleo et al. (1994) of oligoclonal expansion of $CD8^+$ T lymphocytes among individuals infected with HIV. The oligoclonal expansions were most notably in a restricted set of variable-domain beta chain families. Cells expressing the expanded V betas predominantly expressed the $CD8^+$ T-cell differentiation antigens and mediated HIV-specific cytotoxicity (Pantaleo et al., 1994).

More effective antiviral regimens and the more accurate picture emerging of the relationship between HIV and $CD4^+$ lymphocyte dynamics suggest that factors other than virus replication can limit $CD4^+$ lymphocyte replenishment (Wei et al., 1995: Ho et al., 1995). The actual mechanism of CD4 depletion in HIV infection remains unknown and given the reported ability to replenish up to 5% of $CD4^+$ cells daily, it remains difficult to explain persistently falling $CD4^+$ lymphocyte counts purely as a consequence of the level of HIV replication. CTL from HIV-infected individuals kill CD4 cells *in vitro* and this could indicate a mechanism of CD4 depletion *in vivo*. Determining whether these CTLs

operate *in vivo* and if their actions contribute to disease progression is critical for understanding the pathogenesis of AIDS and for incorporating rational CD8-based approaches into the management of HIV infection.

1. 14. IS AIDS AN AUTOIMMUNE DISEASE?

Since HIV disturbs the balance of the immune system, it is not surprising that autoimmune diseases like Reiter's syndrome, systemic lupus erythematosus, Sjogren's syndrome,vasculitis and polymyositis accompany this viral disease (Calabrese et al., 1989). Vasculitis in HIV-infected patients has been linked to deposition of immune complexes (Calabrese et al., 1989) but immune complex glomerulonephritis is rare.

In terms of humoral immune responses, in early studies of AIDS, antibodies, often associated with clinical disorders, were detected against platelets, T cells, and peripheral nerves (Morrow et al., 1991). The reasons for these sequelae are not known.

A lack of T cell regulation in HIV infection can lead to a proliferation of B cells with resultant polyclonal proliferation and antibody production. These kinds of reactions have been reported in other viral infections like EBV infection, in which hypergammaglobulinemia and autoimmune disorders have been documented (Henle and Henle, 1979). Polyclonal B cell activity has been observed in HIV infected individuals and is associated with high levels of antibody production (Shirai et al., 1992).

When a microrganism shares either sequence or amino acid homology with a normal cellular component, molecular mimicry can exist. In this regard, similarities between HIV proteins and normal cellular proteins could elicit antiviral antibodies or cellular immune responses that cross react with normal cells. Evidence in favour of this possibility include the presence of IL-1, IL-2 receptor, MHC

class I and class II molecules and interferon like sequences in several HIV genes, as well as other potential cross-reacting epitopes within the Env proteins of the virus (Levy, 1989).

One popular mechanism for autoimmunity involves the production of a network of antibodies produced after an antigen is introduced into the host. Besides making antibodies to the incoming antigen, the antibodies to these anti-antigen antibodies are induced. These so-called anti-idiotype antibodies could be mirror images of the epitope against which the initial antibody was produced. Thus, antibodies to HIV envelope gp120 might induce autoantibodies to the CD4 protein to which the gp120 attaches. Although the possibility for these antibodies to form and be detrimental for the host is proposed, evidence for such a phenomenon has not yet been reported.

Although a role for autoimmunity in HIV-induced disease is as yet unsubstantiated, the presence of autoantibodies in HIV should be considered as a potential co-factor in AIDS pathogenesis. An autoimmune response has been linked to the loss of neutrophils and platelets and to the induction of peripheral neuropathy (Kiprov et al., 1988).

There is potential danger that vaccination with HIV proteins could elicit, via molecular mimicry, immune responses that deplete $CD4^+$ cells, compromise the immune system, and further induce autoimmune pathology in other tissues. Measuring this pathological response could therefore form part of the evaluation of any therapeutic approach to HIV infection.

I. 1. 15 COFACTORS IN HIV INFECTION

Following the discovery of HIV, came appreciation that many other factors, besides itself, the virus itself might influence the outcome of the disease. The major observation supporting an important role

for cofactors is the variation in the time from infection to development of symptoms and AIDS among different individuals. Host genetic differences and age have been recognized as important variables influencing the progression of disease. In addition, the T cell activation for efficient HIV infection and spread must be considered. A co-factor role for other viruses like herpesviruses and papovaviruses, antigens, and cytokines that increase immune activation has been proposed. Finally, additional immune suppression, resulting from other infectious agents, drugs, or toxins was considered as a possible contributor.

One potentially important co-factor in HIV pathogenesis is infection by another virus. When herpesviruses, adenoviruses, hepatitis B virus, or specific genes from these viruses were introduced into cells transfected with a construct of HIV LTR linked to the chloramphenicol acetyl transferse (CAT) gene, an increase in production of the CAT protein occurred (reviewed in Laurence, 1990). In spite of various *in vitro* results suggesting the role of other viruses as co-factors in HIV pathogenesis, clinical studies of individuals have not yet indicated a contributory role for specific viruses. Certain viruses may contribute to opportunistic infections or tumours observed in some patients, however an association with enhanced progression to disease has not been well documented.

Certain studies have suggested that agents other than viruses could play a role in the pathology observed in HIV infection. Lo and coworkers in 1991showed that a mycoplasma that was found associated with a Kaposi's sarcoma tissue (*Mycoplasma incognitas* a strain of M. *fermentens*), induced immune deficiency and death when injected into macaques. T cells from HIV-infected individuals have been shown to actively respond to mycoplasma antigens (Lemaitre et al., 1990) and the potential role of mycoplasma (in T cell depletion) as superantigens has also been proposed.

HIV is the ultimate cause of AIDS, but other infectious agents or environmental factors could influence the progression to disease. How these cofactors collaborate in the infected individual is not

very clear. They may induce cytokines or intracellular factors that either promote HIV replication, compromise immune responses or both. They may also reduce the production of cellular products such as cytokines and affect immune function in that way. They could stimulate the immune system abnormally and trigger autoimmune responses. Alternatively, they may reduce the cellular antiviral activity and permit the escape of HIV from host immunologic control. It is possible that other factors, including opportunistic infections, can affect the overall health of the individual. Genetic factors can influence cell susceptibility and host immune response. Either by direct infection of cells or by its indirect effect on the immune system of the host, HIV appears primarily responsible for the disease progression observed.

I. 1. 16 FEATURES OF HIV PATHOGENESIS

Given the viral and immunological factors in HIV infection, the proposed pathogenesis is summarised here. The virus initially enters an individual primarily by infecting either activated T cells, resident macrophages, or mucosal cells in the bowel or uterine cavity. In the initial days after the acute infection, high levels of virus replication will take place in the lymph nodes and will be reflected by p24 antigenemia and viremia (Blomberg and Schooley, 1985). Soon after, the viremia is reduced substantially, as a result of immune reactions against the virus and the $CD4^+$ lymphocyte count falls. Cellular immune responses could be the first effective antiviral activity, since in many cases $CD8^+$ cell HIV responses have been detected prior to seroconversion (Clerici et al., 1992). Over the next few years, the $CD8^+$ cell number remains elevated. Virus replication in the body persists particularly in the lymph nodes and PBMC in very low levels as the virus is effectively suppressed. $CD4^+$ cell number usually returns to near normal levels after acute infection resolves, but tends to fall steadily during the persistent period at an average rate of 25 to 40/ μl of peripheral blood per year (Lang et al., 1989). By

the time the individual develops symptoms, $CD4^+$ cell counts are usually below 300 per μl and the levels of HIV are higher than during the asymptomatic period. At this stage, a reduction in the antiviral activity of $CD8^+$ cells can be demonstrated (Mackewicz and Levy, 1992). When the individual advances to AIDS, the virus usually has characteristics distinct from that recovered soon after infection. It takes on properties associated with virulence in the host, including an enhanced cellular host range, rapid kinetics and $CD4^+$ cell cytopathicity.

Because of an ongoing reduction in immunologic control of HIV infection, the more virulent variants replicate to higher levels and cause destruction of a large number of $CD4^+$ cells. They eventually eliminate the potential for any kind of immune response to control opportunistic infections. In the very last stages, the $CD8^+$ cells decrease in number, perhaps in part because of the loss of IL-2 production by $CD4^+$ cells.

Whether emergence of these cytopathic viruses or the suppression of immune responses occurs first is not yet clear. Cellular immune responses appear to be similar against all strains of HIV, suggesting that the loss of $CD8^+$ cell activity may be a major factor in the progression of the disease. A drop in the $CD8^+$ cell antiviral response occurred in three subjects just prior to a fall in $CD4^+$ cell number (Mackewicz and Levy, 1992). The importance of $CD8^+$ cells in controlling the infection over time must be explored in greater detail.

Direct virus infection of $CD4^+$ cells, a compromise in cytokine production, and aberrant immune reactions like ADCC, CTL, autoreactive T cells, autoantibodies, and apoptosis could all play a role in the immunopathogenesis of the disease. The induction of apoptosis in $CD4^+$ cells as well as $CD8^+$ cells particularly requires further study.

The major co-factor influencing delay in disease progression is the inherited genetic makeup of the host that determines both the susceptibility of cells to HIV replication and the extent of

effectiveness of the antiviral immune response. Moreover, the relative sensitivity of the host immune system to destructive effects of viral proteins or cytokines could be important in determining whether there is rapid progression to disease or long term survival.

I. 1.17 FACTORS AFFECTING PROGNOSIS

A 14-year study of HIV infection in a defined cohort of subjects in San Francisco has shown that about 80% of the individuals develop symptoms and that 55% have AIDS (Lifson et al., 1991). These findings indicate that 20% of the infected people remain healthy after 10 years and 12 % retain normal $CD4^+$ cell counts (Lifson et al., 1991).

The major factors responsible for long term survival are summarised here. First, while the $CD8^+$ cell responses decrease with time as individuals progress to disease, they remain strong in long-term survivors and weaker in progressors (Gotch et al., 1992). Second, relatively noncytopathic HIV strains are found in the PBMC of long term survivors with strong $CD8^+$ cell responses (Mackewicz and Levy, 1992). Third, these infected individuals have a low viral load as measured by the number of infected $CD4^+$ cells and free infectious virus in the peripheral blood. Finally, neutralizing and not enhancing antibodies to the virus are found in the blood of long term survivors (Homsy et al., 1990).These findings can be explained by the inability of the HIV strains to replicate in the presence of $CD8^+$ cell antiviral activity.

The most important question in determining the prognosis of HIV infection may be what causes the alteration in the antiviral response of $CD8^+$ cells. A decrease in antiviral activity of the $CD8^+$ cells permits increased virus replication and progression of disease. A major influence can be the genetic makeup of the individual; protection can come from strong immune responses and reduced

inherent sensitivity of the host cells to virus replication. Progression does not reflect a reduction in the total number of $CD8^+$ cells since the level of this subset often remain elevated until the late stages of the disease (Lang et al., 1989). In cell culture, $CD28^+$ $CD8^+$ cells demonstrate high antiviral activity against all HIV strains whether cytopathic or not (Mackewicz and Levy, 1992). Thus. it appears that an intrinsic loss of $CD8^+$ cell activity is involved. This is an important area, which has to be explored so as to shed light on the prognosis of HIV infected individuals.

I. 1.18 GENERAL PURPOSE AND HYPOTHESIS

The objective of this thesis was to assess functional CTL activity in HIV-seropositive individuals and to monitor CTL function over the course of disease progression. The assays were designed in a way to study the nature of CTL activity and to find out whether this may contribute to the $CD4^+$ T cell loss in HIV-infected patients. The first step was to design an assay to define the nature of cytotoxicity among the $CD8^+$ T lymphocytes in HIV-infected individuals. It has been proposed that there are two primary mechanisms of cell-mediated cytotoxicity, one mediated by interactions between FasL on the effector cell and Fas antigen on the target cell and the other mediated by directed release of perforin and granzymes by the effector cell. In the next chapter, I describe the assay, which was designed to distinguish between FasL-mediated and perforin-mediated cytotoxicity. This *in vitro* assay uses murine P815 cells, that express Fas antigen and FcγR receptors as targets. IgG anti-CD3 antibodies bind FcγR receptor of P815 cells and non-specifically sensitize them to CTL-mediated lysis. Human FasL interacts with murine and human Fas antigen and, therefore, P815 cells are sensitive to FasL-mediated killing by human CTL. The cytotoxicity is assessed by a five-hour chromium release assay.

The results as shown in the next chapter will demonstrate that this is an accurate and easy way to measure the cytotoxicity of CTLs in HIV infection.

When cytotoxicity assays were performed using the assay system described in chapter II, it was observed that cytotoxicity was reduced when PMA and ionomycin were used to activate T cells. This phenomenon was studied further in chapter III. As previously reported, I found that T lymphocytes from HIV-infected individuals are more prone to undergo activation-induced cell death than T lymphocytes from uninfected individuals. However, we observe surprisingly, that the PMA and ionomycin-induced death of the T lymphocytes from HIV-infected individuals was predominantly non-apoptotic cell death. The results of chromium release assays, flow cytometry studies, electron microscopy studies and nuclear fragmentation assays clearly demonstrated that this novel form of activation-induced cell death is not mediated by Fas and does not involve nuclear fragmentation.

Cytotoxicity assays were done using PBMC from HIV-infected individuals as effectors at fixed intervals. Total cytotoxicity was measured using P815 cells as targets and autoreactive CTL activity was measured using PHA-activated uninfected T lymphocytes as targets. The phenotype of the autoreactive CTLs was determined by flow cytometry and depletion/selection experiments. Occurrence of CTL-mediated killing of uninfected lymphocytes was then evaluated in the context of clinical and laboratory parameters associated with disease progression in HIV infection. In chapter IV, I describe the results of these experiments, which indicate that the development of autoreactive $CD8^-$ $CD28^-$ T cells is a fundamental component of the immunopathogenesis of HIV infection.

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CHAPTER II

The Differential Sensitivity of P815 Cells to Anti-Fas Antibody and Fas Ligand Illustrates the Mechanism of Cytotoxicity of Diverse Effector Cells

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Running Head: Cytotoxic mechanism of diverse effector cells

Abbreviations used: Chx, cycloheximide; mAb, monoclonal antibody; PMA, phorbol myrystic acetate; PHA, phytohemagluttinin; CTL, cytotoxic T lymphocyte; HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; NK, natural killer; FCS, fetal calf serum; IL-2, interleukin 2; PBS, phosphate buffered saline; PBMC, peripheral blood mononuclear cells; E:T, effector:target ratio.

Most methods for discriminating between Fas ligand and perforin-dependent cytotoxic mechanisms exploit the sensitivity of the effector cell to agents that selectively block one or the other mechanism. We have developed an assay system with P815 cells as generic targets that discriminates perforin-based from Fas ligand-based killing by blocking the Fas receptor with an antagonistic monoclonal antibody (mAb). Over a five-hour assay period, P815cells are completely insensitive to direct induction of apoptosis by anti-Fas mAb Jo2, but are sensitive to Fas ligand expressed on effector cells. Thus, treatment of P815 target cells with Jo2 blocks Fas ligand-mediated killing while allowing effector cell activation by whatever means necessary to trigger cytotoxicity. P815 cells express Fas and Fcγ receptors, therefore, IgG anti-CD3 antibodies, lectins such as phytohemagglutinin (PHA) and pharmacologic agents such as phorbol myristic acetate (PMA) and ionomycin can non-specifically trigger killing of P815 cells by a variety of effector cells. Since Fas ligand interacts across species with human and murine Fas and perforin shows no species selectivity, P815s are sensitive to both types of cytotoxicity mediated by effector cells from various species. Comparable inhibition of cellular cytotoxicity against P815 cells by Jo2 or by cycloheximide, a protein synthesis inhibitor preventing Fas ligand induction, confirmed that the different levels of killing of Jo2 treated and untreated P815 cells reflected the extent that perforin and Fas ligand, respectively were utilized in target cell killing. We used murine T cell hybridomas, a human T cell clone and human and woodchuck peripheral blood mononuclear cells to show that P815 cells can be used to determine dependence on Fas ligand and perforin-mediated killing pathways by virtually any effector cell population, regardless of cell type or species of origin.

Key words: Fas ligand, Fas, perforin, cytotoxic T lymphocytes

INTRODUCTION

Research with perforin knockout mice and with mutant mice lacking functional expression of Fas or Fas ligand suggests that Fas ligand-based cytotoxcity is important in immune regulation, whereas perforin-based cytotoxcity is critical for controlling infections (1-5). This proposed dichotomy has implications in understanding the evolution of protective and pathological cytotoxic T cell (CTL) responses in chronic infections and in chronic inflammation (5, 6). Perforin-mediated killing depends on extracellular calcium but not on new protein synthesis, while cellular Fas ligand-mediated killing requires new protein synthesis but not extracellular calcium (7, 8). Although Fas ligand-based killing can be distinguished from perforin-based killing in calcium free medium (9), induction of Fas ligand expression is calcium dependent and under many circumstances, calcium free medium abrogates both types of killing. Cycloheximide selectively blocks Fas ligand-based killing by preventing *de novo* protein synthesis, but in certain instances it may be cytotoxic to target or effector cells or may reduce cytolytic effector function by inhibiting synthesis of proteins other than Fas ligand. Concanamycin, an inhibitor of vacuolar type H^+-ATPase, selectively inhibits perforin-mediated cytotoxcity, but this requires pre-treatment of the effector cells and it is unclear what effects concanamycin would have when pharmacologic activation of the effector cells is required to trigger cytotoxicity (10). An *in vitro* assay system without effector cell pre-incubation, metabolic inhibitors or calcium free medium would be more broadly

applicable to discriminating between perforin and Fas ligand-based cytolysis mediated by a wide variety of effector cells from different species, triggered in different ways.

Cytolysis by perforin is receptor independent and Fas ligand and Fas are relatively conserved across species. Therefore, if a cytolytic effector cell can be activated to express Fas ligand or release its perforin containing cytolytic granules, species differences between effector and target cell would not prevent killing. Murine P815 cells express Fcγ receptors and can be sensitized to CTL-mediated lysis with IgG anti-T cell receptor (TCR) antibodies (11). P815 cells can also be rendered sensitive to other types of cytolytic effector cells using lectins to bridge target and effector cells or using pharmacologic agents to activate the effector cells. P815 cells express Fas constitutively (12) and are sensitive to Fas ligand and perforin-mediated killing by murine and human effector cells, but resistant to direct lysis by Jo2, an anti-Fas antibody. P815 cells thus make a suitable generic target cell for discriminating between Fas ligand-based and perforin-based killing mediated by diverse types of effector cells from different species, under a variety of conditions.

We incubated P815 cells with Jo2 mAb prior to their use as targets in various cell-mediated cytolysis assays to selectively block killing triggered through Fas/Fas ligand interactions. Cycloheximide, a protein synthesis inhibitor which blocks Fas-dependent killing by preventing *de novo* expression of Fas ligand (9, 13, 14), was used in parallel with anti-Fas antibody to substantiate the selective blocking of Fas-based killing in this assay. There was good agreement between inhibition of killing by cycloheximide and inhibition by anti-Fas

antibody treatment with both NK and T lymphocyte effector cells from 3 different species, triggered by 3 distinct mechanisms. This assay system can be applied to discriminate between perforin and Fas ligand-based killing by virtually any effector cell population or even the same effector cell poulation triggered by different methods.

MATERIALS AND METHODS

Cell lines and antibodies. P815, LS102.9, K-562, Jurkat, J.RT3-T3.5 and OKT3 (IgG_1 anti-human CD3) cell lines were all obtained from American Type Culture Collection, Rockville, MD (ATCC # TIB-64, HB-97, CCL-243, TIB-152, TIB-153, and CRL-8001 respectively). MT-2 cells and the HIV gp120-specific human $CD4^+$ T cell clone Een217 (15), were obtained from the National Institute for AIDS and Infectious Diseases (NIAID) AIDS Research Reference Reagent Program. 6D9, 2F5 and 2H4 are murine T cell hybridomas specific for thyroglobulin peptides that were generated and generously provided by Dr. G. Carayanniotis, Memorial University. All cell lines were maintained in lymphocyte medium; RPMI 1640 supplemented with 10% fetal calf serum (FCS), 10 mM HEPES, 2 mM L-glutamine, 1% penicillin and streptomycin (all from Gibco, Grand Island, NY) and 2×10^{-6} M 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO) in a 5% CO_2 humidity controlled incubator. Een217 cells were restimulated every 10 days with irradiated (3000 Rad) allogeneic peripheral blood lymphocytes and 0.25 μg/ml purified phytohemagglutinin

(PHA-P) (Wellmark Diagnostics, Guelph, ON) in lymphocyte medium supplemented with 50 U/ml recombinant human interleukin-2 (IL-2) (Hoffmann-La Roche Inc., Nutley, NJ).

Hamster IgG anti-murine Fas antibody Jo2 and hamster anti-murine CD3 antibody 2C11 were from Pharmingen Canada, Mississauga, ON. IgG_1 anti-human Fas antibodies BMS 138 and ZB4 were obtained from Biowhittaker USA, Walkersville, MD and Kamiya Biomedical Comany, Thousand Oaks, CA respectively.

Peripheral blood mononuclear cell (PBMC) isolation. Human blood was collected from a forearm vein into heparinized vacutainers and woodchuck blood was collected under general anesthetic from a femoral vein. Blood samples were diluted 1:1 with phosphate buffered saline (PBS) pH 7.2, layered over Ficoll-Paque gradient separation medium (Pharmacia Chemicals, Dorval, Quebec) and centrifuged at 400*g* for 30 minutes. Interface cells were collected, washed three times in PBS containing 1% FCS, counted and used immediately in cytotoxicity assays.

Activation of effector cells. Murine T cell hybridoma cells were activated to kill P815s by including 1 μg/ml hamster anti-murine CD3 antibody 2C11 in the assay medium and were activated to kill P815, LS102.9 and human Fas-expressing Jurkat, J.RT3-T3.5 and MT-2 T cells by including 10 ng/ml PMA and 500 ng/ml ionomycin (Calbiochem-Novabiochem Corporation, LaJolla, CA) in the assay medium. Killing of human and murine Fas-expressing target cell lines by the murine hybridomas also occurred following 3 hours incubation in medium with 10 ng/ml PMA and 500 ng/ml ionomycin prior to carrying out the

assay in plain lymphocyte medium. The human T cell clone Een217 was triggered to kill P815s by adding 1 μg/ml OKT3 anti-CD3 antibody to the assay medium. Other IgG anti-CD3 antibodies such as UCHT1 and HIT3a generally work as well as OKT3. Killing of K-562 cells by the Een217 clone and killing of P815cells by human and woodchuck PBMC was triggered by adding 3 μg/ml PHA to the assay medium.

Chromium release assays. Approximately $2x10^6$ target cells were incubated for 90 minutes at 37^0C with 100 μCi $Na_2{}^{51}CrO_4$ (Amersham, Boston, MA) in approximately 500 μl of lymphocyte medium. Labeled cells were then washed 4 times in PBS containing 1% FCS and counted. For discrimination of Fas ligand-based killing, a sufficient number of labeled P815 cells were incubated for 30 min in a small volume of medium with 5 μg Jo2/10^6 cells, washed once and resuspended at $2x10^5$/ml in medium. Untreated target cells were resuspended at the same concentration and effector cells were then tested against both untreated and antibody-treated P815 cells at the indicated effector to target (E:T) ratios. Target cells were added at $1x10^4$/well in 50 μl of medium to round bottom microtitre plates (ICN Pharmaceuticals Canada Inc. Montreal, Quebec). Effector cells were added to duplicate wells in 50, 25 and 12.5: 1 volumes for 3 different E:T ratios. Cycloheximide (Calbiochem-Novabiochem Corporation, La Jolla, CA) and colchicine (Sigma Chemical Co. St. Louis, MO) were added to certain test wells at final concentrations of 50 μg/ml and 1 mM respectively to confirm the nature of cytotoxicity mediated by different effector cells studied. The volume in each test well was made up to 300 μl with medium and targets were also added to duplicate

test wells containing either medium alone (spontaneous release) or 1 N HCl (maximum release). Once both effector and target cells were added, the assay plates were incubated for 5 hours at 37^0C in a humidified 5% CO_2 incubator. After 5 hours, 100 µl of cell-free supernatant was transferred to tubes and counted in a gamma counter. Percent specific lysis mediated by the effector cells was calculated using the following formula:

(Experimental ^{51}Cr release - Spontaneous release / (Maximum release - Spontaneous release) X 100. In all assays reported, spontaneous ^{51}Cr release was < 25% of total ^{51}Cr release.

RESULTS

P815 cells are resistant to anti-Fas mAb-mediated killing. To assess the relative sensitivities of 2 Fas-expressing murine cell lines, LS102.9 and P815, we labeled both cells with ^{51}Cr and incubated them for 5 hours with different amounts of soluble Jo2, a hamster IgG anti-murine Fas antibody. Percent ^{51}Cr release triggered by the antibody was used to indicate sensitivity to direct killing by Jo2. Whereas incubation of LS102.9cells with less than 5 ng/ml of Jo2 increased ^{51}Cr release above background levels, ^{51}Cr release by P815 cells remained near background at levels of Jo2 up to 5 µg/ml (Fig. 1). This demonstrates that Fas expressing cell lines differ markedly in susceptibility to anti-Fas antibody-induced lysis, that P815cells

are relatively resistant to direct killing by Jo2 over a five-hour incubation period and that LS102.9 cells are extraordinarily sensitive to rapid induction of apoptosis by Jo2.

Fas ligand-mediated killing of P815cells is inhibited by Jo2. The sensitivity of LS102.9 and P815 to Fas ligand-mediated lysis was tested using murine T cell hybridomas that express Fas ligand upon activation by specific antigen or treatment with PMA and ionomycin. These hybridomas efficiently kill LS102.9 cells pulsed with specific peptide and this killing is completely abrogated by adding cycloheximide to the assay medium (data not shown). Since P815 cells do not express IA^s, we could not use specific peptide to activate the hybridoma. Surprisingly, the LS102.9 cells and P815cells were equally sensitive to Fas ligand-mediated killing by the murine T cell hybridoma 6D9 activated by PMA and ionomycin (Fig. 2). PMA and ionomycin were not toxic to the target cells and the hybridoma was not cytotoxic without activation by PMA and ionomycin. Since the P815cells were sensitive to Fas ligand-mediated killing and resistant to anti-Fas antibody-mediated lysis, Jo2 was used to selectively block murine Fas ligand-mediated killing of P815 cells (Fig. 2). Lysis of P815 cells was reduced by approximately 80% when the target cells were pre-incubated with Jo2 (Fig.2). The same 6D9 hybridoma activated with PMA and ionomycin was tested against 3 human Fas-expressing T cell lines, Jurkat, J.RT3-T3.5 and MT-2. The activated hybridoma killed the 3 human T cell lines, confirming a productive interaction between murine Fas ligand and human Fas. This killing was effectively blocked (~80% inhibition) with the anti-Fas antibodies BMS138 (Fig. 3) or ZB4 (data not shown). Two additional

murine T cell hybridomas, 2F5 and 2H4 (designated 9 and 10 respectively in this experiment), were triggered to kill P815cells by anti-murine CD3 mAb. Neither hybridoma killed P815 cells without anti-CD3 and killing was effectively blocked either by including cycloheximide in the assay medium or by pre-incubating P815 cells with Jo2 anti-murine Fas mAb (Fig. 4)

Fas ligand-mediated killing of P815 cells was also studied using PBMC from healthy woodchucks. Since antibodies against the woodchuck T cell receptor are not available, PHA was used to cross-link effectors and targets and activate the effector cells. Effector cells from woodchucks were tested against untreated P815 cells and P815 cells pre-incubated with anti-Fas antibody in the presence of PHA and against untreated P815 cells in the presence of PHA and cycloheximide. The reduction in killing of P815 cells pre-treated with anti-Fas antibody indicates that the majority of the cytotoxic activity within circulating woodchuck PBMC is mediated via expression of Fas ligand (Fig. 5).

Killing of P815 cells by PBMC from woodchucks 293 and 297 was reduced by 67% and 80% respectively when target cells were pre-incubated with Jo2. A similar reduction in the level of killing observed in the presence of cycloheximide confirms the predominance of Fas ligand-mediated killing (Fig. 5).

Perforin-mediated killing of P815cells is unaffected by Jo2. The mechanism of cytotoxicity of circulating PBMC from two healthy humans was studied using the same assay system. Neither pre-treatment of target cells with Jo2 nor inclusion of cycloheximide in the assay medium had a major effect on the level of killing (Fig. 6). This illustrates that the circulating

cytotoxic cells in healthy humans are predominantly perforin-dependent. These are presumably NK cells, which previously have been reported to mediate both perforin and Fas-dependent killing (16, 17). Results with our assay system suggesting predominantly perforin-dependent killing were confirmed by using Fas-negative K-562 cells as targets and EGTA to chelate extracellular calcium required for perforin polmerization and by using colchicine to inhibit the microtubule dependent degranulation required for NK cell perforin release. Both colchicine and EGTA reduced the level of PHA-triggered killing of P815cells by human PBMC practically to background levels (Fig. 7).

Cytotoxicity assays carried out with the human Een217 $CD4^+$ T cell clone also showed that treating P815 cells with Jo2 does not affect perforin-mediated killing. This clone mediated little cytotoxicity against either P815 cells or K-562 cells in the absence of anti-CD3 or PHA (Fig. 8). Anti-CD3 triggered killing of P815 cells by the $CD4^+$ clone and surprisingly, this killing was not inhibited by cycloheximide or anti-Fas antibody. Utilization of the Fas-independent mechanism of cytotoxicity by this clone was confirmed by its ability to kill Fas-negative K-562 cells in the presence of PHA. Killing of K-562 cells by Een217 was also unaffected by cycloheximide (Fig. 8).

DISCUSSION

We have developed a simple, broadly applicable assay system that discriminates between perforin and Fas ligand-based cell mediated cytotoxicity by selectively blocking

interaction between Fas ligand and Fas. The keys to this assay system are the promiscuous cross-species productive interaction between Fas and Fas ligand and resistance of the Fcγ receptor-positive P815 cell line to direct killing by the anti-Fas antibody Jo2 coupled with sensitivity to Fas ligand-mediated killing. Presumably, expression of Fas ligand on the fluid membrane of an effector cell allows for localization into the area of cell to cell contact and efficient aggregation and cross-linking of Fas antigen. With P815 cells, it appears that this is necessary for apoptosis induction, whereas very low levels of soluble divalent Jo2 are sufficient to induce apoptosis of LS102.9 cells. Despite this discrepancy, both cell lines were equally sensitive to Fas ligand-mediated cytolysis by murine T cell hybridomas. These observations reiterate the important role of poorly understood extra- and intracellular factors in modulating the ability of the Fas receptor to transduce signals leading to apoptosis.

Using anti-receptor antibodies, lectins or pharmacologic agents to trigger a wide variety of effector cells, murine P815 cells can serve as universal targets in this assay system. We utilized murine P815 cells to analyze the cytotoxic mechanism employed by various types of effector cells from 3 different species. Killing of P815 cells by murine T cell hybridomas that express Fas ligand when activated with PMA and ionomycin was almost completely inhibited when P815 target cells were pretreated with Jo2. Killing by murine T cell hybridomas triggered with anti-CD3 was inhibited to virtually the same extent with Jo2 as with cycloheximide, indicating that blocking the Fas receptor with Jo2 prevents Fas ligand-mediated killing as effectively as blocking Fas ligand expression. Killing of P815 cells

by freshly isolated PBMC from healthy woodchucks triggered with PHA was markedly inhibited either by cycloheximide or by pretreating the P815 cells with Jo2, indicating predominant use of the Fas ligand-mediated pathway of cytotoxicity by these effector cells. In contrast, using the same assay system, we showed that PBMC from healthy humans predominantly lysed P815 cells via perforin release. We also used the assay system to show that a human $CD4^+$ T cell clone mediated Fas-independent killing of P815 cells and K-562 cells. This is apparently a rare example of perforin-mediated killing by human $CD4^+$ T cells.

The advantage of this discriminatory assay system over other methods of selectively blocking one or the other mechanism of cell-mediated cytotoxicity is its broad utility and the lack of any possible interference with different methods employed to trigger killing of P815 cells by diverse effector cells. Calcium chelation can only be used in a system where the effector cells are pre-activated to express Fas ligand. This requires preincubation of effector cells and expression of Fas ligand will then decay over the time course of the assay. Inhibition of perforin-mediated killing by concanamycin also requires pre-treatment of effector cells (10). Cycloheximide can be added in some assay systems to prevent Fas ligand expression, but cycloheximide may be toxic to cells under certain conditions and generalized inhibition of protein synthesis can affect cytotoxicity in other ways. In this assay system, activation of effector cells and protein synthesis are allowed to occur normally, but the target cells are rendered insensitive to Fas ligand-mediated killing by blocking the Fas receptor. Therefore, whatever stimulus is necessary to trigger the effector cells can be freely incorporated into the

assay and the relative conservation of Fas and Fas ligand across species allows analysis of clones, hybridomas, cell lines and fresh PBMC from diverse species. We showed that circulating cytotoxic cells in PBMC from healthy woodchucks, presumably NK cells, killed primarily via Fas ligand expression, whereas circulating human NK cells primarily kill via perforin release. Murine T cell hybridomas were shown to kill susceptible human and murine targets via Fas ligand expression and a human $CD4^+$ T cell clone was shown to mediate Fas-independent cytolysis. The accuracy of this assay system in discriminating perforin from Fas ligand-mediated killing was confirmed by using Fas-negative K-562 cells as targets and by using cycloheximide and colchicine to inhibit Fas ligand expression and microtubule-dependent perforin release respectively.

Since Fas/Fas ligand interactions occur between species, this assay system with murine P815 target cells can readily be applied to study the role of CTL subsets both in human diseases and in various animal models of human disease. For example, infection of woodchucks with woodchuck hepatitis virus is an accepted model of human hepatitis B infection and this assay system can be used to characterize CTL activity in the woodchuck hepatitis model during different stages of disease or following infection protocols that lead to resolving, chronic latent or chronic active hepatitis. This assay system can be equally well applied to monitor the level and Fas ligand-dependence of CTL activity over the course of HIV infection using anti-human CD3 antibodies to trigger killing.

In summary, we used P815 cells as generic target cells to discriminate between Fas ligand and perforin-based cytotoxicity mediated by three different types of cytotoxic cells from humans, woodchucks and mice using three different methods to trigger effector cell function. Therefore, using P815s as targets and comparing levels of killing with or without pre-treatment with Jo2 anti-murine Fas mAb, the nature of cytotoxicity mediated by virtually any lymphocyte population immediately *ex vivo*, following different modes of activation, or after various selection, cloning, and transformation procedures can be rapidly and easily determined.

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Fig. 1

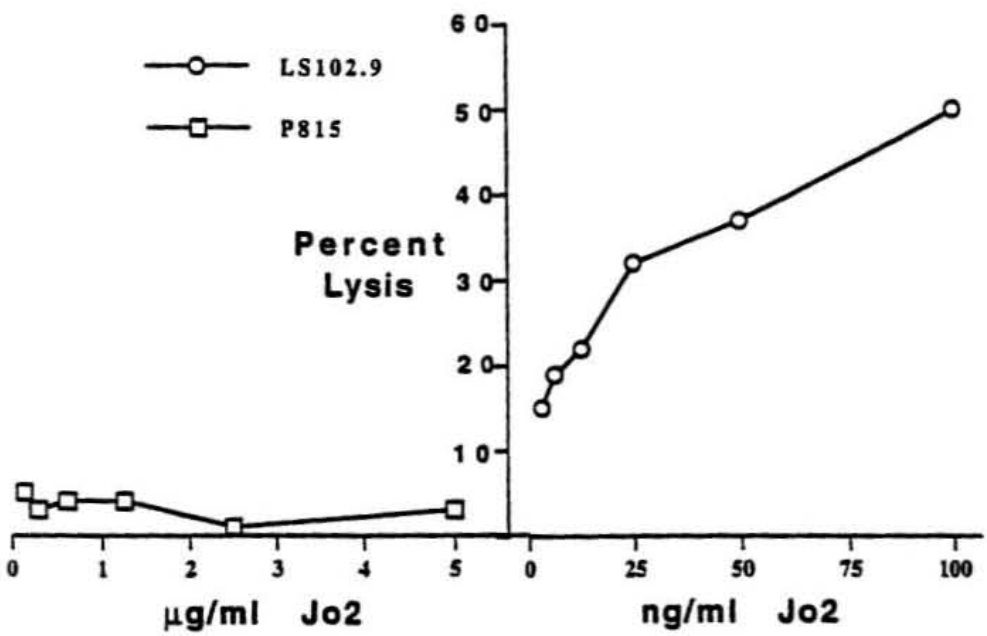

LS102.9
P815
Percent Lysis
60
50
40
30
20
10
0
1
2
3
4
5
0
25
50
75
100
µg/ml Jo2
ng/ml Jo2

Fig. 2

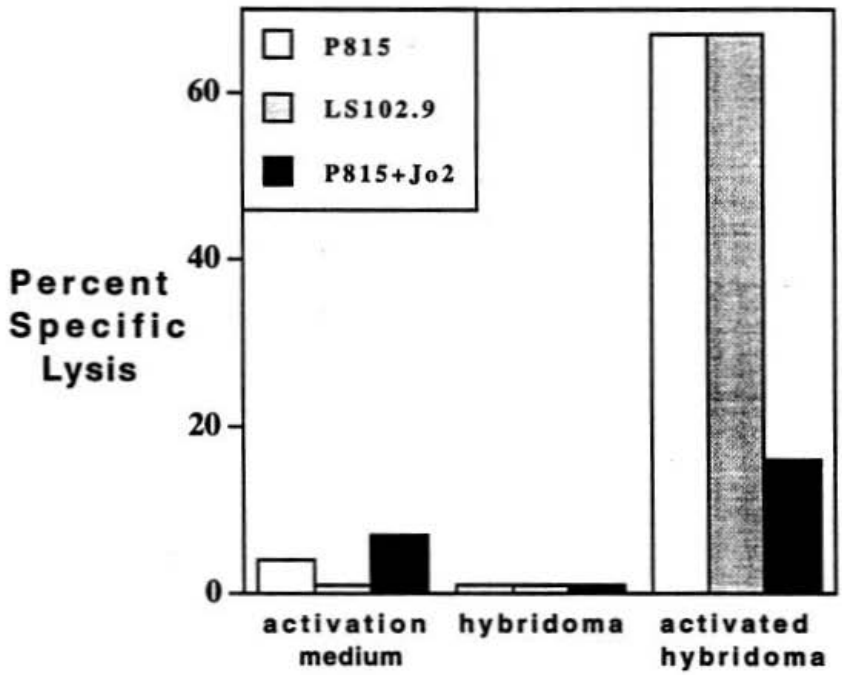

P815
LS102.9
P815+Jo2
60
40
20
0
Percent Specific Lysis
activation medium
hybridoma
activated hybridoma

Fig. 3

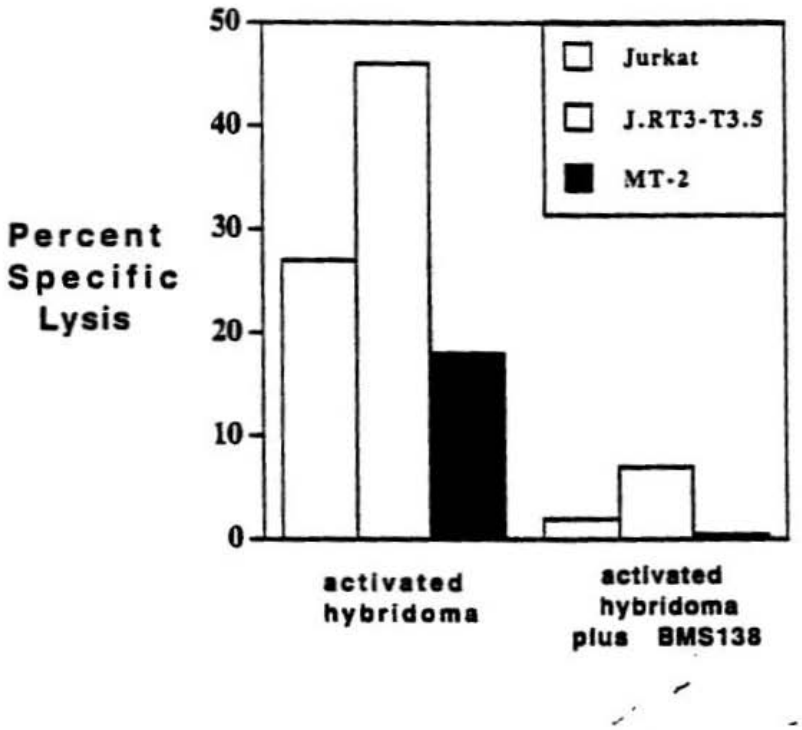

Percent Specific Lysis
50
40
30
20
10
0
Jurkat
J.RT3-T3.5
MT-2
activated hybridoma
activated hybridoma plus BMS138

Fig. 4

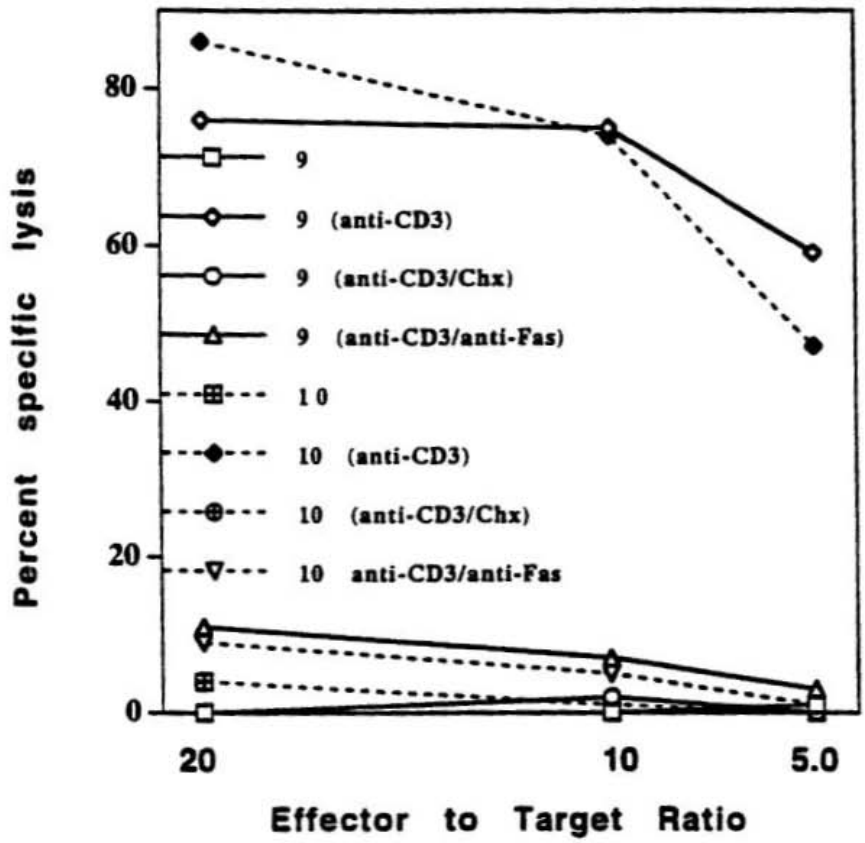

Percent specific lysis
80
60
40
20
0
9
9 (anti-CD3)
9 (anti-CD3/Chx)
9 (anti-CD3/anti-Fas)
10
10 (anti-CD3)
10 (anti-CD3/Chx)
10 anti-CD3/anti-Fas
20
10
5.0
Effector to Target Ratio

Fig. 5

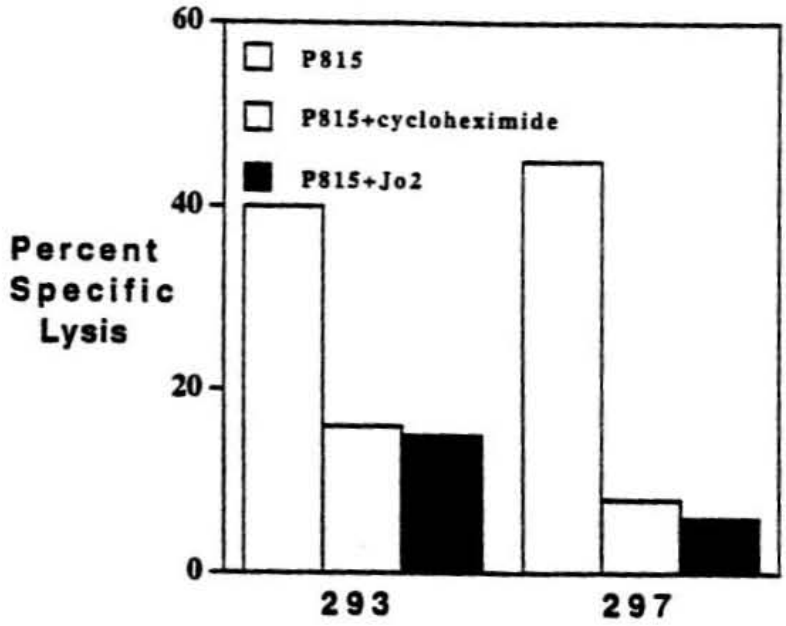

60
40
20
0
Percent Specific Lysis
P815
P815+cycloheximide
P815+Jo2
293
297

Fig. 6

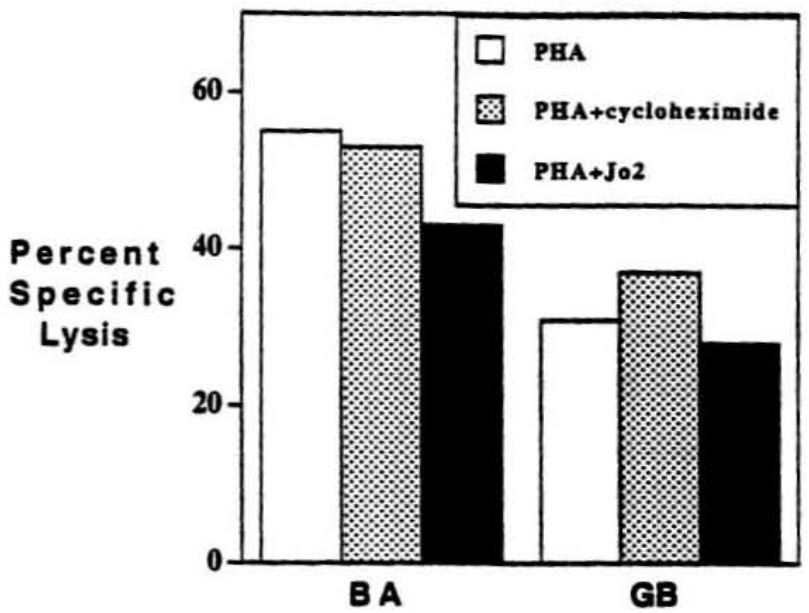

PHA
PHA+cycloheximide
PHA+Jo2
Percent Specific Lysis
60
40
20
0
BA
GB

Fig. 7

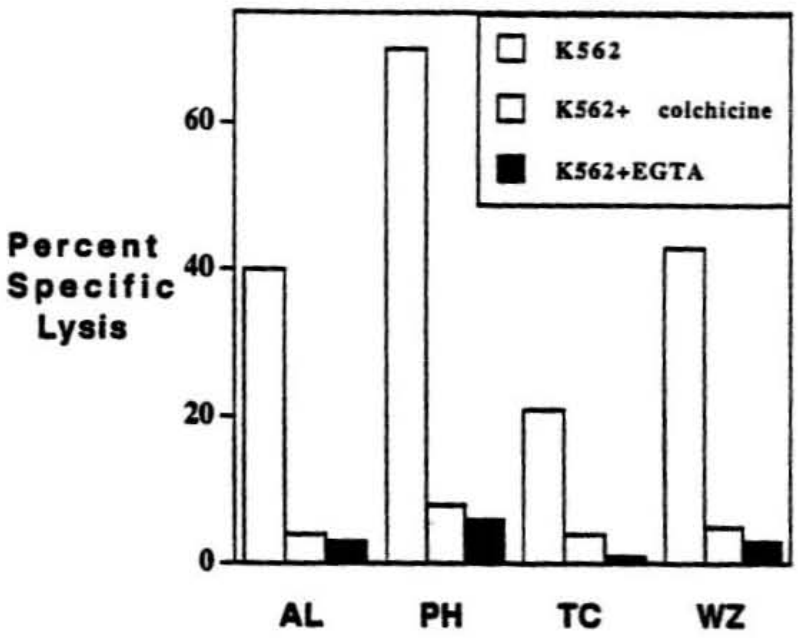
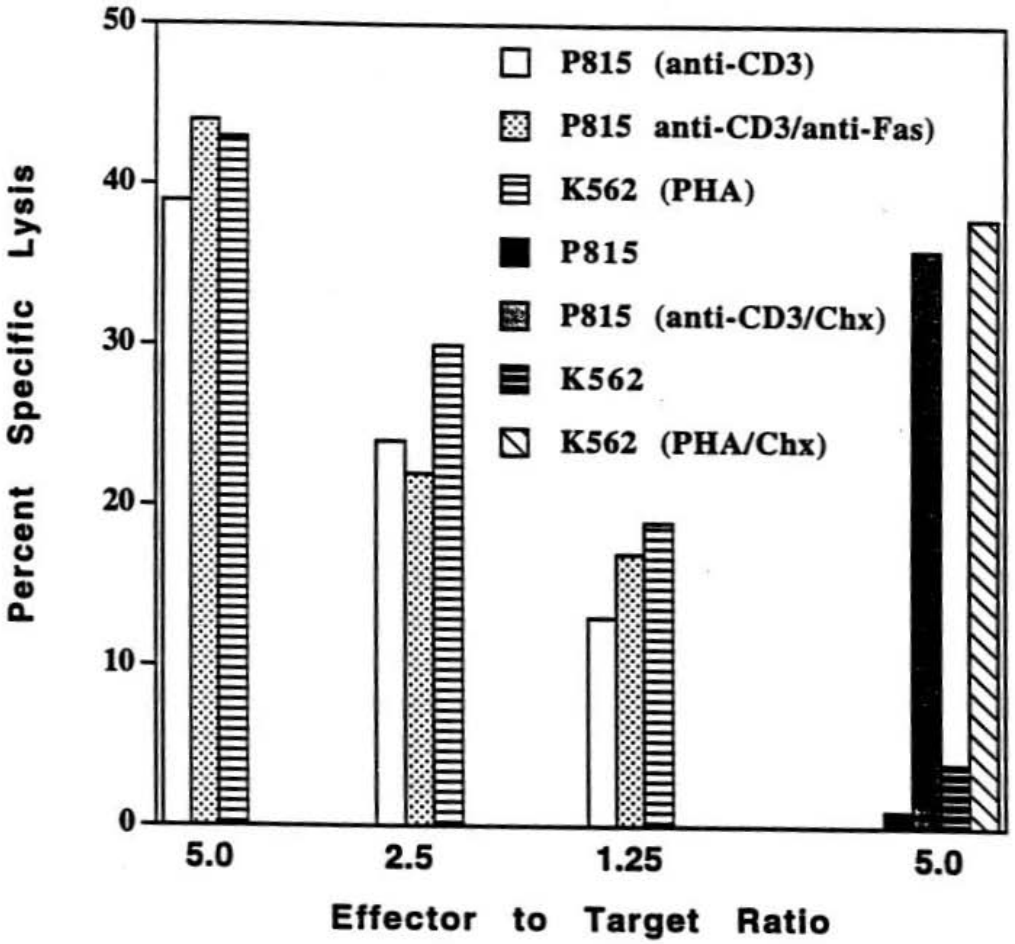

K562
K562+ colchicine
K562+EGTA
Percent Specific Lysis
60
40
20
0
AL
PH
TC
WZ

Fig. 8


50
40
30
20
10
0
Percent Specific Lysis
P815 (anti-CD3)
P815 anti-CD3/anti-Fas)
K562 (PHA)
P815
P815 (anti-CD3/Chx)
K562
K562 (PHA/Chx)
5.0
2.5
1.25
5.0
Effector to Target Ratio

CHAPTER III

A Novel Form of Activation-Induced Cell Death Without DNA Fragmentation in T Lymphocytes from HIV-infected Individuals

A Novel Form of Activation-Induced Cell Death

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Key words: activation-induced cell death, HIV, T lymphocytes

SUMMARY

Abnormally high numbers of T cells from HIV-infected individuals undergo spontaneous and activation-induced cell death (AICD). T cells from HIV-infected individuals also are especially sensitive to Fas-mediated apoptosis, suggesting that Fas/Fas ligand (FasL) interactions might contribute to AICD in HIV infection. Treatment of T cell lines, clones and hybridomas with phorbol myrystic acetate (PMA) and ionomycin induces FasL expression, therefore, we used this treatment to investigate the possible role of Fas/FasL interactions in AICD in HIV infection. PMA/ionomycin-induced AICD was examined by Chromium (^{51}Cr) release, DNA analysis, propidium iodide (PI) uptake and electron microscopy. PMA and ionomycin acted synergistically to induce up to 70% release of incorporated ^{51}Cr from fresh PBMC of HIV-infected individuals, compared with up to 26% ^{51}Cr release from fresh PBMC of healthy uninfected volunteers. Cr release increased in a linear fashion throughout the 5 hour assay period, consistent with the kinetics of cell-mediated cytotoxicity. Inhibition of Cr release by addition of cold targets and the lack of cytotoxicity of supernatants from PMA/ionomycin treated PBMC also suggested that cell-to-cell contact was required to trigger AICD. Chelating extracellular Ca^{2+} reduced AICD to background levels, but neither antagonistic anti-Fas antibodies nor cycloheximide inhibited AICD. Fas^+ P815 cells and Fas^- K-562 cells were equally effective in cold target inhibition experiments, also suggesting that Fas/FasL interactions were not involved in this AICD. Electron microscopy revealed disruption of the plasma membrane while the nuclear membranes of damaged cells remained

intact. Analysis of DNA isolated from PMA/ionomycin treated PBMC revealed no fragmentation, while PI uptake confirmed loss of plasma membrane integrity and identified the majority of cells undergoing AICD as T lymphocytes. Treatment with PMA and ionomycin induces a novel form of AICD with no associated DNA fragmentation in T lymphocytes from HIV-infected individuals, and to a lesser extent, in T lymphocytes from non-HIV-infected volunteers.

INTRODUCTION

In recent years, AICD has been considered a possible factor in the depletion of $CD4^+$ T lymphocytes and degeneration of $CD8^+$ T cell-mediated immunity in HIV infection. Spontaneous and activation-induced apoptosis has been observed for $CD4^+$ and $CD8^+$ T cells in human HIV infection and in non-human primates infected with pathogenic strains of simian immunodeficiency virus (SIV) [1-4]. *In vitro* HIV gp120-induced apoptosis of uninfected $CD4^+$ T cells and apoptosis of HIV-infected T cells, apoptosis of uninfected $CD4^+$ T cells within lymph nodes, HIV-infected macrophage-mediated T cell apoptosis and modulation of T cell apoptosis by cytokines with altered expression patterns in HIV infection illustrate the possible relationship between T cell apoptosis and progressive HIV infection [5-9]. AICD of mature T cells occurs largely through Fas/Fas ligand (FasL) interactions [10]. Thus, increased expression of Fas on PBL in HIV infection, increased sensitivity of $CD4^+$ and $CD8^+$ T cells from HIV-infected individuals to anti-Fas antibody-mediated apoptosis, FasL-

mediated apoptosis of T cells by macrophages from HIV-infected individuals and anti-Fas antibody production in HIV infection suggests Fas/FasL interactions may play a major role in T cell apoptosis in HIV infection [8, 11-14].

Since sensitivity to exogenous anti-Fas antibody-mediated apoptosis increases in parallel with disease progression in HIV infection [12], we stimulated PBMC from 60 HIV-infected individuals with PMA and ionomycin to induce FasL expression and investigated whether increased sensitivity to autocrine or paracrine cellular FasL expression occurs in HIV infection. While this treatment produced significant AICD, we found no dependence on Fas/FasL interactions and no evidence of DNA fragmentation. Three different techniques characterized this AICD as manifest primarily through loss of membrane integrity. These findings raise the possibility that a novel pathway of AICD unrelated to Fas/FasL interactions and not associated with DNA fragmentation may contribute to physiological and pathological T cell regulation.

MATERIALS AND METHODS

Subjects

HIV-infected individuals participating in this project were recruited through the Infectious Disease Clinic of the St. John's General Hospital, St. Johns, Newfoundland, Canada. Subjects were assessed clinically concurrent with flow cytometric measurement of

$CD4^+$ and $CD8^+$ T lymphocyte counts at each visit. Non-HIV-infected volunteers were recruited from laboratory personnel. All subjects gave informed consent and the study received local ethics approval from the Memorial University Human Investigation Committee. Blood was collected in vacutainers with EDTA anticoagulant. Whole blood was diluted 1:1 with phosphate-buffered saline (PBS), underlaid with Ficoll-Paque gradient separation medium (Pharmacia Chemicals, Dorval Quebec) and centrifuged at 400 *g* for 30 min. Interface cells were collected, washed 3 times in PBS plus 1% fetal calf serum (FCS) (Gibco, Grand Island, New York), and counted.

Chromium release assay

Approximately $2x10^6$ freshly-isolated PBMC were incubated for 90 minutes in about 500 :l total volume lymphocyte medium (RPMI plus 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 1% penicillin/streptomycin, $2x10^{-5}$ M 2-mercaptoethanol, all from Gibco) with 100 µCi $Na_2{}^{51}CrO_4$ (Amersham Life Sciences, Arlington Heights, IL). Labeled cells were washed 4 times with PBS plus 1% FCS and resuspended in 1 ml medium. Minimum and maximum release wells were set up in duplicate with 250µl of medium or 1N HCl respectively in 96 well round bottom microtitre plates (ICN Canada Inc., Montreal, Quebec). 50 µl of the labeled PBMC suspension was added to the control wells and to duplicate test wells containing 250µl of medium with 10 nM phorbol myrystic acetate (PMA) and 500 nM ionomycin (Calbiochem, La Jolla, CA). For some samples, duplicate test wells were

supplemented with 5 μg/ml anti-CD3 antibody OKT3 (ATCC CRL-8001) or HIT3a (Pharmingen Canada, Mississauga, ON) or 5 μg/ml anti-Fas antibody ZB4 (Kamiya Biomedical Co., Thousand Oaks, CA), 5 μg/ ml anti-HLA-A, B and C antibody PA2.6 (ATCC HB-118), 50 μg/ml cycloheximide (Calbiochem, La Jolla, CA), 1 mM colchicine (Sigma Chemical Co., St. Louis, MO), 50 U/ml recombinant human interleukin-2 (rIL-2) (Hoffmann-La Roche, Nutley, NJ), 1 mM EGTA with 1.5 mM $MgCl_2 6H_20$ or $2.5x10^5$ unlabeled K-562 (ATCC CCL-243) or P815 (ATCC TIB-64) cells, always in a final volume of 300 μl lymphocyte medium. Cells were incubated for 5 hrs at 37^0 C in a 5% CO_2 humidity controlled incubator and 100 μl supernatant was removed from each well for counting in a Wallac 1480 gamma counter. Percent specific ^{51}Cr release was calculated by the following formula:

$$\frac{\text{(experimental } ^{51}\text{Cr release - spontaneous } ^{51}\text{Cr release)}}{\text{(maximum } ^{51}\text{Cr release -spontaneous } ^{51}\text{Cr release)}} \times 100$$

Spontaneous ^{51}Cr release was less than 30% of maximum release in all assays.

DNA fragmentation analysis

1 $x10^6$ PBMC from HIV-infected individuals were incubated either for 5 or 16 hours in lymphocyte medium with 10 nM PMA and 500 nM ionomycin. DNA was extracted from these cells as previously described [15]. Briefly, pelleted cells were washed once in PBS with 1% FCS, lysed with 0.2% sodium dodecyl sulfate and incubated with 0.16 mg/ml proteinase

K (Gibco, Grand Island, New York) for 1 hr at 37^0 C. DNA was isolated from the digest by phenol extraction (Gibco, Grand Island, New York), followed by extraction with chloroform/isoamyl alcohol 24:1 (Sigma Chemical Co., St. Louis, MO). DNA was then precipitated with 100% ethanol (Sigma Chemical Co., St. Louis, MO) and washed once in 70% ethanol. As a positive control for visualization of DNA fragmentation, DNA was extracted as above from LS102.9 (ATCC HB-97) cells incubated for 5 hours with 100 ng/ml hamster anti-murine Fas antibody, Jo-2 (Pharmingen Canada, Mississauga, ON). Isolated DNA was separated by electrophoresis on 1.6% agarose gels for 45 min at 50 mA and visualized with ethidium bromide (Sigma Chemical Co., St. Louis, MO).

Flow cytometry

Approximately $2x10^6$ PBMC from HIV-infected individuals were incubated for 16 hrs at 37^0 C in a 5% CO_2 humidity controlled incubator in lymphocyte medium with or without 10 nM PMA and 500 nM ionomycin. These cells were pelleted and washed in PBS plus 0.1% BSA and 5 mM EDTA. Cells were incubated for 30 min at 4^0 C with FITC-conjugated murine IgG1 isotype control or FITC-conjugated anti-CD3, anti-CD4, or anti-CD8 (all from DAKO Co., Carpinteria, CA) or anti-CD28 (Immunotech S. A., Marseille, France). Samples were washed once and incubated at 4^0 C for 15 min with 10 μg/ml PI (Sigma Chemical Co., St. Louis, MO) in PBS and cells were analyzed for PI uptake at 576±26 nm and FITC-

conjugated antibody binding at 530±30 nm with a FACStar^{PLUS} analyzer (Becton Dickinson, Mississauga, ON) after excitation at 488 nm with an argon laser.

Electron microscopy

$5x10^6$ freshly isolated PBMC were incubated for 5 hr at 37^0 C in a 5% CO_2 humidity controlled incubator in lymphocyte medium with or without 10 nM PMA and 500 nM ionomycin. These cells were then centrifuged at 400*g*, washed with PBS plus 1% FCS and resuspended in 1 ml Karnovski's fixative (4 g paraformaldehyde, 5% glutaraldehyde in 0.2 M sodium cacodylate buffer). After 6 hours in fixative, the cell pellet was treated with 1% osmium tetroxide for 20 min and the cells were then washed and dehydrated with ascending concentrations of alcohol from 70-100%. The cells were then washed with acetone and suspended in epoxide resin overnight at 70^0 C. Ultra thin (90 nμ) sections were cut, counterstained with uranite acetate and examined with a Jeol 1220 X electron microscope.

RESULTS

Chromium release assays

To assess AICD in PBMC from HIV-infected individuals, freshly isolated PBMC were labeled with ^{51}Cr and incubated in lymphocyte medium with 10 nM PMA and 500 nm ionomycin. Percent ^{51}Cr release over a 5 hour assay period was measured with PBMC from 60 HIV-infected individuals and 15 healthy non-infected volunteers. The percent ^{51}Cr release

ranged from 5% to 26% (mean ± SD = 16.1% ± 5.7%) for the controls and from 5%-70% (mean ± SD = 29.3% ± 13%) for the HIV-infected individuals (fig. 1). Mean AICD triggered by PMA and ionomycin-induced activation as measured by ^{51}Cr release was significantly higher in the HIV-infected group (Student's *t* test, $p<.001$). Both the PMA and ionomycin contributed to AICD. With PBMC from subjects 10, 12, 13, 27, 36, 50, 51 and 81 there was a strong synergistic effect of combining the 2 agents, whereas with subjects 5, 45, 46 and 64, the effects of the 2 agents were approximately additive (fig. 2). A time course study with labeled PBMC from 5 HIV-infected individuals showed that ^{51}Cr release from the PMA and ionomycin treated PBMC increased in a roughly linear fashion over the five hour assay period (fig. 3).

Since the linear increase in ^{51}Cr release over the 5 hour assay period was consistent with cell-mediated killing, we investigated the role of cell-cell contact in this form of AICD by adding cold target K-562 or P815 cells at a ratio of 10:1 to the labeled PBMC. Both the Fas-expressing P815s and Fas-negative K-562s reduced the level of Cr release to near background levels (fig. 4), suggesting that cell-to-cell contact is involved in this form of AICD and that Fas is not a primary mediator of signals leading to AICD. Supernatants from the PBMC of HIV-infected individuals incubated with PMA and ionomycin were not cytotoxic even when substantial AICD occurred during the 5 hour incubation (data not shown). This apparent requirement for cell-to-cell contact suggested that cell-mediated killing might be involved and when the AICD assay was carried out in calcium free medium, Cr

release fell to background levels (fig 5). IL-2 did not rescue cells from AICD and neither cycloheximide nor colchicine, which inhibit FasL expression and granule exocytosis respectively, significantly reduced the level of AICD. Antagonistic anti-Fas or anti-perforin antibodies also did not reduce AICD. Monoclonal anti-CD3 antibody OKT3 significantly reduced AICD in this system, whereas HIT3a, another IgG2a anti-CD3 antibody with similar reported characteristics, did not inhibit AICD at the same concentration of 5 μg/ml (fig 5). At 5 μg/ ml, PA2.6, an anti-HLA class I antibody that blocks HLA-A, B and C-restricted cytotoxic T cell-mediated killing also did not reduce the level of AICD (data not shown).

DNA fragmentation analysis

To test for DNA fragmentation associated with this form of AICD, DNA was extracted from the PBMC of HIV-infected individuals following incubation in lymphocyte medium with PMA and ionomycin for 5 or 16 hours. Agarose gel electrophoresis of the DNA isolated from the PBMC of four subjects with high levels of AICD as measured by Cr release revealed no breakdown into fragments characteristically spaced 180 base pairs apart after either 5 or 16 hours of incubation with PMA and ionomycin (fig. 6). At both time points, the isolated DNA appeared completely intact, indicating that this form of AICD does not involve DNA fragmentation.

Flow cytometry

Cr release data and DNA analysis suggested this form of AICD was associated primarily with reduced plasma membrane integrity. Therefore, we analyzed PI uptake through flow cytometry in order to confirm the loss of membrane integrity and also to phenotype the PBMC subsets undergoing AICD. Lymphocyte gates based on cell forward and side scatter were expanded to detect and analyze the majority of PI^+ cells (fig 7). Flow cytometric quantitation of cells failing to exclude PI following overnight incubation in lymphocyte medium or medium with PMA and ionomycin clearly demonstrated that PMA and ionomycin treatment substantially increased the number of PI^+ cells. The correlation coefficient between % Cr release after 5 hours and the percentage of cells taking up PI following overnight incubation with PMA and ionomycin was 0.74, suggesting that these 2 measures were both representative of the cell death induced by PMA and ionomycin. Counter staining the cells with FITC-labeled anti-CD3 identified the majority of PI^+ cells as T cells (fig. 7 and Table 1). Although PMA and ionomycin has been reported to down modulate CD4 and CD8 (16), and we did observe this with several controls, increased numbers of $CD4^+$ and $CD8^+$ cells appeared in the PI^+ population following treatment with PMA and ionomycin. With most HIV-infected individuals, no $CD4^+$ T cells remained in the lymphocyte population that excluded PI after treatment, while a significant portion of the $CD8^+$ T cell population was usually spared (Table 1). Both $CD28^+$ and $CD28^-$ T cells appeared equally susceptible to this form of AICD (data not shown).

Electron microscopy

In order to visualize cellular ultrastructural changes related to the loss of plasma membrane integrity, PBMC treated with PMA and ionomycin for 5 hours were fixed and analyzed by electron microscopy. Treated cells showed a pale cytoplasm, excess vacuolization, membrane elongation and marked plasma membrane disruption relative to cells cultured for 5 hours in unsupplemented medium (figs. 8a and 8b). Very few cells were seen with the nuclear degeneration and disintegration into discreet vesicles characteristic of classical apoptosis. The vast majority of the cells with plasma membrane damage showed no loss of integrity to the membrane or interior of any intracellular organelle, including the mitochondrion and the nucleus. Although rounding of the nucleus and some chromatin condensation against the nuclear membrane was often observed, the nuclear membranes themselves appeared normal even at 40,000x magnification (fig. 8c). Ultrastructural changes such as vacuolization and plasma membrane damage did not occur to any significant extent when PBMC from uninfected individuals were incubated with PMA and ionomycin (not shown).

Discussion

In this study we showed that T cells from HIV-infected individuals are susceptible to a novel form of AICD induced by stimulation with PMA and ionomycin. PMA and

ionomycin-induced AICD was initially detected by above background Cr release from freshly isolated PBMC over a 5 hr incubation period. The elevated release of ^{51}Cr suggested a loss of plasma membrane integrity, which was confirmed both by flow cytometric assessment of PI uptake and by visualization through electron microscopy. The failure of either antibodies that block Fas signaling or an inhibitor of protein synthesis to prevent AICD indicated that the Fas/FasL pathway played no role in this form of AICD. Furthermore, no evidence of nuclear degeneration or DNA fragmentation was seen by electron microscopy or by agarose gel electrophoresis of DNA isolated from stimulated cells. Inhibition of cell lysis with cold target inhibitor cells indicated that cell-to-cell contact was required for AICD, but an anti-class I antibody that blocks class I-restricted CTL did not block AICD. Chelating free calcium in the assay medium prevented AICD, but neither anti-perforin antibodies nor inhibition of lymphocyte degranulation with the microtubule poison colchicine reduced AICD.

Counter staining the cells that failed to exclude PI after incubation with PMA and ionomycin with FITC-labeled anti-CD3, anti-CD4, anti-CD8 and anti-CD28 demonstrated that the dead and damaged PI^+ cells were predominantly T cells, that both $CD28^+$ and $CD28^-$ T cells were susceptible to AICD and that there was relative sparing of $CD8^+$ T cells over $CD4^+$ T cells. T cells were not rescued by IL-2, but AICD was substantially reduced by the anti-CD3 antibody OKT3. Electron microscopy revealed cellular vacuolization with plasma membrane rupture, while the nucleus and intracellular organelles remained intact.

This AICD observed in these studies is novel in the absence of DNA fragmentation and in the effects of different inhibitors of known mechanisms of contact dependent cell death. The requirement for cell-to-cell contact and inhibition with calcium free medium or anti-CD3 antibodies is consistent with cell-mediated cytotoxicity through perforin release, but the lack of inhibition by other anti-CD3 antibodies that block CTL-mediated killing, or by anti-perforin antibodies, or by colchicine argues against this being the primary mechanism of cell destruction. We considered the possibility that the PMA and ionomycin might induce non-targeted degranulation of CTL resulting in the death of neighboring cells in such close contact that anti-perforin antibodies could not reduce killing. However, we found in several perforin-dependent CTL systems that inclusion of PMA and ionomycin in the assay medium substantially reduces killing. The effect of calcium free medium could be through antagonism of the effects of ionomycin or PMA on activation of the PBMC, rather than through inhibition of perforin polymerization in the cytotoxic effector phase. Absence of nuclear fragmentation and lack of inhibition with cycloheximide or anti-Fas antibodies clearly exclude a role for TNF or FasL-mediated cytotoxicity in this system. Therefore, the role of cell-to-cell contact, the nature of the intercellular interactions leading to T cell death and the mechanism by which OKT3 reduces cell death in this system are unknown.

Non-apoptotic death of activated lymphocytes was previously reported following treatment with a mAb, RE2, that reacts with an MHC class I-associated determinant on activated lymphocytes (17). Scanning electron micrographs of cells treated with RE2 showed

plasma membrane damage similar to what we observed following incubation of PBMC from HIV-infected individuals in PMA and ionomycin. Killing by RE2 was independent of extracellular Ca^{2+}, which differs from our observations, but the requirement for Ca^{2+} in our system may relate to lymphocyte activation and acquisition of sensitivity rather than to the actual killing mechanism. Antibodies are unlikely to play any role in our system, but the absence of DNA fragmentation and ultrastructural similarities in both instances raise the possibility that the target of the RE2 antibody may have a corresponding cellular ligand that triggers AICD or that the intracellular pathway leading to AICD may be common to both systems. A novel type of lymphocyte cell death involving apoptosis-like nuclear morphology and mitochondrial swelling without DNA fragmentation was also recently reported within the lymph nodes of HIV-infected and uninfected individuals with chronic lymphadenopathy (18).

The relevance of AICD to lymphocyte depletion and disease progression in HIV infection is presently unknown. It seems likely that susceptibility of the T lymphocytes from the HIV-infected individuals to AICD relates to the history of *in vivo* activation and current activation status of the T cells. In general, AICD was highest with PBMC isolated from HIV-infected individuals with high $CD8^+$ T cell counts and low $CD4^+$ T cells counts and was low at later stages of disease once absolute T lymphopenia occurred. Elevated levels of plasma β-2 microglobulin and shortened telomeres in the $CD8^+$ T cells of HIV infected individuals indicate a high rate of lymphocyte turnover (19, 20). Although it remains unclear just how such sensitivity is acquired *in vivo*, sensitivity to both apoptotic and non-apoptotic forms of

AICD may contribute to T cell turnover and depletion and to the loss of effective immunological surveillance in progressive HIV infection.

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Figure 1

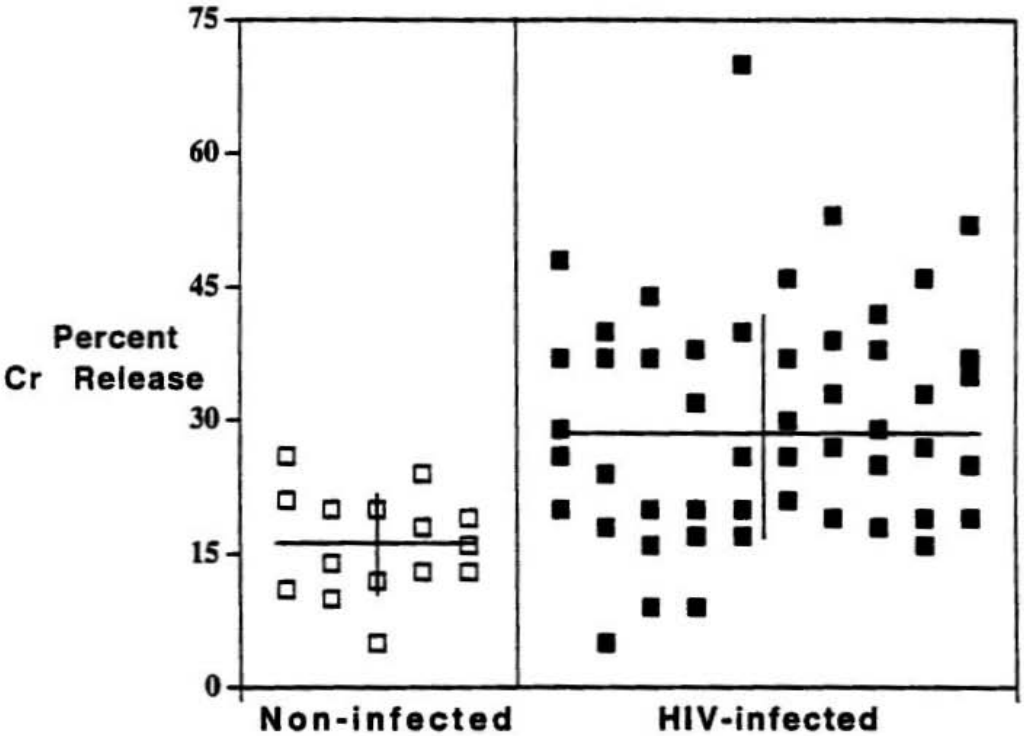

Figure 2

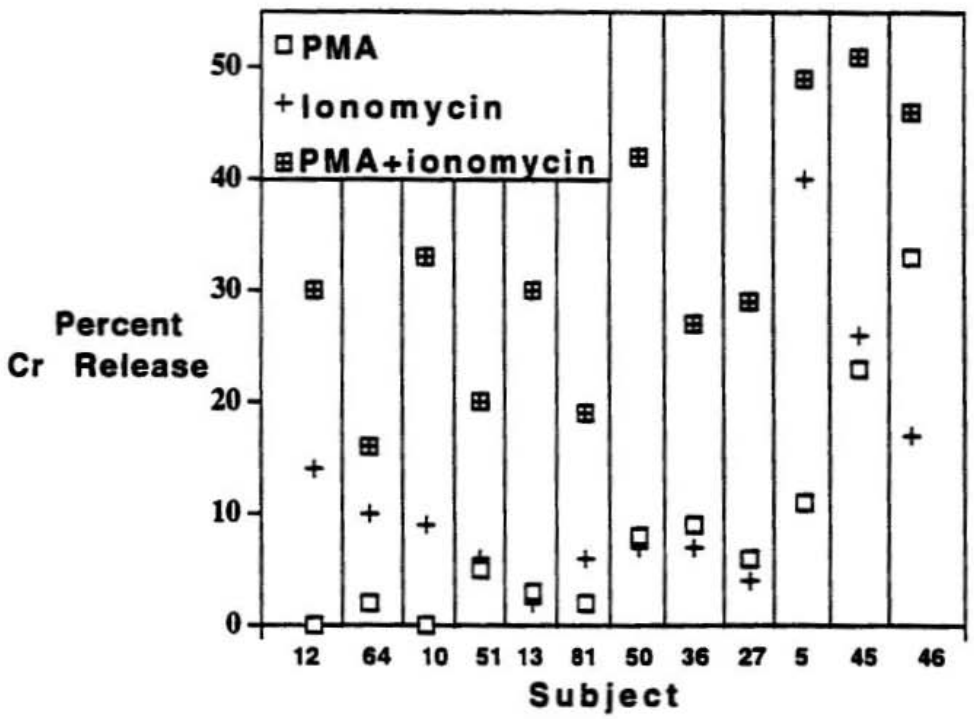
PMA
Ionomycin
PMA+ionomycin
Percent Cr Release
0
10
20
30
40
50
12
64
10
51
13
81
50
36
27
5
45
46
Subject

Figure 3

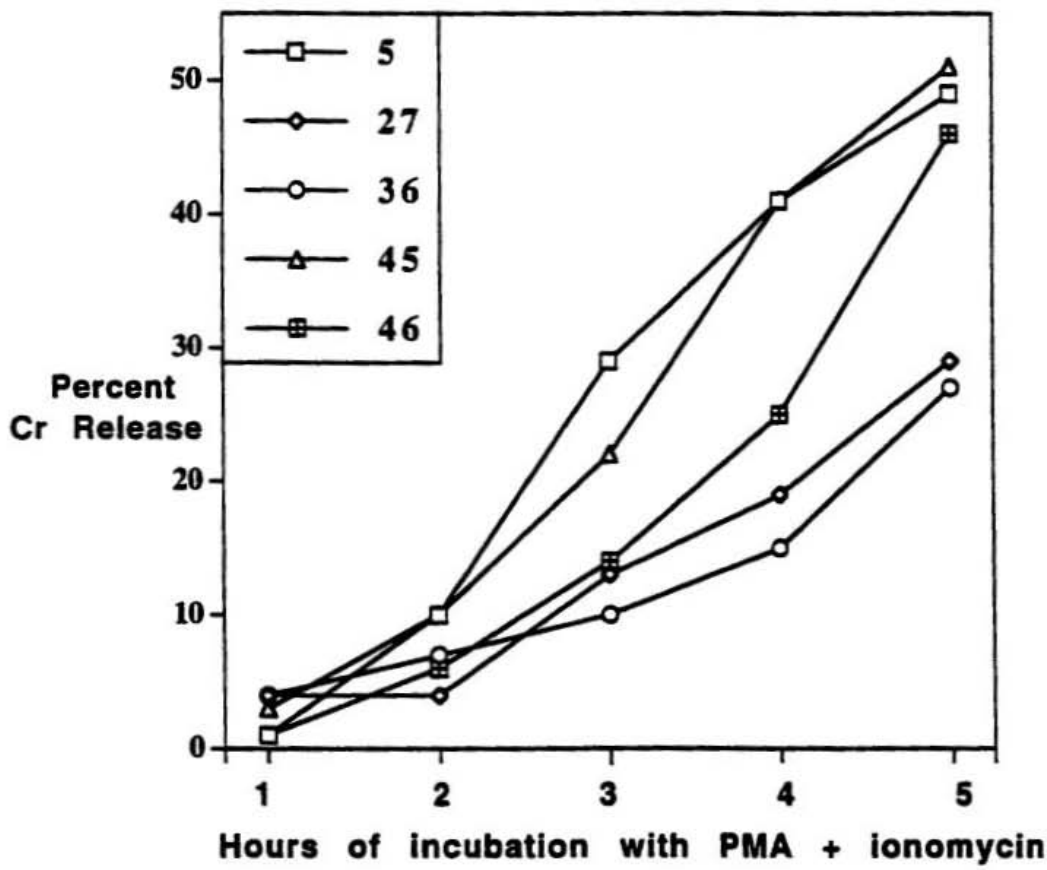
5
27
36
45
46
50
40
30
20
10
0
Percent
Cr Release
1
2
3
4
5
Hours of incubation with PMA + ionomycin

Figure 4

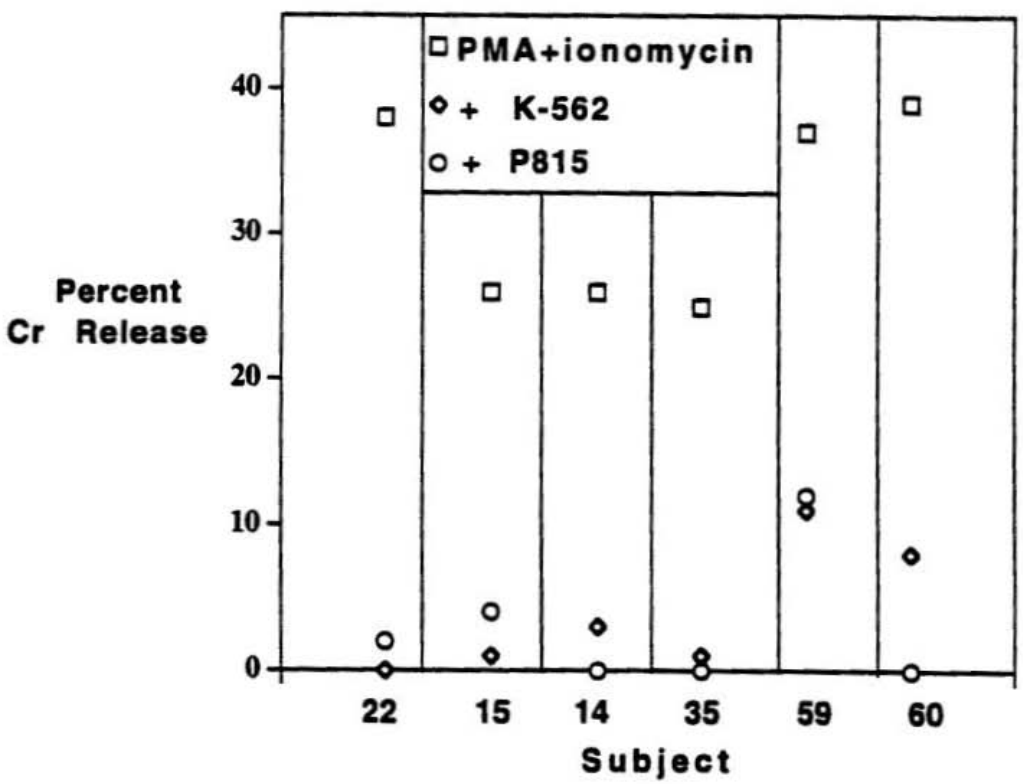
PMA+ionomycin
+ K-562
+ P815
Percent
Cr Release
40
30
20
10
0
22
15
14
35
59
60
Subject

Figure 5

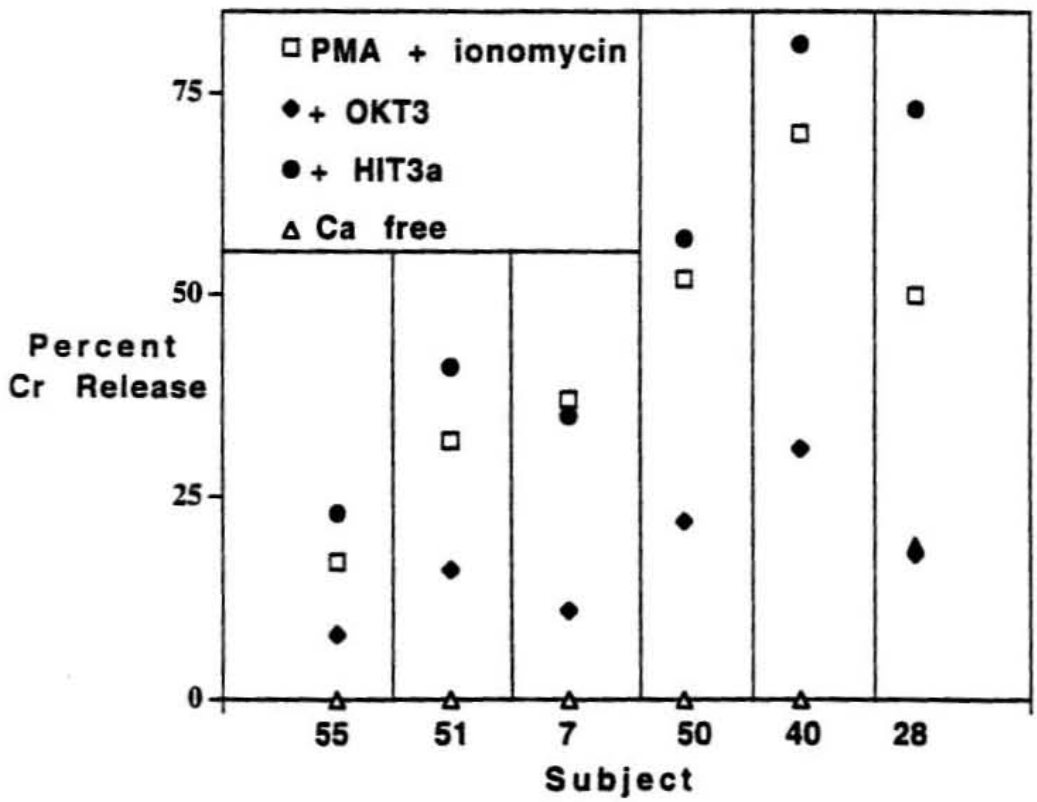
□ PMA + ionomycin
◆ + OKT3
● + HIT3a
△ Ca free
75
50
25
0
Percent
Cr Release
55
51
7
50
40
28
Subject

Figure 6

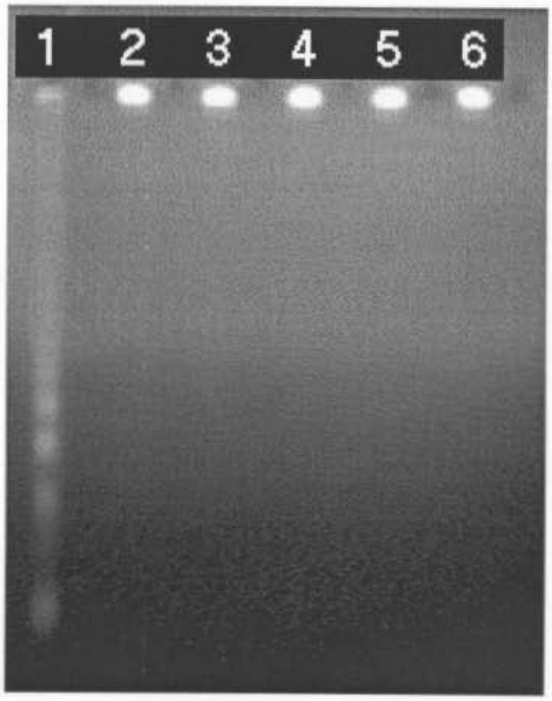

Fig. 7a

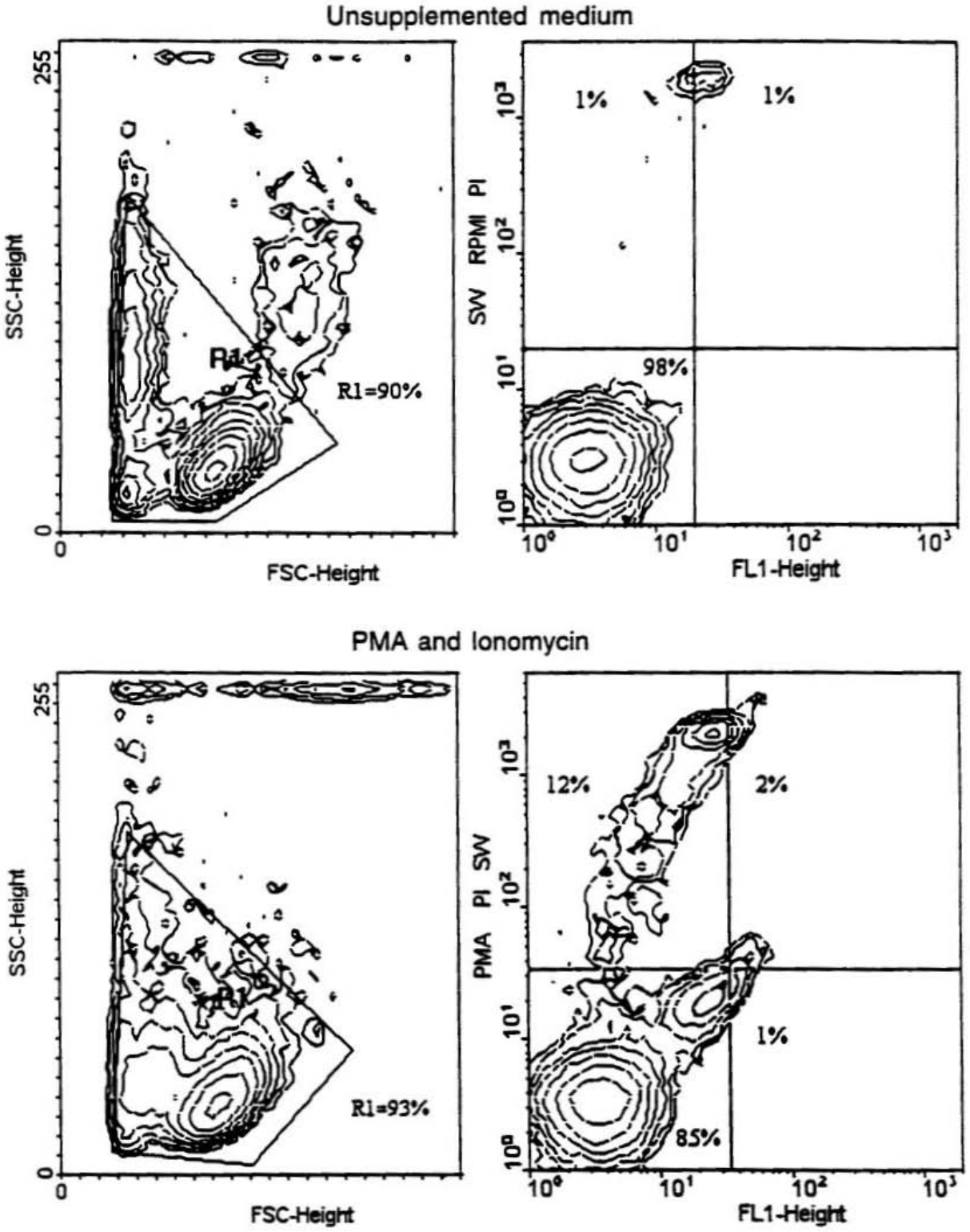
Unsupplemented medium
SSC-Height
255
0
0
FSC-Height
R1
R1=90%
SW RPMI PI
1%
1%
98%
10^3
10^2
10^1
10^0
10^0
10^1
10^2
10^3
FL1-Height
PMA and Ionomycin
SSC-Height
255
0
0
FSC-Height
R1=93%
PMA PI SW
12%
2%
1%
85%
10^3
10^2
10^1
10^0
10^0
10^1
10^2
10^3
FL1-Height

Figure 7b

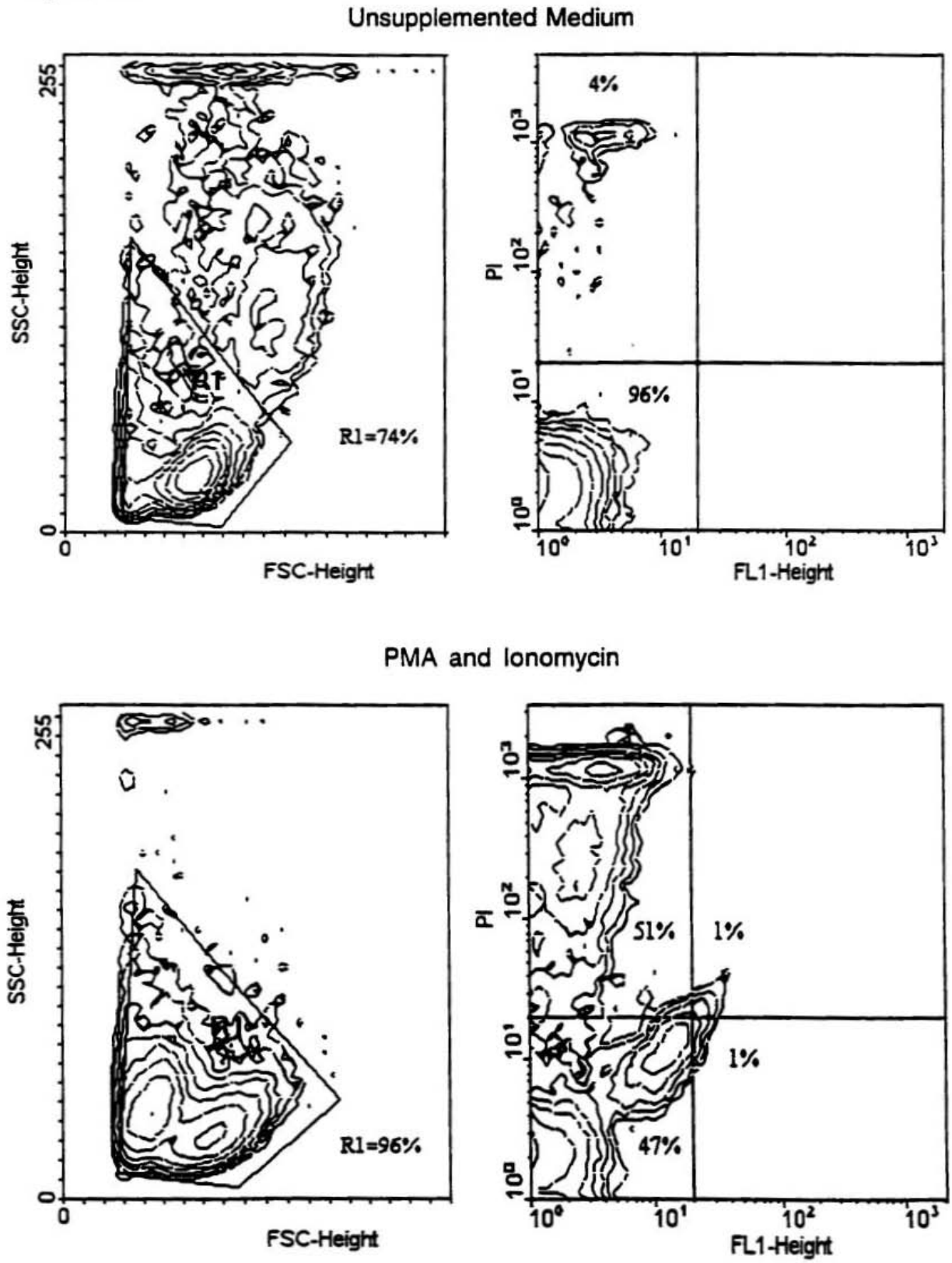
Unsupplemented Medium
SSC-Height
255
0
FSC-Height
R1=74%
PI
4%
96%
FL1-Height
PMA and Ionomycin
R1=96%
51%
1%
1%
47%

Figure 7c

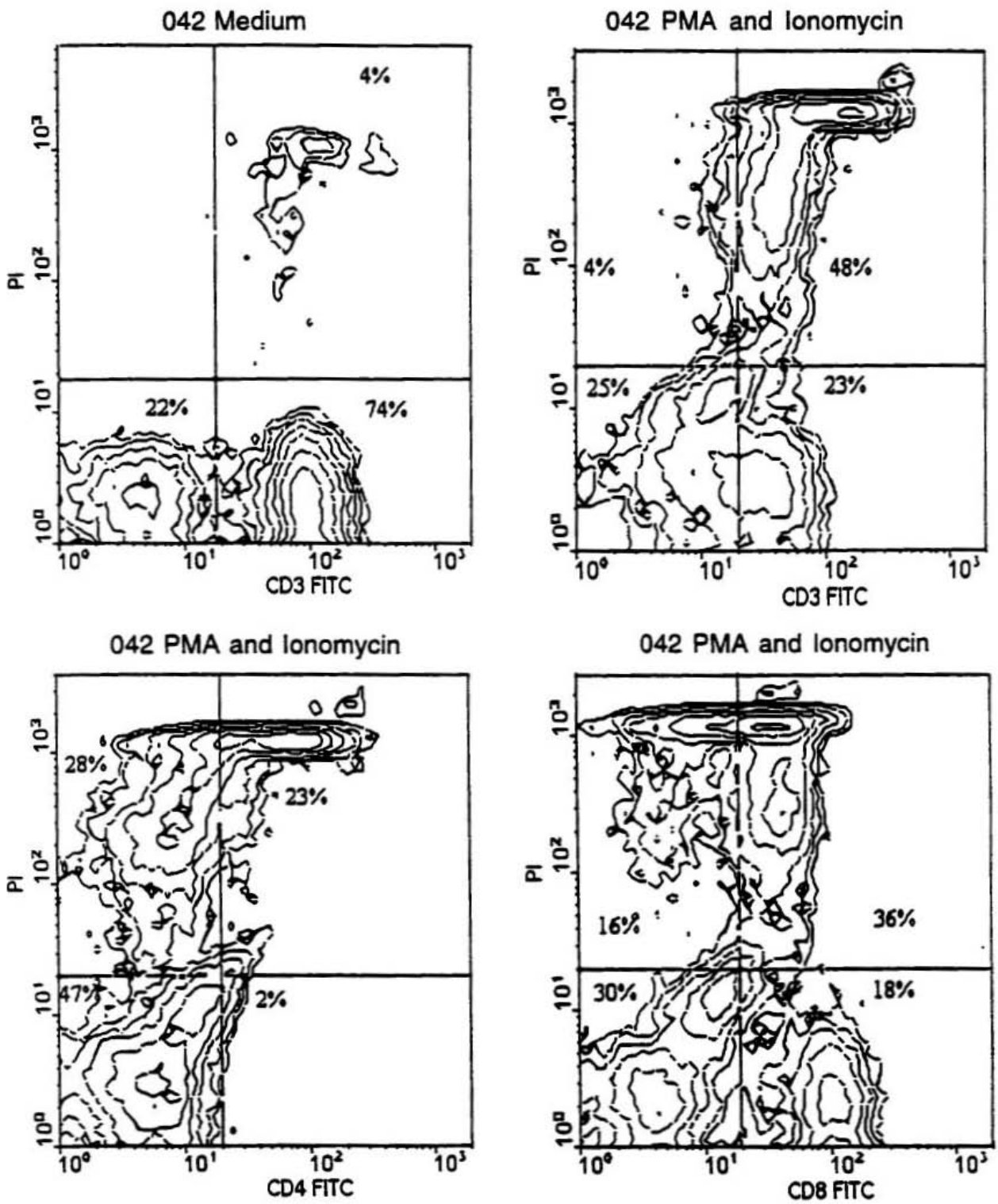
042 Medium
4%
22%
74%
PI
CD3 FITC
042 PMA and Ionomycin
4%
48%
25%
23%
PI
CD3 FITC
042 PMA and Ionomycin
28%
23%
47%
2%
PI
CD4 FITC
042 PMA and Ionomycin
16%
36%
30%
18%
PI
CD8 FITC

Figure 8a

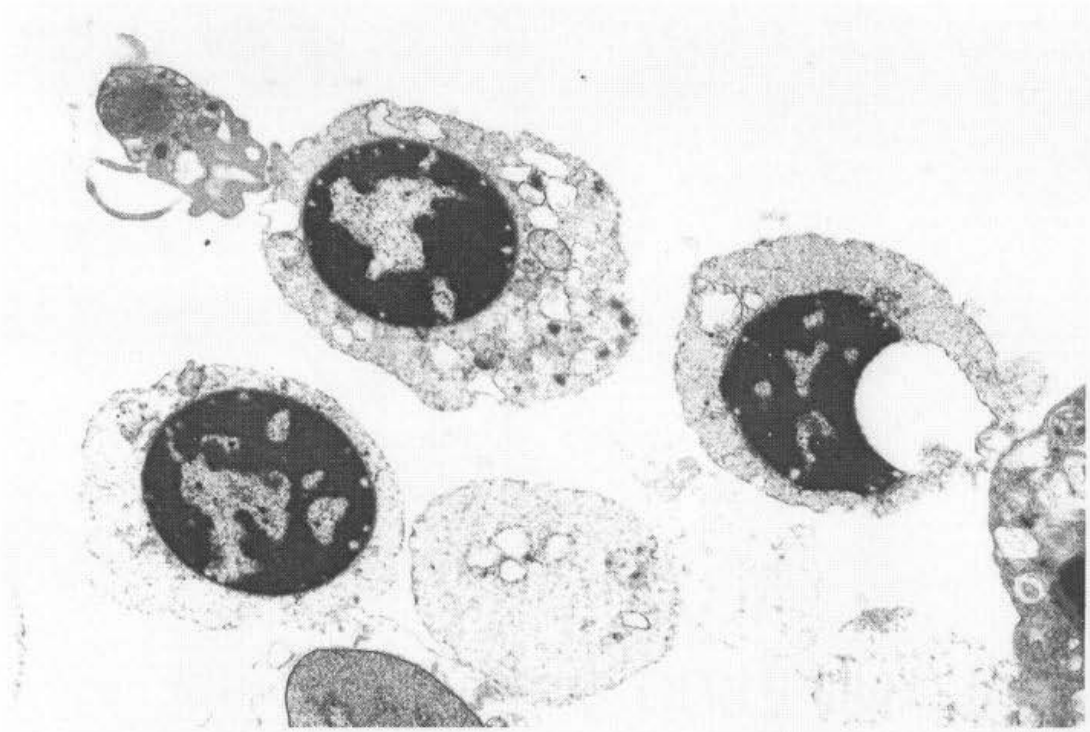

Figure 8b

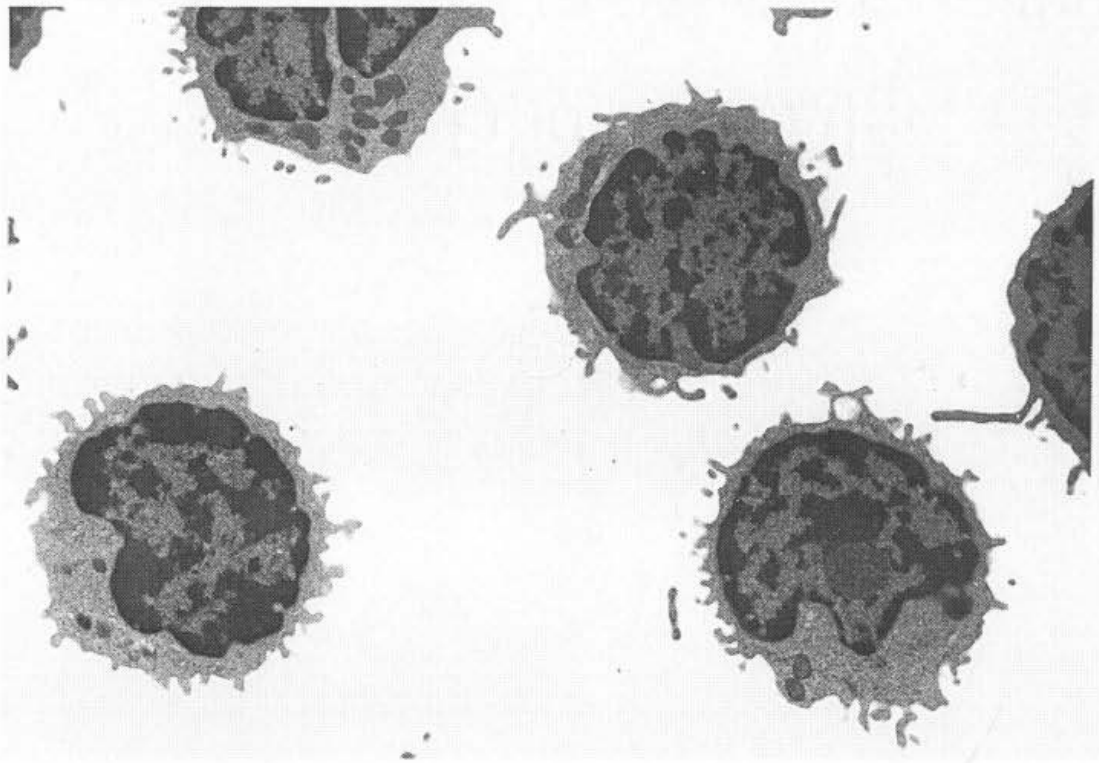

Figure 8c

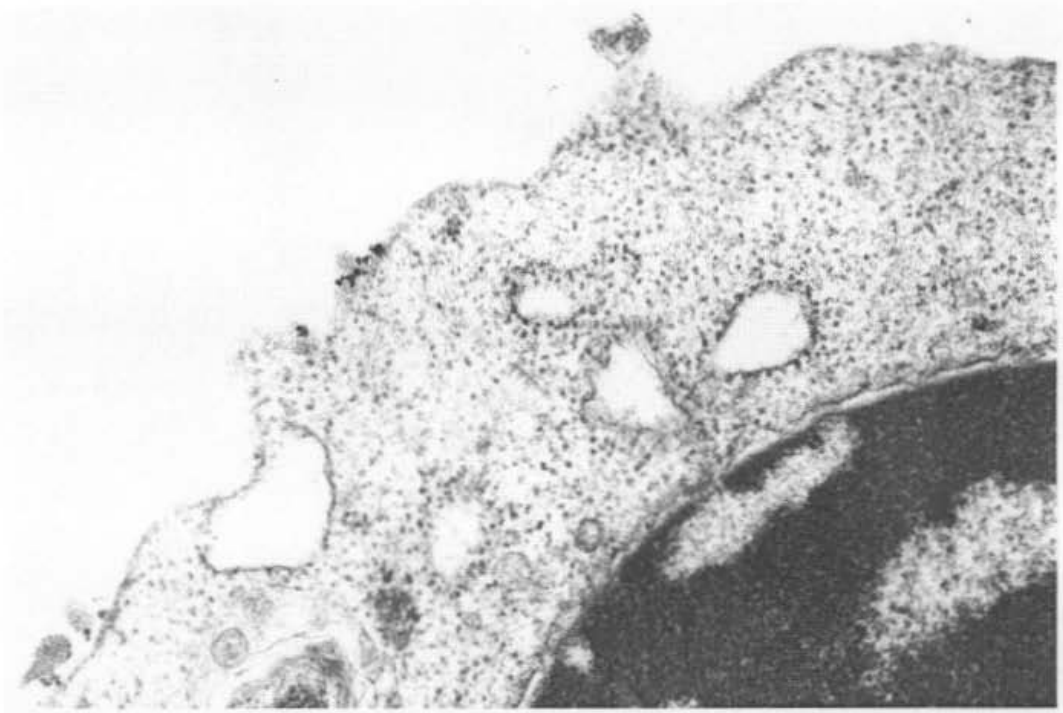

Figure Captions

Fig. 1. AICD induction by PMA and ionomycin. The level of cell death induced by PMA and ionomycin treatment of PBMC from 60 HIV-infected individuals and 15 uninfected volunteers was estimated by Cr release. Mean Cr release values ± standard deviation for both groups are indicated by the solid horizontal and vertical lines bisecting the data points.

Fig. 2. Contributions of PMA and ionomycin to AICD of PBMC from HIV-infected individuals. AICD triggered by PMA or ionomycin either alone or in combination was estimated by Cr release from freshly-isolated PBMC of 12 HIV-infected individuals.

Fig. 3. Time course of AICD triggered by PMA and ionomycin as estimated by Cr release from the PBMC of HIV-infected individuals. Cr release from the PMA and ionomycin-treated PBMC of 5 HIV-infected individuals was measured at 1 hour intervals for 5 hours.

Fig. 4. The effect of cold target cells on AICD triggered by PMA and ionomycin in PBMC from 6 HIV-infected individuals. AICD was measured by Cr release in the absence of cold target cells and with a 10:1 ratio of either Fas-negative K-562 cells or Fas-expressing P815 cells added as cold targets.

Fig. 5. The effects of Ca free medium and 2 different anti-CD3 antibodies on PMA and ionomycin-triggered AICD of freshly-isolated PBMC from 6 HIV-infected individuals. AICD was induced as described and measured by Cr release in the presence of 5 □g/ml OKT3, in the presence of 5 μg/ml HIT3a and in Ca free medium.

Fig. 6. Agarose gel electrophoretic analysis of DNA isolated from PBMC undergoing PMA and ionomycin-triggered AICD as indicated by Cr release. Lane 1 contains positive control DNA isolated from the LS102.9 cell line triggered to undergo apoptosis by 5 hour incubation with 100 ng/ml of the anti-Fas antibody Jo2. Lanes 2 through 5 contain DNA isolated from PBMC of 4 HIV-infected individuals after 5 hours of incubation with 10 nM PMA and 500 nM ionomycin. Cr release values after 5 hours for these subjects were 38%, 39%, 48% and 40% respectively. Lane 6 contains DNA isolated from the PBMC of subject four after 16 hours of incubation with PMA and ionomycin.

Fig. 7. Flow cytometric analysis of cells undergoing AICD triggered by PMA and ionomycin. PBMC cultured for 16 hours in lymphocyte medium with or without PMA and ionomycin were harvested, washed and stained with FITC-conjugated isotype control antibodies or antibodies against CD3, CD4 or CD8 before incubation with propidium iodide (PI). Lymphocyte gates based on cell forward and side light scatter characteristics were expanded

to include most of the PI^+ cells in the analysis (a and b). The proportions of PBMC that failed to exclude PI are compared for SW, an uninfected control (a) and 042, an HIV-infected individual (b), after overnight incubation in lymphocyte medium with or without PMA and ionomycin. Determination of the proportion of $CD3^+$ lymphocytes undergoing AICD in medium alone, and the proportion of $CD3^+$, $CD4^+$ and $CD8^+$ lymphocytes undergoing AICD following stimulation with PMA and ionomycin is also shown for the HIV-infected individual 042 (fig. 7c).

Fig. 8. Visualization of ultrastructural changes in PBMC undergoing AICD triggered by PMA and ionomycin. PBMC were harvested after 5 hours incubation in medium alone or medium supplemented with PMA and ionomycin and processed for transmission electron microscopy. PBMC of an HIV-infected individual are shown at 3000x magnification after incubation in medium with PMA and ionomycin (a) or medium alone (b). A single PBMC undergoing AICD is shown at higher magnification (40,000x) to reveal the nuclear membrane (c). These micrographs are representative of results observed with electron microscopy of treated PBMC from more than 15 HIV-infected individuals.

Table 1. Phenotype of PBMC failing to exclude propidium iodide (PI^+) after 16 hr incubation with PMA and ionomycin*

Subject	5 hr ^{51}Cr Release	CD4s	CD8s	PBMC PI^+ PBMC	$CD3^+PI^+$ PI^+	$CD3^+PI^+$ $CD3^+$	$CD4^+PI^+$ $CD4^+$	$CD8^+PI^+$ $CD8^+$
004 RPMI				.28				
004 PMA/I	38%	0	207	.61	.78	.82	.97	.71
005 RPMI				.15				
005 PMA/I	9%	21	383	.43	.64	.82	1.0	.54
014 RPMI				.10	.73	.16	.83	.03
014 PMA/I	26%	4	335	.26	.81	.73	1.0	.48
022 RPMI				.07				
022 PMA/I	38%	3	704	.46	.92	.80	1.0	.63
023 RPMI				.08				
023 PMA/I	26%	7	372	.28	.66	.75	1.0	.50
029 RPMI				.18				
029 PMA/I	21%	7	373	.44	.70	.75	1.0	.43
042 RPMI				.03	1.0	.05	.14	.04
042 PMA/I	40%	384	1225	.51	.92	.69	.95	.65
051 RPMI				.11				
051 PMA/I	32%	40	802	.21	.76	.53	.88	.28

055 RPMI				.11				
055 PMA/I	17%	367	3051	.21	.88	.30	.71	.26
067 RPMI				.19	.61	.30	.61	.19
067 PMA/I	40%	279	813	.65	.55	.95	1.0	.78
068 RPMI				.19	.84	.25	.35	.20
068 PMA/I	44%	683	855	. 77	.70	.87	1.0	.81
SW RPMI		Control			.02			
SW PMA/I	5%				.14			

Table 1. Legend

*The percentage of PBMC that did not exclude PI after incubation in medium alone or after treatment with PMA and ionomycin was estimated by flow cytometric analysis and the phenotype of the PI^+ cells determined by co-staining with FITC-conjugated anti-CD3, anti-CD4 or anti-CD8.

Five hour Cr release data is shown for the same time point for comparison with PI exclusion data and $CD4^+$ and $CD8^+$ T lymphocyte counts at the time of testing are presented for each individual to illustrate the composition of the starting PBMC population.

CHAPTER IV

Circulating Autoreactive Cytotoxic T Lymphocyte Activity is Associated with Disease Progression in HIV-1 Infection[1]

Running head: Autoreactive CTL in HIV Infection

text = 4963 words

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Objective: To investigate the relationship between circulating autoreactive cytotoxic T lymphocytes (CTL) and disease progression in HIV-1 infection.

Design and methods: Peripheral blood mononuclear cells (PBMC) from 75 HIV-infected individuals at various stages of disease were tested directly *ex vivo* for T lymphocyte-mediated killing of uninfected activated T lymphocytes. $CD4^+$ and $CD8^+$ T lymphocyte counts were measured for each subject when blood was drawn for cytotoxicity testing and in certain samples, the proportion of $CD8^+$ T cells expressing CD28 was determined. Plasma β-2 microglobulin and HIV RNA were also measured in selected samples. Mean levels of each of these parameters were compared in groups of HIV-infected individuals separated on the basis of whether at any time over the study period their freshly isolated T cells killed uninfected activated T lymphocytes. The prevalence of detectable autoreactive CTL activity was also compared in groups stratified by levels of markers associated with an increased relative risk of rapid progression to AIDS.

Results: Circulating autoreactive CTL activity was detected in >50% of the individuals tested over the period of study. As a group, HIV-infected individuals with autoreactive CTL activity had significantly more $CD8^+$ T cells, fewer $CD4^+$ T cells, a higher proportion of $CD28^- CD8^+$ T cells, and higher plasma levels of HIV RNA and β-2 microglobulin. Autoreactive CTL prevalence was higher in groups of HIV-infected individuals with immunological and virological parameters indicating an increased relative risk of rapid disease progression.

Depletion and enrichment experiments showed the autoreactive CTL were predominantly, if not exclusively, $CD28^-$.

Conclusions: Circulating autoreactive CTL activity in HIV infection is associated with $CD4^+$ T cell loss, immune activation, $CD8^+$ T cell expansion, selective expansion or accumulation of $CD28^-$ $CD8^+$ T cells and inadequate suppression of HIV replication.

Key words: CTL, CD28, HIV, autoimmunity

Introduction

Eventual depletion of virtually all $CD4^+$ T lymphocytes in HIV infection, despite the low percentage of HIV-infected $CD4^+$ T cells *in vivo*, implies that HIV indirectly targets uninfected $CD4^+$ T cells. Marked activation of $CD8^+$ cytotoxic T lymphocytes (CTL) is a prominent immunological feature of HIV infection and several mechanisms have been proposed through which persistent $CD8^+$ CTL activation and expansion might contribute to $CD4^+$ T cell depletion. The potential for HIV-specific CTL to kill HIV-infected $CD4^+$ T cells and antigen presenting cells offers an active mechanism whereby $CD8^+$ CTL could mediate CD4 depletion and immunodeficiency, but this mechanism would target only infected cells [1, 2]. Another possibility is that if total T cell numbers were regulated by a homeostatic mechanism blind to T cell subset proportions, the extent of $CD8^+$ T cell proliferation and accumulation commonly triggered by HIV infection could depress new $CD4^+$ T cell

production [3]. Through this passive mechanism, $CD8^+$ T cells might exacerbate HIV-related $CD4^+$ T cell loss independent of any specificity for HIV as a byproduct of persistent expansion of the $CD8^+$ T cell population as a whole.

Although $CD8^+$ T cells may contribute to CD4 depletion through one or both of these proposed mechanisms, neither can account for the increased death of uninfected CD4s directly illustrated by *in situ* analysis of apoptotic $CD4^+$ T cells within lymph nodes of HIV-infected humans and SIV-infected macaques [4]. Several investigators have reported CTL that kill activated uninfected T lymphocytes within the peripheral blood lymphocytes (PBL) of HIV-infected individuals and the relationship between expansion of distinct $CD8^+$ T cell subsets and disease progression in HIV infection suggests that some uninfected $CD4^+$ T cell death might reflect $CD8^+$ T cell-mediated immunopathology [5-10]. Development of these autoreactive CTL is generally associated with SIV or HIV infections progressing to AIDS as they do develop in SIV-infected macaques, but don't develop in HIV-infected chimpanzees [5, 11]. In an early study of autoreactive CTL in HIV infection, Zarling *et al.* reported circulating CTL against activated uninfected T lymphocytes in 11/13 HIV-infected individuals, but provided no other information on the individuals tested [5]. Subsequent studies addressing the prevalence, character and role of these autoreactive CTL in cohorts of HIV-infected individuals following *in vitro* activation of circulating PBMC, have produced conflicting interpretations of their role. In one study of the prognostic significance of these CTL, more short term $CD4^+$ T cell loss was observed in HIV-infected individuals with

autoreactive CTL [12]. In contrast, a subsequent study following more subjects over a longer time period found the presence of these CTLs conferred a more favourable prognosis for $CD4^+$ T cell loss [13]. Since levels of these autoreactive CTLs following *in vitro* stimulation may bear little relation to their concomitant eminence *in vivo*, we carried out a cross-sectional study comparing immunological and virological markers of disease in HIV-infected individuals grouped on the basis of circulating autoreactive CTL activity. Direct detection of the autoreactive CTL within circulating PBL should indicate that these CTL are active *in vivo* or at least that the conditions underlying their *in vivo* activation in HIV infection currently exist within the test subject.

We found the presence of circulating autoreactive CTL activity in HIV infection was associated with more severe immunological and virological signs of disease progression. Our results establish development of autoreactive $CD8^+$ CTL as a prominent feature of progressive HIV infection and suggest a causal relationship between development of this CTL activity and the immunopathogenesis of AIDS. Therefore, we propose that a destructive synergy between HIV replication and immune activation drives the development of autoreactive CTL, underlies the relationship between development of autoreactive CTL and disease progression, and links $CD8^+$ T cell-mediated immunopathology with the degeneration of effective anti-HIV immunity.

Methods

Study Subjects and Clinical Laboratory Evaluation

HIV-infected individuals were recruited through the Infectious Diseases Clinic of the St. John's General Hospital, St. John's. NF, Canada. Seroconversion was detected by ELISA testing with commercial kits (Abott Laboratories, Chicago, IL) and confirmed by western blot. Depending upon disease stage, subjects visited the clinic every 1, 3 or 6 months. At each visit, clinical evaluation and blood work, including measurement of peripheral blood $CD4^+$ and $CD8^+$ T lymphocyte numbers was carried out. Over the last part of the study, plasma HIV RNA was measured at each visit using Amplicor HIV-1 Monitor quantitation kits (Roche Diagnostic Systems Inc., Mississauga, ON). Ethical approval for this study was obtained from the Memorial University Faculty of Medicine Human Investigation Committee and informed consent for drawing blood samples and accessing medical records was obtained from all study participants.

Sample preparation

Whole blood was drawn by venipuncture into vacutainers containing heparin or ethylene diamine tetra acetic acid (EDTA) to prevent clotting and all samples were processed within 4 hours of withdrawal. Whole blood samples were centrifuged at 500*g* for 10 min and plasma was collected, labelled and immediately stored at -80^0 C. The packed blood was

diluted to twice the original volume with sterile phosphate buffered saline (PBS; pH 7.2), transferred to sterile 50 ml centrifuge tubes and underlaid with an approximately equal volume of Ficoll-paque lymphocyte separation medium (Pharmacia Chemicals, Dorval, Que). After centrifuging for 30 min at 400*g*, peripheral blood mononuclear cells (PBMC) were collected from the gradient interface, washed 3 times in PBS with 1% fetal calf serum (FCS; Gibco, Grand Island, NY), resuspended in lymphocyte medium (RPMI 1640 with 10% FCS, 10 mM hepes, 2 mM L-glutamine, 1% penicillin/streptomycin and $2x10^{-5}$ M 2-mercaptoethanol; all from Gibco) and counted. Freshly-isolated cells were tested immediately in cytotoxicity assays or in some cases, cultured for 7 days in lymphocyte medium supplemented with 10 μg/ml Concanavalin A (Con A; Difco, Toronto, ON) and 5 U/ml interleukin-2 (IL-2; Hoffmann La Roche, Nutley, NJ) before cytotoxicity assays.

To measure the proportion of $CD8^+$ T cells expressing CD28, PBMC were washed once in PBS with 5 mM EDTA and 0.1% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO) and incubated for 20 min at 4^0 C with either fluorescein isothiocyanate (FITC)-conjugated anti-CD3 and phycoerythrin (PE) conjugated anti-CD8, anti-CD8 FITC and anti-CD28 PE or isotype controls. Anti-CD28 was from Becton Dickinson, Mississauga, ON and all other antibodies for flow cytometry were from Dako Diagnostics, Mississauga, ON. Samples were washed once after staining, resuspended in 0.5% paraformaldehyde in PBS and analyzed on a FACStar^{Plus} analyzer (Becton Dickinson, Mississauga, ON) after excitation at 488 nm with an argon laser.

Lymphocyte separations

In some cases, specific T cell subsets were removed before cytotoxicity assays. Cells were pelleted, washed in PBS with 0.1% BSA and 5 mM EDTA and incubated in a small volume for 30 min at 4□C with 5 μg/10^6 cells of anti-CD4 (OKT4; ATCC CRL8002), anti-CD8 (OKT8; ATCC CRL8014) or anti-CD28 (Becton Dickinson, Mississauga, ON). After incubation with primary antibodies, the cells were washed and incubated at 3x10^6/ml in PBS plus 0.1% BSA and 5 mM EDTA at 4^0 C for 45 min on a rotating shaker with goat anti-mouse IgG magnetic beads (Dynal Inc., Great Neck NY) at a 10:1 bead to target cell ratio. Cells bound to the beads were removed by positioning the tubes against a magnet, removing supernatant, washing the beads gently in PBS and repeating the process. For some assays, the unbound cells were centrifuged, resuspended and used directly in cytotoxicity assays while in others, they were counted and effector:target (E:T) ratios were set with the purified cells. Analysis by flow cytometry showed that this method removed 98% of the target cell population (data not shown).

Cytotoxicity assays

Target cells were PBMC isolated from an HIV-seronegative individual and cultured for 7 days in lymphocyte medium with 5 μg/ml purified phytohemagluttinin (PHA-P; Wellmark Diagnostics, Guelph, ON). Previous studies suggest the target of the autoreactive

CTL is non-polymorphic and relatively uniformly expressed on activated $CD4^+$ T lymphocytes from different individuals, however, we always generated target cells from individuals previously shown in multiple assays to provide sensitive target cells [14]. On the day of assay, the PHA-activated cells were harvested, washed and incubated in a small volume of lymphocyte medium for 90 min at 37^0 C with 100 μCi of $Na_2{}^{51}CrO_4$ (Amersham Life Sciences, Arlington, IL). The labelled target cells were washed 4 times with PBS plus 1% FCS, counted and resuspended in medium at $1x10^5$/ml for use as targets. In most cases, effector cells were freshly-isolated PBMC from HIV-infected individuals, but we also used further purified fresh cell populations and cultured cells. Effector cells were washed, counted and resuspended in fresh medium at $5x10^6$/ml. For depletion studies, E:T ratios were based on the starting cell number and effectors were not recounted, while for enrichment studies, the E:T ratio was established with purified cells. Killing of uninfected activated T cells was tested at E:T ratios of 50, 25 and 12.5:1 and at 50:1 in the presence of 5 μg/ml OKT3 to confirm that killing was T cell-mediated. Assays were carried out in duplicate in microtitre plates (ICN Canada Inc., Montreal, Que). Fifty μl of target cells were added to each well for a total of 5000 targets, while 50, 25 or 12.5 μl of effector cells were added to set the E:T ratio. Final volume in each well was adjusted to 300 μl with medium. Minimum and maximum release wells were generated by incubating target cells in medium alone or 1N HCl respectively. Once effector cells and target cells were added, the assay plates were incubated for 5 hr in a 5% CO_2 humidity controlled incubator. One hundred μl of cell-free supernatant

was then removed and counted in a Wallac 1480 gamma counter. Percent killing of the uninfected lymphocytes by the effector cells was calculated by the following formula:

$$\frac{\text{(experimental } ^{51}\text{Cr release - spontaneous } ^{51}\text{Cr release)}}{\text{maximum } ^{51}\text{Cr release - spontaneous } ^{51}\text{Cr release}} \times 100$$

Spontaneous ^{51}Cr release was less than 25% of maximum release in all assays.

Measurement of plasma β-2 microglobulin

For measurement of β-2 microglobulin, plasma was separated from freshly drawn whole blood by 10 min centrifugation at 500*g* and immediately stored at -80^0 C. Immulon-2 ELISA plates (VWR Scientific, Mississauga, ON) were coated overnight at 4^0 C with 250 ng/well goat anti-mouse IgG (Bio/Can Scientific, Mississauga, ON) in 100 µl carbonate buffer. The following morning the plates were washed once with PBS plus 0.5% Tween (Sigma Chemical Co., St. Louis, MO) and blocked for 60 min with 200 µl/well 1% BSA in PBS. The plates were then washed twice and 100 ng/well monoclonal anti-β-2 microglobulin (MC115, Serotec Canada, Mississauga, ON) was added for 60 min in 100 µl PBS with 0.1% BSA. The plates were then washed 6 times and 100 µl of purified β-2 microglobulin (Sigma Chemical Co., St. Louis, MO), resuspended in PBS with 0.1% BSA at concentrations ranging from 1-10 ng/ml or plasma samples diluted between 1:500 and 1:2000 were added for 90 min. The plates were again washed 6 times and 100 µl of a 1:1000 dilution of horseradish peroxidase (HRP)-conjugated rabbit anti-human β-2 microglobulin (Dako Diagnostics,

Mississauga, ON) in PBS with 0.1% BSA was added for 60 min. The plates were washed a final 6 times and 100 µl/well HRP substrate was added. After 30-min colour development, the reaction was stopped with 50 µl/well 2.5 N H_2SO_4 and the optical density (OD) read at 490 nm on an ELISA reader. The level of β-2 microglobulin in each sample was calculated from the sample OD 490 and the standard curve constructed from the OD 490 of the standards made with purified β-2 microglobulin.

Statistical analysis

Differences in the mean levels of continuous parameters in groups of HIV-infected individuals separated on the basis of circulating autoreactive CTL activity were assessed by Student's *t* test. Differences in the prevalence of autoreactive CTL activity in groups sorted by levels of various continuous parameters previously associated with risk of rapid progression to AIDS were assessed by c^2 analysis of contingency. A normal distribution of the parameters measured within the cohort was assumed from the percentage of measures falling within the mean ± 2 standard deviations (SD). Correlations between different parameters were assessed by linear regression analysis.

Results

Overall incidence of circulating autoreactive CTL activity

Over the course of this study, 43/75-HIV-infected individuals tested for circulating

autoreactive CTL activity demonstrated <10% killing of uninfected activated T lymphocytes on 1 or more occasions. The level of killing observed at an E:T ratio of 50:1 ranged from 0 to 55%. When freshly-isolated PBMC mediated between 10 and 15% killing, individuals were considered indeterminate in terms of circulating autoreactive CTL activity unless <10% killing was observed on another occasion. Six individuals fell into this indeterminate category, therefore, we report the overall incidence of circulating autoreactive CTL against uninfected lymphocytes within our cohort as 37/69 or 54%. Two distinct groups without circulating autoreactive CTL were revealed from the distribution of $CD4^+$ and $CD8^+$ T cell counts in the cohort (fig. 1). One group of 20 (solid diamonds), clustered towards the lower right region of the scatter plot, generally had high $CD4^+$ T cell counts and low $CD8^+$ T cell counts signifying limited disease progression. The other group of 12 (open circles), tightly clustered within the extreme left hand lower corner of the scatter plot, occupied the opposite end of the spectrum with very low $CD4^+$ and total T cell counts signifying end-stage disease. The T cell counts of these 12 individuals indicated a terminal, rather than active stage of disease progression and they likely had already passed through the progressive stage of infection wherein autoreactive CTL activity might be relevant to disease progression. Therefore, we excluded HIV-infected individuals with less than 500 total T cells/μl peripheral blood from our analysis of associations between circulating autoreactive CTL activity and markers of disease activity or disease progression. The upper left region of the scatter plot, representing individuals with high $CD8^+$ T cell counts and low $CD4^+$ T cell counts and active

progressive HIV disease, was densely and exclusively populated with individuals demonstrating circulating autoreactive CTL activity over the course of the study.

Mean levels of immunological and virological markers of disease progression in groups with or without circulating autoreactive CTL

Mean levels of peripheral blood $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, plasma β-2 microglobulin, plasma HIV RNA and the mean proportion of $CD8^+$ T cells expressing CD28 were calculated after separating the cohort into groups with or without circulating autoreactive CTL and excluding those individuals with >500 total T cells. Significant differences between the 2 groups were then assessed using Student's *t* test. The group of HIV-infected individuals with circulating autoreactive CTL activity had a higher mean $CD8^+$ T cell count ($p<.006$), a higher mean level of plasma HIV RNA ($p<.011$), and a higher mean level of plasma β-2 microglobulin ($p<.025$). This group also had a lower mean proportion of circulating $CD8^+$ T cells expressing CD28 ($p<.003$) and a lower mean $CD4^+$ T cell count ($p<.002$) (Table 1). As a group, HIV-infected individuals with circulating CTL activity against uninfected activated lymphocytes have significantly higher levels of markers of immune activation, HIV replication and disease progression.

Frequency of autoreactive CTL activity in HIV-infected individuals stratified by level of immunological and virological markers of disease progression

Individuals clearly classified as positive or negative in terms of having circulating CTL against uninfected lymphocytes were stratified by $CD4^+$ T lymphocyte count as an indication of disease progression to compare the prevalence of circulating CTL activity against uninfected activated T lymphocytes in different groups. Two of 6 HIV-infected individuals with $CD4^+$ T cell counts >500/μl peripheral blood were positive for circulating autoreactive CTL activity compared to 18/38 with $CD4^+$ T cell counts between 200 and 499 and 15/17 with $CD4^+$ T cell counts <200. Due to the scarcity of HIV-infected individuals with >500 $CD4^+$ T cells in our cohort, we combined this group and those with between 200 and 499 $CD4^+$ T cells into a single group with <200 $CD4^+$ T cells/μl peripheral blood. Chi-square analysis of contingency was then used to determine if $CD4^+$ T lymphocyte counts affected the proportion of HIV-infected individuals with circulating autoreactive CTL activity. We subdivided the cohort into 2 groups of <200 (n=17) and >200 (n=40) $CD4^+$ T cells/μl peripheral blood and the number of individuals with circulating CTL activity against uninfected activated T lymphocytes in each group was 15 and 22 respectively. With Yate's correction, the c^2 statistic of 6.5 indicated that autoreactive CTL occurred more frequently in HIV-infected individuals with $CD4^+$ T lymphocyte counts <200/μl peripheral blood ($p<.025$). Similar analyses were done based on CD8 count, proportion of $CD8^+$ T cells expressing CD28, plasma β-2 microglobulin and viral load. Autoreactive CTL also occurred more

frequently in HIV-infected individuals with $CD8^+$ T lymphocyte counts >600/μl peripheral blood ($p<.025$), with <40% of their $CD8^+$ T cells expressing CD28 ($p<.005$), with plasma β-2 microglobulin >3 μg/ml ($p<.01$) or with plasma HIV viral load $>10^4$ copies/ml ($p<.025$).

Correlations between immunological and virological markers of disease progression in groups with or without circulating autoreactive CTL

The distribution of values of the parameters measured for this cohort within groups of HIV-infected individuals with or without autoreactive CTL is shown in figures 2a, b and c. In figure 2a, the level of HIV RNA in the plasma is plotted versus $CD8^+$ T cell counts to show the direct correlation (Pearson product-moment correlation coefficient, r) between $CD8^+$ T cell counts and virus load in the group without circulating autoreactive CTL activity ($r=.625$, $p<.05$). Note that no HIV infected individuals with circulating autoreactive CTL within our cohort had a plasma virus load below the assay detection limit of $\log_{10} 2.3$. The percentage of circulating $CD8^+$ T cells expressing CD28 was plotted against $CD4^+$ T cell counts in fig. 2b to show the direct correlation between $CD4^+$ T cell counts and the percentage of $CD8^+$ T cells expressing CD28 in the group with circulating autoreactive CTL activity ($r=.463$, $p<.05$). In this case, a region defined by <300 $CD4^+$ T cells and <40% $CD8^+$ T cells expressing CD28 completely excludes individuals without autoreactive CTL in our cohort. Fig. 2C shows a preponderance of high plasma β-2 microglobulin levels in HIV-infected individuals with autoreactive CTL activity, but many HIV-infected individuals with

autoreactive CTL activity also had β-2 microglobulin levels in the normal range.

Phenotype of autoreactive CTL

The relationship we observed between circulating autoreactive CTL activity and the proportion of $CD8^+$ T cells expressing CD28 prompted phenotypic analysis of the autoreactive CTL. Depletion experiments with freshly isolated PBMC from HIV-infected individuals showed the circulating autoreactive CTL found in HIV-infected individuals were predominantly, if not exclusively $CD28^-$ (fig 3). It is also noteworthy that the effector cells were all T cells as the killing is completely abrogated by addition of OKT3 to the assay (fig 3). In each of 4 cases shown, removal of $CD28^+$ cells prior to setting the final E:T ratio produced at least a modest increase in killing and in no case did removal of $CD28^+$ cells reduce killing. This is consistent with the low (<30%) level of $CD8^+$ $CD28^+$ cells present within the circulating T cell population of these individuals (data not shown). Depletion and enrichment experiments were carried out with effector cells cultured for 7 days in Con A and IL-2, which have a much higher proportion of $CD28^+$ $CD8^+$ T cells than freshly isolated PBMC (generally >80%, data not shown). Depletion experiments without re-establishing the original E:T ratios show that removal of $CD28^+$ or $CD4^+$ cells does not reduce killing, but removal of $CD8^+$ cells reduces killing to background levels (fig. 4a). Removal of $CD28^+$ cells from cultured effector cells followed by re-establishment of E:T ratios enriches autoreactive CTL activity as the $CD28^-$ population mediates higher killing of uninfected activated

lymphocytes at equivalent E:T ratios (fig 4b).

Discussion

Autoreactive CTL against uninfected $CD4^+$ T lymphocytes were first described in HIV-infected individuals nearly 10 years ago, but neither the origin, nor the role of these CTL has been clarified [5, 6]. The absence of autoreactive CTL in HIV-infected chimpanzees, then thought completely resistant to disease following HIV infection, prompted initial speculation these CTL might contribute to $CD4^+$ T cell depletion and disease progression [5]. Other investigators did not confirm the high prevalence of circulating autoreactive CTL initially reported, therefore, subsequent studies focussed on *in vitro* activation of autoreactive CTL and produced conflicting data concerning the association between autoreactive CTL and disease progression [12, 13]. The discrepancies observed may reflect the diminished relevance of *in vitro* stimulated CTL activity compared to circulating CTL activity in terms of accurately representing the *in vivo* situation. Since circulating autoreactive CTL against uninfected lymphocytes were originally detected by routine means in 11/13 HIV-infected individuals, while subsequent investigators were unable to detect them at all, it seemed plausible that circulating autoreactive CTL would be concentrated within a particular subset of HIV-infected individuals [5]. In this cross-sectional study, we confirmed this was the case and investigated immunological and virological characteristics of HIV infection associated with development

of autoreactive CTL.

After excluding individuals with terminal disease (<500 total T cells/μl peripheral blood), we found that 15/17 HIV-infected individuals with <200 $CD4^+$ T cells/μl peripheral blood had circulating autoreactive CTL activity. This is much higher than the overall frequency of circulating autoreactive CTL in our cohort (37/69), but similar to the frequency reported by Zarling *et al*, suggesting the participants in their study were at a similar stage of disease [5]. A $CD4^+$ T cell count of <200/μl peripheral blood was clearly enough associated with an increased risk of rapid progression to clinically-defined AIDS that it now serves as a laboratory-defined criterion for AIDS in the United States [15]. We also saw an increased frequency of circulating autoreactive CTL in groups defined by the level of other parameters associated with increased risk for rapid progression to AIDS, including a $CD8^+$ T cell count >600/μl peripheral blood, a plasma β-2 microglobulin level >3 μg/ml and an HIV plasma virus load $>10^4$ copies/ml [16-18]. Although the proportion of circulating T lymphocytes expressing CD28 is not commonly recognized as a prognostic indicator in HIV infection, this proportion decreases overall and especially within the $CD8^+$ T cell population in parallel with disease progression [9, 10]. We also observed an increased prevalence of circulating autoreactive CTL in HIV-infected individuals with <40% of their $CD8^+$ T cells $CD28^+$. The observed differences in autoreactive CTL prevalence and the difference in mean levels of virological and immunological markers associated with disease progression in HIV-infected individuals with circulating autoreactive CTL activity all support a relationship between

active disease progression and development of autoreactive CTL activity.

The association between different markers of disease progression and development of autoreactive CTL activity in HIV infection does not address mechanisms underlying their activation or prove a role for these CTL in disease progression, but the various relationships observed outline the conditions under which these autoreactive CTL develop *in vivo*. Previous studies showed the autoreactive CTL express the αβ form of T cell receptor and that killing is T cell receptor-mediated even though it is not classically HLA-restricted [7]. This suggests some form of antigen-specific activation triggered by HIV, but not necessarily involving HIV as an antigen. Although loss of CD28 expression in some cases may reflect extensive previous proliferation, the predominant $CD28^-$ phenotype of the CTL is also consistent with autoreactivity and recognition of antigens through non-conventional presenting molecules [20-22]. T cells lacking CD28 expression appear rapidly during primary HIV infection and are present shortly after birth in vertically-infected infants, therefore, if loss of CD28 reflects a previous extent of proliferation mitigating replicative senescence, this can apparently occur in a matter of weeks [23, 24]. One alternative explanation proposed for the rapid emergence of $CD28^-$ T cells in HIV infection is mobilization of resident $CD28^-$ T cells from mucosal sites of HIV replication [25]. The constitutive cytotoxicity, autoreactivity, oligoclonality and limited proliferative potential of the $CD28^-$ $CD8^+$ T cells found in the peripheral circulation in HIV infection are also features of the resident $CD28^-$ intestinal epithelial lymphocyte (IEL) population [26-28]. Surprisingly, anti-SIV CTL activity is

detectable in IEL from SIV-infected macaques, despite the marked oligoclonality of this population [29]. Although there is no direct evidence for emigration of IEL to the periphery, there are intriguing phenotypic, functional and molecular genetic similarities between this population and the circulating $CD8^+$ T cells predominating in progressive HIV infection.

The results of this study lead us to speculate that the appearance of $CD28^-$ T cells in HIV infection reflects conditions driving intense $CD8^+$ T cell proliferation. Whether they originate from mucosal sites, selective expansion of circulating $CD28^-$ precursors or proliferation-dependent transformation of circulating $CD28^+$ precursors remains to be determined. The $CD28^-$ $CD8^+$ T cells of HIV-infected individuals have shorter telomeres than $CD28^+$ cells, consistent with loss of CD28 expression as the number of cell divisions reaches a critical limit [30]. Rapid clonal exhaustion of T cells stimulated in primary HIV infection has also been reported, however, the telomeres of $CD28^-$ T cells in uninfected individuals are longer than those in HIV-infected individuals, suggesting that inherently $CD28^-$ cells may be selectively proliferating in HIV infection [30, 31]. In either case, the cells that are $CD28^-$ in HIV infected individuals are undergoing, or have undergone selective proliferation. We and others have found that the proportion of $CD28^-$ T cells in the circulation increases with disease progression, either because responding cells are approaching senescence or conditions change to favour the selective accumulation of $CD28^-$ cells [9, 10]. The general relationship between immune activation, development of autoreactive CTL, HIV replication and accumulation of $CD28^-$ T cells is consistent with a destructive synergy between HIV replication and immune

activation that underlies the $CD8^+$ T cell-mediated immunopathology associated with HIV infection. We have recently shown that autoreactive CTL are indistinguishable from those found in the circulation of HIV-infected individuals can be generated *in vitro* from seronegative individuals by stimulation of PBL with autologous activated $CD4^+$ T lymphocytes (unpublished data). HIV replication is dependent upon T cell activation and through its antigenic potency and expression of transactivating gene products (tat and possibly nef), HIV replication could drive T cell activation in a self-amplifying cycle. Over a certain threshold, the activated T cells themselves could compete with HIV as antigens for $CD8^+$ T cells and shift the balance from a predominance of effective $CD28^+$ anti-HIV CTL towards a predominance of $CD28^-$ autoreactive immunoregulatory CTL and inefficient anti-HIV CTL. This shift could occur from primary infection onwards and may be susceptible to bi-directional modulation by immune activation or antiretroviral therapy. In the absence of effective antiviral therapy, the shift would likely be reflected by increases in plasma virus load, emergence of a syncytia-inducing (SI) viruses, and increased rates of $CD4^+$ T cell loss.

In summary, the development of autoreactive CTL activity is a prominent feature of HIV infection associated with viral replication, immune activation, $CD4^+$ T cell loss and accumulation of $CD28^-$ T cells. We propose that HIV capitalizes on immune activation by triggering an immunoregulatory pathway that diverts the $CD8^+$ T cell response away from efficient suppression of HIV replication and towards immunopathology. Thus, immune activation may be an integral component of HIV disease progression that could be carefully

targeted in constructive synergy with antiretroviral therapy to develop increasingly efficient combination therapies.

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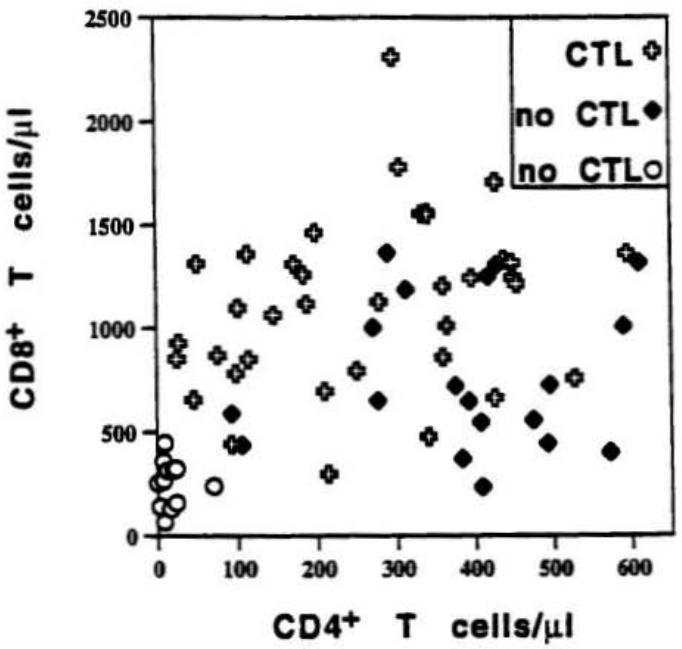
Fig. 1


2500
2000
1500
1000
500
0
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0
100
200
300
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CD4+ T cells/µl
CTL
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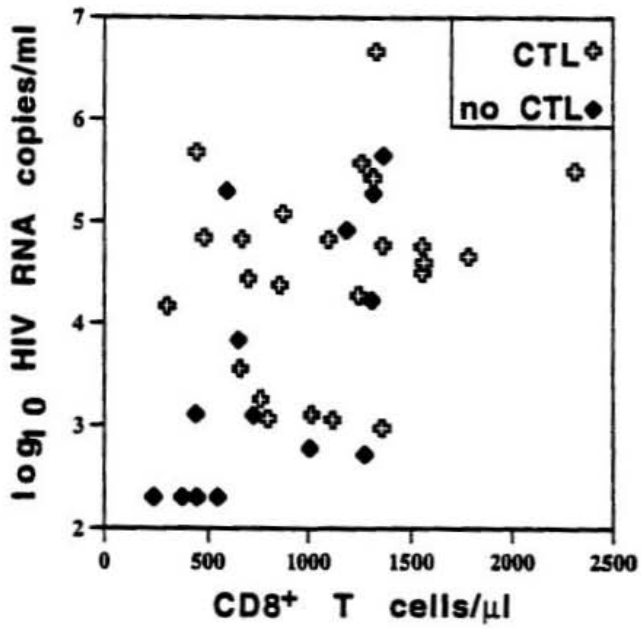
no CTL

Fig. 2a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6
5
4
3
2
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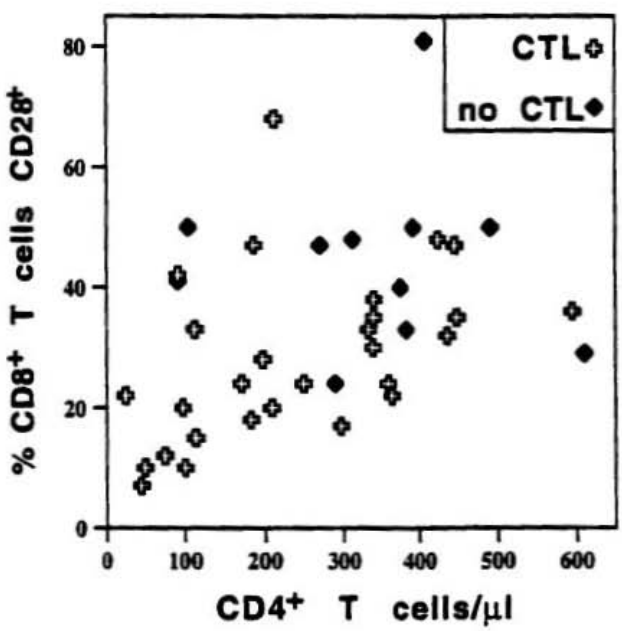
1000
1500
2000
2500
CD8+ T cells/µl

Fig. 2b


CTL
no CTL
% CD8+ T cells CD28+
CD4+ T cells/µl
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20
40
60
80
0
100
200
300
400
500
600

Fig. 2c

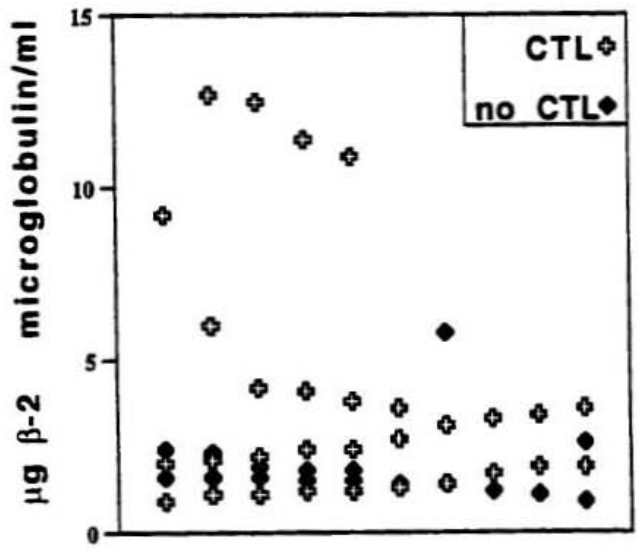
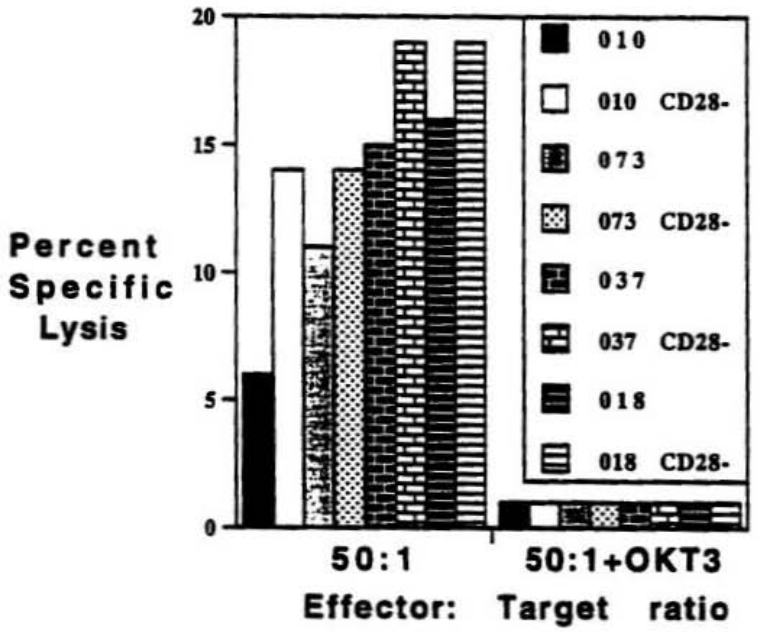


Fig. 3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10
5
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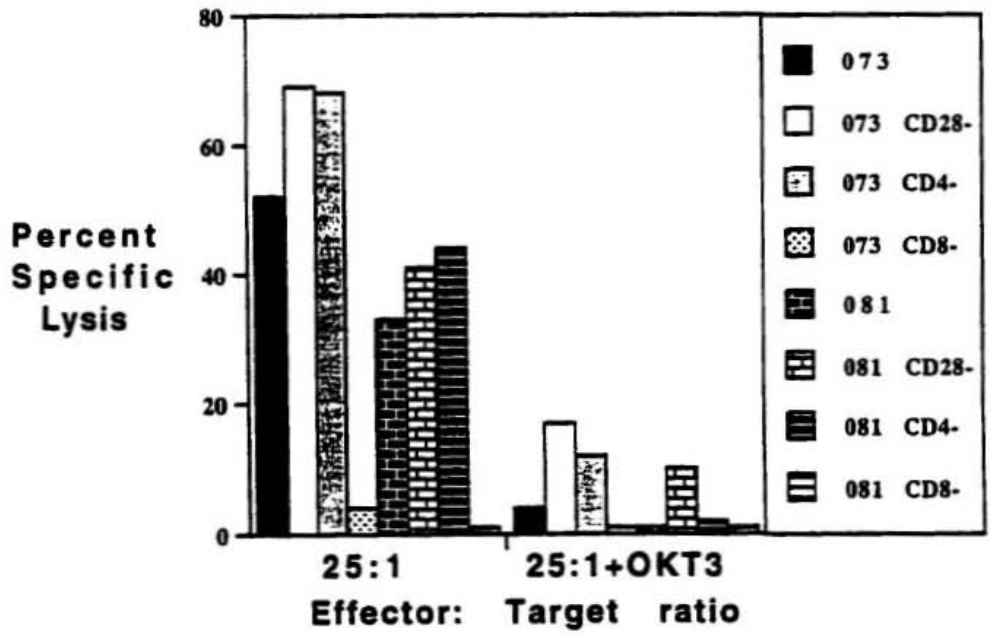
50:1+OKT3
Effector: Target ratio

Fig 4.a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40
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073 CD28-
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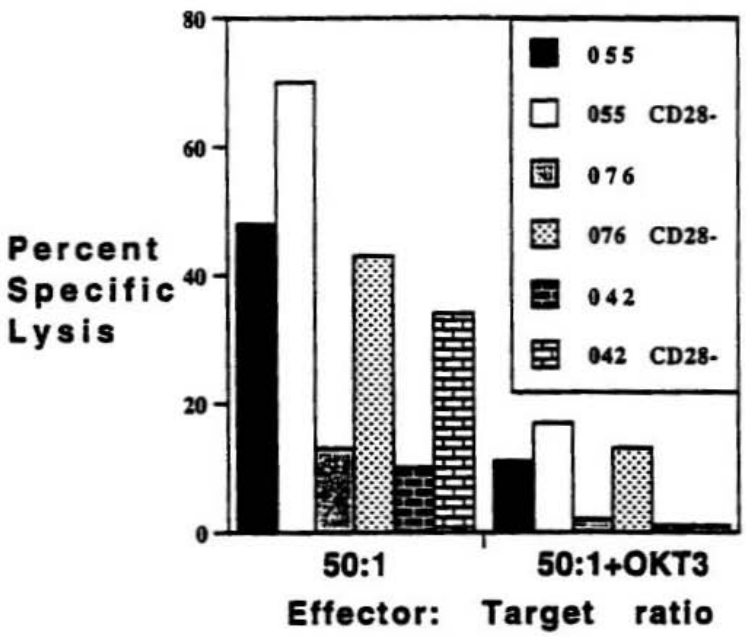
25:1+OKT3
Effector: Target ratio

Fig. 4b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60
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20
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055
055 CD28-
076
076 CD28-
042
042 CD28-
50:1
50:1+OKT3
Effector: Target ratio

Table 1. Different mean levels of parameters associated with disease progression in groups of HIV-infected individuals with or without circulating autoreactive CTL

	$CD4^+$ T cells/μl blood±sem	$CD8^+$ T cells/μl blood±sem	$CD28^+CD8^-$ T $CD8^-$ T	β-2 μglobulin μg/ml plasma	HIV RNA copies/ml plasma (log_{10})
Group 1					
CTL against uninfected CD4s	264±25 n=37	1121±67 n=37	0.28±.03 n=28	3.98±.65 n=30	4.54±.19 n=25
Group 2					
no CTL against uninfected CD4s	436±57 n=20	803±84 n=20	0.45±.05 n=11	1.91±.27 n=17	3.58±.34 n=14
t test $\mu_1>\mu_2$	p=.002	p=.006	p=.003	p=.025	p=.011

HIV-infected individuals tested for circulating autoreactive CTL activity were grouped according to presence or absence of these CTL over the observation period and mean levels of certain parameters were compared between groups by Student's *t* test.

CHAPTER V

SUMMARY AND FUTURE DIRECTIONS

The purpose of this study was to analyze the functions of CTL in HIV infection. Many investigators have described the cellular immunological signs of HIV infection as a decreased CD4/CD8 ratio in the peripheral blood, a progressive quantitative and qualitative reduction of $CD4^+$ lymphocytes, and also an elevated level of $CD8^+$ lymphocytes (Pantaleo et al. 1990). This expansion of $CD8^+$ T lymphocytes persists until the very late stages of the disease, when their depletion allows uncontrolled replication of HIV as well as overwhelming infections with opportunistic pathogens (Pantaleo et al. 1990). Although attempts were made to study the functional significance of $CD8^+$ T lymphocytes in HIV-1 infection (Grant et al. 1992), many aspects of CTL function like the mechanism of cytotoxicity, changes in the overall specificity of cytotoxicity and relationship with established prognostic markers like plasma viral load were not clearly demonstrated. Determining the role of $CD8^+$ T cells in HIV-1 infection and AIDS, with regard to control of infection, immunopathology and immune system dysregulation, is absolutely necessary for understanding the immunopathogenesis of AIDS.

Many investigators agree that apoptosis plays an important role in the pathogenesis of HIV infection. HIV-infected chimpanzees and African green monkeys infected with the simian immunodeficiency virus (SIVagm) do not develop disease and do not show abnormal levels of $CD4^+$ T-cell apoptosis. However, rhesus macaques infected with a pathogenic strain of SIV develop simian AIDS and show an increase in the number of $CD4^+$ T-cells that undergo apoptosis (Estaquier et al. 1994). Interestingly, the number of apoptotic $CD4^+$ T cells found in the peripheral blood lymphocytes

is greater than the number of infected cells, indicating that uninfected cells are dying by apoptosis (Carbonari et al. 1995). One major pathway of lymphocyte apoptosis is mediated through the tumour necrosis receptor family, particularly Fas. Ligation of Fas by Fas ligand present on the same or a neighbouring cell can induce apoptosis (Anderson et al. 1993) and it has been shown that in HIV-seropositive individuals, a higher proportion of PBLs express Fas (Katsikis et al. 1995) and also more Fas is expressed per cell (Gougeon et al. 1996). More significantly, both the $CD4^+$ and $CD8^+$ T-cell subpopulations from HIV-infected persons have been shown to be more susceptible to death induced by Fas ligation (Silvestris et al. 1996). Since most of these studies did not investigate *vis a vis* FasL and perforin-mediated cytolytic properties of $CD8^+$ T cells, we decided to develop an assay system to clearly and easily distinguish between Fas-mediated and perforin-mediated killing. The assay system described in chapter 2, uses no metabolic inhibitors, is simple and is less time consuming than many other assays, which rely on biochemical and molecular features of cell death. In this assay, we use an anti-Fas antibody, Jo-2 to block Fas-mediated cytolysis of P815 cells rendered sensitive to CTL redirected lysis. The results obtained clearly demonstrate we can distinguish between both types of CTL cytotoxicity using a five-hour chromium release assay.

During this study we have tried to analyze the functional significance of $CD8^+$ CTL from HIV-infected subjects. The major aspect of this study includes the nature of CTL cytotoxicity, the phenotypic characteristics of CTL, and the ability of CTL to lyse PHA-activated uninfected lymphocytes. When we used PMA and ionomycin to induce expression of Fas ligand on peripheral blood lymphocytes, we observed that the lysis of Fas-expressing target cell line, P815, was significantly reduced. We followed up on this observation by incubating ^{51}Cr-labelled fresh PBL from HIV-infected individuals in the presence or absence of PMA and ionomycin for five hours. The results described in chapter 3 demonstrate that fresh PBL from HIV-seropositive individuals are highly susceptible to

activation-induced cell death. The nature of this cell death was further investigated and the results showed that this cell death involved the loss of cell membrane integrity without prior or coincident nuclear fragmentation. The cells susceptible for activation-induced death were mainly lymphocytes. The results are in agreement with many others who also have suggested that lymphocytes from HIV-infected individuals are highly susceptible to activation-induced cell death. Our results clearly ruled out any involvement of Fas or FasL in the cytolysis. We have found that the AICD was more prominent in HIV-seropositive individuals with low CD4 counts and higher than normal CD8 counts, but the exact role played by the $CD8^+$ T cells in this type of cell death is not known. Our results showed that $CD8^+$ T cells are also susceptible to activation-induced cell death and this might be one underlying mechanism of $CD8^+$ T-cell loss in the late stages of HIV-infection. This possibility is supported by the observation that activation-induced lymphocyte death is less prominent in HIV-seropositive subjects with absolute lymphopenia. An interesting observation during these experiments was the capacity of an anti-CD3 antibody, OKT3 to rescue the lymphocytes from cell death. This was found to be a specific property of OKT3, as similar results were not obtained with other mitogenic anti-CD3 antibodies such as HIT3a and UCHT1. If there is an *in vivo* significance to the activation-induced death triggered by phorbol esters in HIV-infection, OKT3 could potentially be used to rescue the T lymphocytes from AICD and block the rapid depletion of T lymphocytes.

Many investigators have shown that the presence, number and proportion of activated $CD8^+$T lymphocytes in the peripheral blood of HIV-infected individuals correlate with disease progression (Giorgi et al. 1989; Kestens et al. 1992; Levacher et al. 1992). This at least partially reflects an adaptive immune response to increasing HIV replication but may also reflect the active involvement of $CD8^+$ T lymphocytes in the pathogenesis of AIDS. Several scientists initially proposed a role for $CD8^+$ T lymphocytes in the pathogenesis of AIDS based on lymph node histopathology and natural

history of disease in HIV-infected individuals (Ziegler and Stites 1986) and Zinkernagel (1988) also proposed that $CD8^+$ T cells cause AIDS based on similar immunopathology in human hepatitis B, murine lymphocytic choriomeningitis virus infection, and HIV infection. Walker et al. (1987) suggested that $CD8^+$ CTL contribute to $CD4^+$ T cell depletion by killing HIV-infected $CD4^+$ T-cells. However, some $CD8^+$ CTL from HIV-infected subjects kill even uninfected $CD4^+$ T lymphocytes never exposed to infectious HIV or HIV antigens (Zarling et al. 1990; Israel-Biet et al. 1990; Lederman et al. 1988; Moody et al. 1988; Grant et al. 1993; Grant et al. 1994). The characteristics of these CTL imply possible autoimmune depletion of activated $CD4^+$ T-lymphocytes in HIV infection.

In this study we examined the associations between these autoreactive CTL in the peripheral blood of HIV-infected individuals and disease progression. A significant percentage of HIV-seropositive persons (>50%) in our study cohort, showed cytolysis of PHA-activated uninfected lymphocytes. Surprisingly, a high level of killing was often mediated by freshly isolated peripheral blood lymphocytes. Gruters et al.(1991) presented data from six HIV-infected individuals indicating a progressive decrease in $CD28^+$ T lymphocyte subset population during the evolution from seroconversion to AIDS. Brinchmann et al. (1994) reported a functional defect within the $CD8^+$ but not $CD4^+$ T cells from HIV-infected individuals. They also reported that this functional derangement was restricted to the $CD28^-$ $CD8^+$ T cells although it was seen in all $CD8^+$ T cells later in the infection. These observations lead us to investigate the phenotypic characteristics of autoreactive CTL. The results as shown in chapter 3, clearly show that $CD28^-$ $CD8^+$ T cells are responsible for the autoreactivity. We have also confirmed the expansion of $CD28^-$ $CD8^+$ T cells during progression of disease. A high % of $CD28^-$ $CD8^+$ T cells was seen in all HIV-infected individuals with demonstrable levels of circulating CTL.

Immunodominance associated with biased TCR Vβ gene repertoires has been identified among blood and tissue lymphocytes of AIDS patients (Pantaleo et al. 1994; Dwyer et al. 1993) and also with *in vitro* response to HIV components (Kalams et al. 1994). Expansion of an autoreactive $CD28^-$ $CD8^+$ T lymphocyte subset seen in HIV-infected subjects of our study suggested that the T cells may be oligoclonal. TCR Vβ gene expression pattern as shown in chapter 3 indicated that the $CD28^-$ $CD8^+$ T-cells were oligoclonal with respect to their TCR Vβ gene usage. The expansion of an oligoclonal autoreactive T cell subset population strongly suggest that these CTL not only play a role in the *in vivo* destruction of the $CD4^+$ T cells but may actually be responsible for the progression of the disease as manifested by the appearance of opportunistic infections.

Based on our hypothesis that these autoreactive CTL actually contribute to disease progression we tried to correlate the autoreactivity with markers of disease progression such as plasma viral load, $CD4^+$ T-cell count, $CD8^+$ T cells count and total T cell counts. The data shown in chapter 5 show that the autoreactive CTL are associated with those disease progression markers, examined in this study. As a group those HIV-infected individuals with autoreactivity had higher plasma viral load, higher β_2 microglobulin, lower CD4 counts and higher CD8 and total T cell counts. The data is in agreement with the proposed hypothesis that these CTL actually contribute to immunodeficiency and clinical progression to AIDS. If they do not actually contribute they likely reflect a fundamental component of disease progression. Further studies based on longitudinal follow up of these patients may help uncover the functional significance of this T cell subset.

FUTURE DIRECTIONS

It has been shown that there is an immunodominance of T cells with biased TCR Vβ gene usage (Pantaleo et al. 1994, Dwyer et al. 1993) indicating a poor prognosis in HIV infection. It has also been shown by many investigators that there is expansion of $CD28^-$ $CD8^+$ T cells in HIV infection with defective function in terms of IL-2 production and cytotoxicity (Brinchman et al. 1994; Gruters et al. 1991). Some investigators have shown that $CD8^+$ CTL from HIV-infected individuals can lyse activated uninfected $CD4^+$ T cells (Israel-Biet et al. 1990; Zarling et al. 1990; Grant et al. 1993; Grant et al. 1994; Lederman et al. 1988). Here we have demonstrated that there is an autoreactive $CD28^-$ $CD8^+$ T cell subset associated with relevant markers of disease progression in HIV infection. This data is highly significant in the present context as most of the therapeutic measures employed target only the virus. The host factors responsible for disease progression in HIV infection have always been obscure and complicated (Fauci 1996) and the role of $CD8^+$ CTL in HIV infection is possibly the most obscure of all immunologic features of HIV infection. The possibility of whether there could be CTL populations which function as suppressors of HIV replication in a lytic fashion or in a non lytic fashion as well as some CTL which possess autoreactivity (Grant et al. 1994) were never properly studied. This study focuses on the possible pathogenic role for CTL in HIV infection. The current data indicate that the $CD28^-$ $CD8^+$ T cell subset is autoreactive and may contribute to disease progression. Further investigations into the nature of this subset are necessary to fully understand the immunopathogenic role of CTL in HIV infection. It will be interesting to further investigate the functional characteristics of $CD28^-$ $CD8^+$ T cells. One study has reported that the $CD28^-$ $CD8^+$ T cells from HIV-infected individuals have shortened telomeres than the $CD28^+$ T cells of age-matched controls (Effros et al. 1996). Shortened telomere length may indicate replicative exhaustion of $CD8^+$ T cells. It is certainly

worthwhile to see whether the autoreactive CTLs from our cohort show signs of senescence. Telomeres can be amplified using a polymerase chain reaction and compared with either $CD28^-$ T cells from HIV-infected individuals or with T cells from uninfected volunteers. Lymphocyte senescence has been proposed as a mechanism for unresponsiveness of T cell subsets in HIV infection (Effros et al. 1996).

Many investigators have reported expansion of $CD28^-$ $CD8^+$ T cells in HIV-infection (Brinchmann et al. 1994; Caruso et al. 1994). Roos et al (1996) have showed that the expansion of $CD28^-$ $CD8^+$ T cells exists in HIV-1 seronegative homosexual control individuals and remained at comparable levels in HIV-1 infected asymptomatic individuals and patients with AIDS. They have proposed an explanation that the immune system of individuals at high risk for HIV-1 infection is continuously stimulated as a consequence of frequent viral infections. This hypothesis can be tested by investigating the proportion of $CD28^-$ T cells in homosexual individuals who are not at high risk for HIV-1 infection. Several other investigators have demonstrated that $CD3^+$ $CD28^-$ T cells accumulate in HIV-infection but are actually unresponsive to anti-CD3 mAb, mitogens, CD28 mAb and staphylococcal superantigens (Borthwick et al. 1994; Vingerhoets et al. 1995; Brinchmann et al. 1994; Azuma et al. 1993). It has been suggested by Azuma et al. (1993) that $CD28^-$ $CD3^+$ T cells are generated as a result of an immunological event in the periphery. Subsequently, $CD28^-$ T cells were supposed to be a population of activated terminally differentiated effector cells that are cytotoxic only in short-term cultures (Borthwick et al 1994; Azuma et al. 1993).

Given the fact that there is actually an expansion of $CD28^-$ T cells in HIV infection, the source of these cells remain a mystery. Kotler et al. (1984) reported that individuals infected with HIV showed characteristic histological features in the intestine. The jejunal biopsy in all HIV seropositive homosexual men showed villous atrophy, crypt hyperplasia and an increased number of intraepithelial lymphocytes (Kotler et al. 1984). Intraepithelial lymphocytes (IEL) are almost entirely T cells and they

differ from peripheral blood lymphocytes by their high proportion (80%) of CD8-postive T cells and lacked CD28 (Lefrancois 1991). The principal *in vitro* functions of IELs appear to be cytolysis and interferon-γ production (Sydora et al. 1993; Mosley et al. 1991; Camerini et al. 1993). In both mice and humans, the TCR repertoire of IELs is oligoclonal, and limited to about 100 clones (Blumberg et al. 1993). IEL oligoclonality in conjunction with the observation that intestinal epithelial cells express nonpolymorphic, nonclassic class I molecules (such as CD1 and thymic leukemia antigen) has led to the hypothesis that these molecules on intestinal epithelial cells can positively select CD8-positive IELs (Balk et al. 1991). The features of IEL are remniscent of the nature of autoreactive CTLs we find in the peripheral blood of HIV-infected individuals. Even though there is no evidence so far that IELs circulate, it is possible that some lymphoid replenishment in HIV infection actually occurs from the gut. In this scenario, the oligoclonal $CD28^{-}$ $CD8^{+}$ T cells from gut reconstitute the continuously disappearing T cells in HIV infection. Being oligoclonal they are unable to respond to a wide range of antigens including HIV. This could explain the slow progression of disease in HIV infection as well as the terminal immunodeficiency state in spite of the absolute increase in $CD8^{+}$ T cells. Schmidt et al. (1996) have reported that $CD28^{-}$ T cells are expanded in rheumatoid arthritis and that they are characterized by autoreactivity. This observation suggests that the expanded autoreactive CTL population seen in HIV infection might be responsible for CD4 loss could also contribute to the development of opportunistic infections.

The fact that these T cells are cytotoxic provokes the question of whether they also possess anti-HIV CTL activity. If they lack anti-HIV activity and at the same time are autoreactive, it could be speculated that they play a major role in the immunopathogenesis of HIV infection. It will be interesting to test different subsets of $CD8^{+}$ T cells from HIV infected individuals for anti-HIV CTL

activity to establish the functional significance of different subsets of T cells in the peripheral blood of HIV-infected subjects.

It has also been argued by some investigators that the expanded $CD28^-$ $CD8^+$ T cells have actually down-regulated their CD28 molecules (Vingerhoets et al. 1995). This argument will certainly have to be addressed before we conclude that the $CD28^-$ autoreactive CTLs are actually gut-derived. Since there are no definite surface markers that discriminate T cells derived from gut from those in the peripheral blood with CD28 down-regulation, we will have to adopt indirect ways to determine the nature of CTLs. Since the gut-derived $CD28^+$ T cells are oligoclonal with respect to their TCR Vβ gene usage, analysis of TCR Vβ gene expression of $CD28^-$ and $CD28^+$ T cells will help to identify whether the cells may be gut-derived. The $CD28^-$ T cells derived from the gut will definitely be oligoclonal whereas the $CD28^+$ T cells will show a polyclonal or unbiased TCR Vβ gene usage. Whereas, T cells having undergone CD28 down-regulation should be no different from their $CD28^+$ T cells with respect to T cell V gene usage. The observations in Chagas disease that Trypanosoma Cruzi probably down-regulates CD28 on the surface of $CD4^+$ T cells as well as $CD8^+$ T cells unravels a different mechanism of host immune evasion by parasites. If the same holds true in HIV infection, this will be a novel mechanism by which the virus could shut down anti-viral responses.

Finally, we have shown a clear association between the presence of autoreactive CTL and many parameters of HIV disease progression, including plasma viral load. This will have to be followed up over the course of disease to further address the role of autoreactive CTLs in the pathogenesis of AIDS. It will also be interesting to look at the influence of anti-viral drugs in controlling or increasing the number and function of autoreactive CTL.

A complete characterization of this subset at the cellular and molecular level is necessary to clearly delineate their functional significance. Correlation of autoreactive CTL activity with disease progression will present a potential immunopathological contribution to the course of disease in HIV-1 infected individuals.

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