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CD8+ T-Lymphocytes in HIV Infection and Resistance

CD8$^+$ Cytotoxic T-Lymphocytes in Hiv-1 Infection and Resistance

By

Dorothee Bienzle, DVM, M.Sc.

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AUTHOR: Dorothee Bienzle, DVM (Guelph), M.Sc. (Guelph)

SUPERVISOR: Dr. Kenneth L. Rosenthal

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ABSTRACT

Cytotoxic T-lymphocytes (CTL) are important in resistance to HIV-1 infection, and modulate disease progression through various effector functions. Conventional CTL recognizing antigenic peptides in the context of major histocompatibility (MHC) class I molecules eliminate virally-infected cells, and have been shown to modulate the course of disease progression. Other functions of $CD8^+$ lymphocytes include non-lytic suppression of viral transcription and replication, and induction of programmed cell death in activated $CD4^+$ cells.

In the work presented here, the interactions of $CD8^+$ lymphocytes with activated $CD4^+$ cells were examined. Lysis of activated $CD4^+$ cells was unique for CTL derived from HIV-1 infected persons, and was not restricted by MHC molecules. The interaction was dependent on target cell activation, correlated with cell proliferation, and was independent of viral replication. Furthermore, the lytic process was biochemically consistent with apoptosis, and resulted in nuclear fragmentation in the target cells.

In vitro susceptibility to infection by primary HIV-1 isolates was assessed in a cohort of couples consisting of an infected and an uninfected, but exposed, partner. These findings were correlated with the genetic integrity of the HIV-1 co-receptors, and with the presence of CTL specifically directed against the partner's primary isolate. Individuals with partial or complete resistance to infection were identified, as well as some individuals

that had CTL recognizing the partner's viral isolate. The latter results suggested that cellular immune responses comprise a major component of resistance to HIV infection, and that they influence resistance even in individuals lacking the main HIV-1 co-receptor.

The later studies were extended by examining the *in vitro* infectivity of $CD4^+$ cells from a group of highly exposed, but persistently uninfected, Kenyan sex workers. No barrier to infection of $CD4^+$ lymphocytes with primary Kenyan HIV isolates was identified, however, $CD8^+$ cells from these subjects were able to efficiently limit viral replication in autologous $CD4^+$ cells.

In summary, the broadly different CTL effector functions described in these studies illustrate the importance of the cellular immune system in resistance to HIV infection, and in modulating the disease pathogenesis.

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PREFACE

This dissertation consists of six chapters. The first chapter, the introduction, provides the background to the experimental work. Chapters two to five are composed of individual manuscripts. The first of these has been published, chapter five is in the process of being submitted for publication, and chapters three and four are currently being prepared for publication. Preceeding each chapter is a brief description of the purpose and the implications of the work. The author of this dissertation has performed all the experimental work described in the chapters except where otherwise indicated. The final chapter of this thesis, the summary, relates the significance of the findings of this research to the overall context of the experimental area.

References cited in the introductory and final chapters are listed at the end of this dissertation.

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Cytotoxic T-Lymphocytes

1. General Background

The cellular immune system consists of two broad categories of effector T cell populations: helper T-lymphocytes and cytotoxic T-lymphocytes (CTL). Helper T cells are generally characterized by expression of the CD4 molecule which interacts with major histocompatability (MHC) class II antigens on antigen-presenting cells (APC). The functional importance of $CD4^+$ cells following antigen recognition resides in providing appropriate help through the production of cytokines for antibody production by B-lymphocytes, for the differentiation and maturation of CTL, as well as for activation of macrophages. CTL are generally distinguished by expression of the CD8 surface molecule, and they interact with MHC class I molecules presenting foreign peptides. The functional characteristics of these cell populations are not exclusive as a proportion of cells expressing the CD4 molecule are able to specifically lyse target cells (Kaplan *et al.*, 1984), however, interaction of MHC class I with $CD8^+$ cells, and MHC class II with $CD4^+$ cells is strictly adhered to.

Effector T cells have evolved to counter antigens in two fundamentally different processes: $CD8^+$ T cells largely monitor the intracellular environment through their ability to recognize cytosolic proteins that have been degraded in the proteasome, have associated with MHC class I molecules in the endoplasmic reticulum, and are subsequently

expressed on the cell surface (Neefjes and Momburg, 1993). This process proceeds in an orderly sequence where proteins that have gained access to the cytoplasm of a cell, such as viruses or intracellular parasites, are degraded by the large ATP-dependent proteasome complex, and the resultant peptides are translocated to the endoplasmic reticulum (ER) by the membrane-spanning heterodimer transporter associated with antigen processing (TAP). Newly synthesized MHC class I molecules, consisting of a heavy chain and β_2 microglobulin, associate with peptides in the ER, and this trimer is transported through the Golgi apparatus to the cell surface (Yewdell and Bennink, 1993). Recently, TAP has been shown to directly associate with MHC class I molecules as well, presumably aiding with the specific association of peptide and MHC in an ER environment with degradative enzymes and competing proteins (Suh *et al.*, 1994; Ortmann *et al.*, 1994). The synthesis and appropriate folding of MHC intermediates is protected by calnexin, a chaperone specifically bound by ER membranes (Jackson *et al.*, 1994). As a result, peptides of a specific length, preferably consisting of nine amino acids, can be displayed in the groove of MHC class I molecules present on all nucleated cells except neurons, and may be recognized by $CD8^+$ CTL.

In contrast, $CD4^+$ T cells orchestrate the response to extracellular pathogens by responding to peptides presented in the context of MHC class II molecules. Such peptides are derived from extracellular proteins endocytosed and degraded in lysosomal compartments of antigen-presenting cells such as macrophages. MHC class II molecules are assembled in the ER from an α and a β chain, and associate with an invariant chain. The latter component appears to protect the newly synthesized MHC II molecules from

prematurely binding peptide (Neefjes and Momburg, 1993). MHC II molecules associated with the invariant chain are transported from the ER to the Golgi as a nine subunit complex, and, following degradation of the invariant chain, bind processed peptide in an as yet poorly defined late endocytic compartment. Class II proteins can bind peptides in excess of nine amino acids (Stern, *et al*, 1994). Thus, soluble proteins bound to surface immunoglobulin (Ig) on B cells, or bound as antigen-antibody complexes to Fc receptors, or endocytosed as a fluid phase component, will be presented to $CD4^+$ cells by MHC II proteins, which in turn respond by producing B and T cell stimulatory cytokines.

These functional divisions within the immune system conceptually limit a CTL response to extralymphoid tissue as professional APC's are considered not to present antigens through the cytosolic pathway in the context of MHC I molecules. However, since naive T cells recirculate only within lymphoid tissue, and adequate co-stimulation is not provided by extralymphoid tissue, a pathway for cytosolic processing of antigens by APC has been considered essential (Bevan, 1995). Indeed, Kovacsovics and Rock (1995) demonstrated the transfer of particulate ovalbumin antigens from phagolysosomes to cytosol, and subsequent presentation by class I proteins. A second route of proteasome-processing of extracellular proteins involving a heat-shock protein (HSP) chaperone has been shown by purification of HSP gp96 with bound peptide from cells which in turn was sufficient to prime CTL (Arnold *et al.*, 1995). These findings suggested that HSP's released from dying infected cells, or from tumor cells, may enter the cytosol of APC's and subsequently elicit a primary CTL response. Though the exact manner in which an *in vivo* primary CTL response with appropriate co-stimulation and stimulatory cytokines

occurs is presently not firmly established, involvement of APC's in lymphoid tissue is likely to be a prerequisite component. Subsequent wide extravascular circulation of memory CTL, and destruction of cells displaying foreign peptides bound by MHC class I molecules, constitute the more commonly observed effector functions of CTL.

A further group of CTL restricted to non-MHC molecules termed CD1 have been described (Strynowski and Lindahl, 1994). CD1 molecules have limited homology to MHC class I proteins, are non-polymorphic, and associate with β_2-microglobulin. They function uniquely in presenting lipid and glycolipid antigens to CTL expressing $\alpha\beta$ or $\gamma\delta$ T cell receptors (TCR), and either lacking the CD4 and CD8 surface molecules, or expressing CD8 (Beckmann *et al.*, 1994). Crystallographic analysis has confirmed this functional preference since the region corresponding to the peptide binding groove of MHC molecules is lined by non-polar amino acid side chains suited for binding hydrophobic ligands (Zeng *et al.*, 1997).

2. Mechanisms of Lysis

Until recently, CTL were presumed to lyse their target cells exclusively following recognition of foreign peptide bound by MHC through a process involving exocytosis of preformed mediators (Atkinson and Bleackley, 1995). In this model, initial conjugate formation involves TCR-MHC-peptide interactions, as well as contributions from accessory molecules. Specifically, TCR stimulation by antigen engagement, or through antibody binding, activated binding of the CD8α chain to a conserved, membrane proximal domain of the class I heavy chain (O'Rourke *et al.*, 1990). Subsequent initiation

of polyphosphoinositide hydrolysis suggested that this interaction was important for T cell responses at low TCR occupancy levels (O'Rourke and Mescher, 1992). An avidity-based contribution from CD8 to the binding of MHC-peptide with the TCR has as well been suggested from recent crystallographic analysis of the interaction of the human CD8αα molecule with the leukocyte antigen (HLA)-A2 with a bound HIV-gag peptide (Gao *et al.*, 1997). In contrast, adhesion of intercellular adhesion molecule (ICAM)-1 to lymphocyte function-associated (LFA)-1 did not result in signalling, but promoted TCR-CD8-MHC I adhesion (Ybarrondo *et al.*, 1994). Interactions between CD28 and B7 in conjunction with TCR stimulation dramatically augmented T cell proliferation, and were considered essential for interleukin (IL)-2 production and induction of cytolytic function in $CD8^+$ cells (Guerder *et al.*, 1995). Thus, accessory molecules contribute to the response elicited from CTL through augmenting conjugate formation and signalling.

Subsequent to conjugate formation, the cellular organelles in CTL become polarized toward the target cell, and components of the cytoplasmic granules are released into the intercellular space (Berke, 1994). The granules contain the pore-forming protein perforin, serine proteases with esterase activity, fragmentins, and proteoglycans such as chondroitin sulfate (Berke, 1994). Perforin monomers are thought to bind and insert into target cell membranes, and to aggregate into a pore-like structure resulting in osmotic lysis of the target cell in a manner analogous to damage induced by the terminal complement complex (Liu *et al.*, 1995). The lytic process mediated by perforin is dependent on the presence of calcium ions (Ca^{++}). The basic concepts of this model of cytotoxicity have been confirmed by biochemical and electronmicroscopic techniques, however, during the

past years it became evident that lysis of target cells by CTL may be more complex than simply induction of pores in target cells. Not all cells were equally susceptible to osmotic lysis, and in some instances nuclear damage was the predominant feature in target cells, and occurred prior to membrane disintegration. Therefore, a hypothesis of perforin being the main mediator of target cell lysis, but acting to deliver other components of CTL granules into cells, has emerged (Liu *et al.*, 1995). *In vitro* observations of the interaction of CTL with target cells demonstrated the presence of a membrane pore, and subsequent nuclear changes consisting of chromatin condensation, membrane blebbing, and ultimately DNA fragmentation (Berke, 1995). Transfection experiments indicated that perforin alone imparted only membranolytic ability, while co-transfection of perforin and granzyme B resulted in cytotoxicity and nuclear disintegration (Nakajima *et al.*, 1995; Nakajima and Henkart, 1994). Granzyme B has a unique proteolytic substrate specificity for cleavage after aspartate, a property shared only with a group of cysteine proteases (caspases) involved in mediating programmed cell death (Smyth and Trapani, 1995). The prototype of the latter proteases is the interleukin-1β converting enzyme (ICE), and enzymatic similarity between granzyme B and ICE suggested that this may be a convergent pathway for CTL-induced apoptosis and other stimuli of programmed cell death (Henkart, 1996). Indeed, granzyme B can cleave FLICE, a precursor caspase to ICE, thus linking two different mechanisms for cell death (Muzio *et al.*, 1996). In turn, granzyme B and FLICE cleave another cysteine protease termed CPP-32, which proceeds to cleave the DNA repair enzyme poly(ADP-ribose) polymerase (PARP) (Darmon *et al.*, 1995). PARP activation is one of the earliest changes detected after delivery of apoptotic signals, and is thought to result from altered endonuclease regulation. Though several key components in

the process of cytolysis through preformed mediators are yet to be discovered, the evolving model suggests that a cascade of events takes place: specific recognition via TCR-MHC-peptide interaction is followed by extrusion of granule contents from the CTL, and by pore formation in target cell membranes. Next, intracellular delivery of proteolytic substances induces apoptotic changes in the target cells through the sequential activation of proteases, and eventually cell death occurs due to a combination of osmotic damage and nuclear disintegration.

The observation that in some circumstances cytolysis with nuclear damage can occur in the absence of Ca^{++}, and that cells devoid of perforin (Walsh *et al.*, 1994) can mediate DNA fragmentation, led to the discovery that interaction of another set of surface receptors can allow induction of death by CTL through apoptosis. Apoptosis is a tightly regulated process of programmed cell death that can be induced by deprivation of growth factors, DNA damaging compounds such as radiation, heat, oxidative stress, glucocorticoids, and by cross linking of specific cell surface receptors (Vaux and Strasser, 1996). The Fas receptor (CD95, APO) and the tumor necrosis factor (TNF) receptor 1 (CD120a) are the main molecules able to transmit a death signal to the cell upon binding their ligands, FasL and TNF, respectively. These receptors have a unique intracellular motif, termed the death domain, that is shared by the cytoplasmic adapter proteins FADD/MORT1, TRADD, and RIP (Rowan and Fisher, 1997). Though the death domain in FADD/MORT1 is required for interacting with Fas, another domain called the "death effector domain" is responsible for signalling cell death (Rowan and Fisher, 1997). Following signal transduction from Fas to FADD/MORT1, this cytoplasmic protein binds

an ICE-like enzyme called MACH/FLICE (Dixit and Krammer, 1996), which in turn binds to another protein most recently identified and termed FLIP/Casper (Irmler *et al.*, 1997; Shu *et al.*, 1997). FLIP/Casper occurs in two splice variants with opposing effects in some cell lines, leading one group to suggest that it acts predominantly as a death inhibitor (Irmler *et al.*, 1997), while the other discovering researchers consider the protein to be a death inducer (Shu *et al.*, 1997). This protein is one of the key molecules in receptor-mediated apoptosis since it intersects the Fas and TNF receptor 1 pathways, and interacts with many of the molecules known to influence death-promoting or death-inhibiting signals. Thus, the signal transduced from activation of Fas may be the most direct path to induction of cell death via a cascade of caspases, but it is subject to many modulating factors. A soluble form of FasL has been detected, and may act in the same manner as the cell-bound molecule (Dhein *et al.*, 1995). Activation of the TNF receptor 1 results in binding of TRADD, and appears to involve FADD/MORT1 as well as MACH/FLICE, and probably several other yet to be identified intermediaries (Cleveland and Ihle, 1995). Both surface receptors are able to bind RIP, however, the downstream molecules interacting with RIP are unknown (Rowan and Fisher, 1997).

Further complicating the elucidation of these pathways is that the TNF receptor 2, like the TNF receptor 1, can activate the nuclear factor κB (NF-κB) via proteins called TRAF1 and TRAF2, which in turn are able to bind to TRADD, thus linking the transcription factor family NF-κB with apoptotic pathways (Rothe, 1994). In turn, inhibitors of apoptosis (IAP) are cytoplasmic proteins interacting with TRAF1 and TRAF2, which may be displaced by FLIP/Casper, and thus may serve as a switch

channeling the TNF receptor signal into either the death pathway, or into a death-resistance mode by activating NF-κB (Wallach, 1997). Though in this brief review only pathways commonly utilized by CTL have been summarized, many other stimuli may induce apoptosis, and numerous other inhibitors and modulators, such as members of the *bcl-2* family and tumor suppressor genes, have been described. The general concept emerging from this rapidly evolving and complex picture is that induction of programmed cell death in a cell is a highly evolved biological process characterized by many modulating factors, and subject to much intercellular variation. However, CTL expressing FasL generally induce death in target cells expressing Fas, and the common effector molecules are cysteine proteases that can be activated by granzyme B or by FLICE/MACH.

The specificity of CTL expressing FasL for cells expressing Fas remains poorly defined. Conceivably, activated CTL armed with FasL, could induce massive cell death on all cells constitutively expressing Fas, as shown for hepatitis B infection (Galle *et al.*, 1995). Similarly, injection of Fas antibodies into mice resulted in widespread hepatocyte death (Wang *et al.*, 1994), begging the question why generation of CTL expressing FasL during an immune response does not result in similar pathology? It appears as if the nature of the signal transmitted from the TCR, and subsequent cytokine secretion, influence the functional ability of CTL to employ the FasL pathway, and that mere expression of FasL is insufficient to lyse every cell with the Fas molecule on the surface. When an MHC-restricted, cytolytic clone was compared to a non-cytolytic variant clone it was noted that TCR signals associated with intracellular Ca^{++} mobilization resulted in perforin exocytosis, while a lack of Ca^{++} increase preserved FasL cytotoxicity, but was associated with

dependence on exogenous IL-2 (Esser *et al.*, 1996). These results were extended by Sad *et al.* who showed that perforin and Fas killing reduced cytokine synthesis by CTL differentially, implying that an initial immune response required predominantly stimulatory cytokines, while induction of cytolytic function proceeded from Fas, with intermediate reliance on IL-2, to perforin-killing that was largely independent of IL-2 (Sad *et al.*, 1996). It has been reported that the FasL, which is expressed exclusively on activated T cells, and constitutively on a few "immune-privileged" tissues, is transcribed in response to specific recognition of MHC with peptide by the TCR, implying MHC restriction for this pathway (Nagata and Golstein, 1995). However, others have reported that FasL-Fas killing is independent of MHC restriction, and that functional FasL-expressing CTL can be induced by non-specific stimulation of the TCR (Ramsell *et al.*, 1994). Presently, the requirement for recognition of self-MHC in this type of killing is unclear. Albeit, consideration that FasL-Fas interactions can mediate apoptosis of infiltrating lymphocytes in the testis and the eye, as well as induce graft-versus-host disease (Abbas, 1996), would suggest that absolute MHC-restriction of this pathway does not occur.

3. Functional Importance

The functional importance of the Fas and FasL molecules is best exemplified by the *gld* (generalized lymphoproliferative disease) and *lpr* (lymphoproliferation) spontaneous mutations identified in mice: *gld* mice have a point mutation near the COOH-terminus of the coding region of FasL which abolishes binding to Fas (Takahashi *et al*, 1994), while insertion of a retrotransposon into the Fas gene causes premature transcriptional termination in *lpr* mice (Watanabe-Fukunaga *et al.*, 1992). Phenotypic alterations in these

mice are influenced by their genetic background, however, all mice with either mutation develop massive lymphadenopathy and splenomegaly with accumulation of $CD4^-/CD8^-$ T-lymphocytes, and are prone to develop autoantibodies (Nagata and Golstein, 1995). These findings strongly suggest that FasL-Fas interactions are essential in regulating the expansion and reduction in lymphocyte numbers associated with an immune response, while thymic development appears normal in the absence of these molecules (Crispe, 1994). Fas is expressed on $CD45RO^+$ T cells (considered to be previously activated), and very little on $CD45RA^+$ cells (naive cells), consistent with up-regulation of expression during antigen encounter (Alderson *et al.*, 1993). In fact, the Fas molecule has co-stimulatory functions in resting T cells, but stimulation of previously activated T cells through the TCR transmits an apoptotic signal. This latter phenomenon has been termed activation-induced cell death (AICD), and is thought to be important for limiting the clonal expansion of antigen-specific T cells, as well as for eliminating autoreactive cells (Brossart and Bevan, 1996). Thus, findings to date strongly implicate Fas-FasL pathways in regulating mature T cell quantity and function. Observations in humans have established that soluble forms of FasL were detectable only in persons with lymphoproliferative disorders involving large granular lymphocytes or natural killer (NK) cells, and this correlated with tissue damage in organs constitutively expressing Fas (Tanaka *et al.*, 1996). Furthermore, a deletion in the intracytoplasmic domain of Fas has been associated with a syndrome reminiscent of phenotypic alterations in *lpr* mice in three related patients who exhibited autoimmune hematologic disease and accumulation of double negative T cells (Rieux-Laucat *et al.*, 1995). In another study, two patients with hypereosinophilia and a moderate proportion of circulating $CD4^-/CD8^-$ T cells were found to either express a

soluble form of Fas thought to antagonize FasL signalling, or to completely lack Fas gene transcription (Simon *et al.*, 1996). Increased levels of soluble Fas have been detected in a number of patients with severe systemic lupus erythematosus (Cheng *et al.*, 1994). Although the later reports are largely of an observational nature, and on a limited number of patients, they strongly suggest that Fas and FasL interactions influence mature T cell dynamics in humans analogously to the situation in mice.

Substantial progress in understanding the roles of perforin and Fas in mediating CTL function came from the generation of perforin knock-out mice. These mice were phenotypically normal, had no reduction in $CD8^+$ or NK cells, but had impaired antiviral and allogeneic cytotoxic activity against fibroblast target cells (Kägi *et al.*, 1994). Null mutants for perforin lacked the cytopathology associated with lymphocytic choriomeningitis virus (LCMV) infection, and had reduced ability to control syngeneic tumor growth (Kägi *et al.*, 1994). However, CTL from the mice were able to lyse hematopoietic cells, and had lytic activity against certain tumor cells (Kägi *et al.*, 1994a). These findings were extended by Lowin *et al.* (1994) and Kägi *et al.* (1994b) who found that Fas-mediated killing accounted for approximately one third of CTL activity against a variety of allogeneic target cells, and that perforin was the predominant effector molecule in anti-viral activity, respectively. Closer examination of chemically or virally-induced carcinogenesis, or injection of tumor cells into perforin null mutant mice, attributed the major role to perforin, and only a minor role to Fas-linked cytotoxicity in tumor surveillance (van den Broek *et al.*, 1996). An interesting differential effect of these two molecules regarding bacterial killing was recently shown: $CD4^-CD8^-$ T cells lysed

Mycobacterium tuberculosis-infected macrophages by Fas-FasL interactions, but did not reduce bacterial viability, while $CD8^+$ T cells killed the macrophages and bacteria in a granule-dependent manner (Stenger *et al.*, 1997). Though Fas and perforin are thought to account for all CTL-mediated death, TNFα was shown to have a role in induction of apoptosis of mature $CD8^+$ T cells (Zheng *et al.*, 1995). In conclusion, the model that has emerged from functional studies utilizing naturally occurring Fas or FasL mutant mice, and perforin knock-out mice, attributes all cellular cytotoxicity directed against antigens recogized by TCR engagement to these two pathways. In addition, some Fas-FasL cytotoxicity can occur non-specifically, devoid of TCR-mediated recognition. Cytotoxicity directed against hematopoietic and lymphoid cells preferentially engages the Fas-FasL pathway, while autocrine or paracrine cell death of mature $CD8^+$ T cells is mediated by TNFα binding to the p55 TNFR, and by Fas in mature $CD4^+$ T cells. Thus, multiple killing pathways are available to an individual cytotoxic cell (Vergelli *et al.*, 1997). Perforin is preferentially employed in classical defense functions, while Fas appears more important for lymphocyte regulation. The different mechanisms may be sequentially engaged by a single cell exposed to different stimuli, hence illustrating the enormous complexity that has evolved in the immune system (Clement and Stamenkovic, 1994).

The Pathogenesis of HIV Infection

1. Viral Entry into $CD4^+$ Cells

The CD4 molecule is used by HIV-1 and HIV-2 to mediate cell attachment and penetration (Klatzmann *et al.*, 1986). Many reports and reviews have been published on the molecular interactions of the CD4 antigen with gp120 and gp41 from the HIV envelope, thus this topic will only be briefly summarized here. More recent developments regarding the interaction of gp120 and CD4 and chemokine receptors will be discussed in greater detail.

The CD4 cell surface glycoprotein belongs to the immunoglobulin superfamily, binds MHC class II molecules with low affinity, and transmits intracellular signals in T cells by associating with the tyrosine kinase $p56^{lck}$ (Janeway, 1989). The immunoglobulin-like domain proximal to the amino terminus, corresponding to the complementarity-determining region (CDR)2, binds gp120 with high affinity, though depending on whether soluble or cell-bound molecules were used, antibody interference with other regions of the CD4 molecule blocked binding as well (Moore *et al.*, 1992). gp120 is a heavily glycosylated protein consisting of five hypervariable (V) regions interspersed with five conserved regions (Willey *et al.*, 1986). Several regions are involved in binding to CD4, however, the V3 loop, though not actually interacting with CD4, is largely responsible for viral tropism and is a main neutralizing epitope (Otteken *et al.*, 1996). Binding of gp120 to CD4 results in progressive conformational changes bringing the viral transmembrane protein gp41 in closer proximity with the CD4 molecule and the cell membrane, and exposing the V3 loop of gp120 (Geleziunas *et al.*, 1994). Dissociation of gp120 from gp41 is thought to be required for actual fusion of the viral membrane with the host cell, and for subsequent viral entry (Eiden and Lifson, 1992). The greater noncovalent

association between gp120 and gp41 is one of the factors thought to be responsible for the decreased susceptibility to neutralization observed in primary HIV strains as compared to T-cell line adapted (TCLA) strains. In addition, greater dependence on cell surface concentrations of CD4 molecules, and subsequent decreased sensitivity to anti-CD4 antibody neutralization, was noted to differentiate primary from TCLA strains (Kabat *et al.*, 1994).

Substantial progress in understanding the biology of HIV was heralded by the landmark discovery that chemokine receptors serve as co-receptors for HIV-1 (Dragic *et al.*, 1996; Deng *et al.*, 1996; Feng *et al.*, 1996). $CD8^+$ cell-derived factors had been known to suppress viral replication in a non-cytolytic manner for many years (Walker *et al.*, 1986), however, only recently were the β-chemokines macrophage inflammatory protein (MIP)-1α, MIP-1β, and RANTES (regulated on activation, normal T cell expressed and secreted) identified as the major factors inhibiting infection by macrophage-tropic HIV-1 strains (Cocchi *et al.*, 1995). Natural infection with HIV-1 is generally due to isolates that preferentially infect macrophages, and that do not induce syncytium formation (NSI) when assayed on susceptible cell lines. Viruses with this phenotype predominate during most of the disease course, only during the terminal phase of AIDS is a switch to syncytium-inducing isolates noted that corresponds to a preferential infection of T cells (Conner *et al.*, 1993). Viral tropism is determined by a very limited number of amino acids in the V3 loop of gp120, and even a single amino acid switch may induce altered infectivity properties (Dittmar *et al.*, 1997). Once β-chemokines had been identified as inhibitors of infection with NSI isolates, discovery of co-receptor usage for

different phenotypic viral variants proceeded rapidly: NSI isolates were found to rely on the CC-chemokine receptor (CCR) 5, and in some instances on CCR3 or CCR2b (Alkathib *et al.*, 1996; Choe *et al.*, 1996; Doranz *et al.*, 1996), while SI isolates achieved cell entry by binding to the CXC-chemokine receptor (CXCR)4, also termed fusin or LESTR (Feng *et al.*, 1996). Although these phenotypic characteristics correlated well with the binding assays employed to determine co-receptor use, it is presently unclear why monocytes/macrophages, that express CCR5 and CXCR4, are only infected by NSI isolates. Investigations of primary SI viral strains have indicated that most are able to use CCR5 if presented on non-human cells in conjunction with CD4, suggesting that primary SI isolates are different from laboratory-propagated TCLA strains (Simmons *et al.*, 1996). In contrast, primary viruses with syncytial characteristics were not sensitive to inhibition by β-chemokines, suggesting they are fully able to substitute CXCR4 for CCR5 in the presence of competing ligands (Jansson *et al.* 1996). Thus, the current consensus is that primary NSI isolates rely exclusively on CCR5 or CCR3 or CCR2b for viral entry, while primary SI isolates may use CCR5 or CXCR4, and TCLA strains have lost the ability to bind to CCR5 in favor of use of CXCR4 (Moore, 1996). Study of viruses with a predilection for infection of nervous tissue, and causing dementia in patients, where found to use CCR3 to a greater extent than CCR5 (He *et al.*, 1997). In concordance, microglia were the only cells except eosinophils to express CCR3 (He *et al.*, 1997). Interestingly, the phenotypic division of co-receptor use is dominant over genetic subtype, as HIV-1 subtypes A, B, C, D, E and O all rely on CCR5 and /or CXCR4 according to syncytium-forming ability (Zhang *et al.*, 1996). The most recent reports on discovery of additional chemokine receptors able to mediate fusion to cells expressing gp120 predict that the

current picture of entry of HIV-1 into $CD4^+$ cells is likely to get more complex (Liao *et al.*, 1997; Deng *et al.*, 1997).

Subsequent to the discovery of chemokine receptor function in viral entry much effort has been spent on delineating the molecular interactions of the CD4, CCR5/CXCR4, and gp120 oligomeric complex. Chimeric constructs consisting of SI envelope genes with specific small insertions from NSI strains indicated that within gp120 the V3 loop confers susceptibility to chemokine inhibition, implying that this part of the HIV envelope competes for binding to the β-chemokine receptor (Cocchi *et al.*, 1996). On the receptor side, extensive domain exchanges between CCR5 and CCR2b, and construction of mutant receptors, did not reveal a specific site responsible for binding to gp120 (Rucker *et al.*, 1996). Instead, no single extracellular domain of CCR5 was indispensable when replaced with a homologous domain from CCR2b, and the amino terminal as well as the first extracellular loop were important, but not absolutely necessary for fusion (Rucker *et al.*, 1996). Similar results were obtained with mouse/human CCR5 chimeric constructs: replacing any extracellular domain of human CCR5 with the corresponding region from the mouse receptor was still permissive for infection, and conversely, inserting human regions into the mouse extracellular CCR5 regions did not significantly alter function (Picard *et al.*, 1997). This conformational complexity, and the lack of a single contact site between CCR5 and gp120, was further confirmed by another research group (Atchison *et al.*, 1996). Thus, despite intense investigation, the precise interaction of the chemokine receptors with their gp120 ligand has not yet been determined. However, results from several studies indicate that coreceptor activity of the seven-transmembrane receptors is

dissociable from ligand-dependent signal transduction as measured by calcium mobilization (Cocchi *et al.*, 1997; Atchison *et al.* 1996).

It has been postulated that HIV-1 entry may sequentially involve binding of gp120 to CD4, interaction of gp120 with the co-receptor to aid in translocating the fusion peptide from gp41 into proximity with the cell membrane, and subsequent fusion of the viral and cell membranes (Dimitrov, 1996). Conceptually favoring this model is that CD4 is an extended molecule, and viral binding occurs relatively far from the cell membrane, while the chemokine receptors, being seven-transmembrane proteins, have short extracellular portions, and could therefore serve to anchor the viral envelope to the cell membrane. Recruitment of fusin into a CD4-gp120 complex at the cell surface has been demonstrated, and supports the notion that in the absence of gp120 there is little contact between CD4 and fusin (Lapham *et al.*, 1996). Thus, viral entry results from complex and sequential interactions of specific regions within the CD4, gp120/gp41, and chemokine receptor molecules, and probably involves additional cell-specific modulating proteins.

2. Viral Integration, Transcription, and Replication

The stages of the viral life cycle are similar for all retroviruses, and will be outlined briefly here, emphasis will be placed on correlating novel findings in viral behaviour with host pathogenesis. Following membrane fusion, the HIV particle enters the cytoplasm, and is uncoated by host cell proteases to yield a nucleoprotein complex consisting of two single-stranded RNA genomes with packaging proteins. Subsequently, in activated T cells,

reverse transcription and viral integration into the host cell DNA proceed quickly, while in resting T cells both steps proceed only inefficiently (Greene, 1991). The activated viral reverse transcriptase initiates DNA synthesis from a tRNA primer bound near the 5' end of the viral RNA template (Gaynor, 1995). Synthesis of the second DNA strand is also dependent on reverse transcriptase, but proceeds only after partial digestion by ribonuclease H of the RNA component of the RNA/DNA complex. Thus, complete reverse transcription yields a double-stranded DNA copy of the original RNA virus including elongated, tandem long terminal repeats (LTR) at both ends.

Next, nuclear translocation and insertion into the host cell DNA, mediated by the viral integrase enzyme, ensue. The integrated provirus is now subject to complex transcriptional regulation mediated by cellular factors in a manner analogous to that of endogenous cellular genes. Constitutively expressed transcription factors, such as SP-1, and the TATA-binding factors, as well as inducible factors such as members of NF-κB family, and nuclear factor of activated T cells (NFAT), exert positive and negative regulatory influences on gene expression (Feinberg and Greene, 1992). In turn, the cellular activation state, and the cytokine milieu of the cell, further modulate the action of inducible factors (Li *et al.*, 1997). Of special importance for transcriptional control is the presence of a RNA stem-loop structure termed the "*trans*-activation response element" (TAR) located at the start of all HIV-1 mRNAs (Gaynor, 1992). Binding of the viral nucleoprotein Tat to this structure potently activates viral expression, and is considered to be essential for transcriptional elongation (Zhou and Sharp, 1995). Variability within the LTR sequence, and subsequent changes in recognition motifs, have been noted among

different patients, and within the same patient over time, however, no correlations with clinical status could be identified (Estable *et al.*, 1996). Large deletions within the LTR that significantly influence pathogenesis have been described in the Sydney blood bank cohort (Deacon *et al.*, 1995), and are probably more common than generally appreciated, however, their importance resides in potentially affecting disease progression in patients, and not in mediating resistance to infection in any form. LTR sequence changes occur exclusive of immune selection pressures, as the LTR itself is insufficiently transcribed or translated to be antigenically recognized, and thus are solely subject to "naturally" induced mutations through the error-prone activity of the reverse transcriptase enzyme, or through recombination events.

LTR-directed HIV-1 gene expression proceeds in an orderly sequence: the first transcripts noted at low levels of transcription are fully-spliced, small mRNA molecules encoding the regulatory proteins Tat, Nef, and Rev. Tat is re-imported into the nucleus, binds to the TAR element, and enhances further transcription. Nef, short for negative factor, has been shown to have many, often contradictory, functions: it is dispensable for, but enhances, *in vitro* viral replication (Spina *et al.*, 1994), it modulates CD4 downregulation (Mangasarian *et al.*,1997), and its absence may be associated with an attenuated disease course (Mariani *et al.*, 1996). Though Nef seems to have distinct effects in cell culture systems, the diversity of the observations suggests that the authentic function of the protein *in vivo* remains to be discovered. On the contrary, Rev is uniquely responsible for mediating the transition from transcription of regulatory proteins to the singly spliced mRNA's for Env, Vpu, Vif, and Vpr, and the unspliced mRNA coding for a

Gag-Pol precursor protein (Feinberg and Greene, 1992). Rev functions similarly to Tat through a specific RNA motif termed the Rev response element located in the *env* gene. Translation of the *env* mRNA results in a gp160 precursor protein that is cleaved by a cellular protease into gp120 and gp41. Similarly, the large Gag precursor protein is cleaved by an HIV-specific protease into the smaller components comprising the nucleocapsid. This particular HIV-specific protease is the target of recently developed anti-HIV therapeutics, the protease inhibitors (Markowitz *et al.*, 1995). For production of the Pol protein a unique ribosomal frame-shifting mechanism is employed since this protein is translated from the same transcript as the Gag precursor (Cullen, 1991). The accessory proteins Vif, Vpr, and Vpu are not required for viral replication, but may modulate in vivo pathogenesis: Vif does not appear to be incorporated into the virion itself, but enhances infection apparently through aiding proper packaging of the viral nucleoprotein core (Subbramanian and Cohen, 1994). The Vpr protein confers enhanced replicative ability to viruses, in particular in macrophages (Hattori *et al.*, 1990). Expression of Vpu has been shown to reduce cytopathic effects presumably due to promoting proper virion maturation and release at the cell membrane (Subbramanian and Cohen, 1994). Altogether, the accessory proteins of the virus are not yet well characterized, and investigation into the viral life cycle will likely yield more definite information regarding their function.

Analysis of the splicing patterns of reverse-transcribed, and polymerase chain reaction (RT-PCR) amplified sequences from patients demonstrated that viral RNA could be detected in patients from all stages, and that disease progression was associated with

increases in the proportion of unspliced (coding for structural viral components) mRNA species (Furtado *et al.*, 1995). This suggested that in HIV-1 infection a true latent stage with no viral transcription either does not exist, or is exceedingly rare. Furthermore, these findings convincingly illustrated the intricacies of lentiviral transcriptional regulation in modulating regulatory versus structural protein production. Similar findings were reported by another group using a complex PCR assay able to distinguish between unintegrated, but reversely transcribed DNA, and integrated, proviral DNA. They concluded that true latency occurred in <0.0033% of resting $CD4^+$ T cells (Chun *et al.*, 1995). However, a pre-integration form of latency consisting of incompletely reverse-transcribed viral RNA, or lack of nuclear import of the DNA copy, has been shown to exist in resting $CD4^+$ T cells, and to present a very limited reservoir for HIV-1 (Chun *et al.* 1995). More recently, a similar analysis applied to lymph node samples indicated that <0.005% of resting $CD4^+$ T cells carrying proviruses could be induced to produce virions, and that the most common form of viral DNA detected in these cells consisted of unintegrated, full-length, linear DNA (Chun *et al.*, 1997). These findings are important since they support the concept that viral integration and replication require cellular activation, and that reversion to a quiescent state is rare for cells with latent proviruses. Furthermore, these truly latent-infected memory $CD4^+$ T cells, though rare, may survive for years, and therefore constitute a significant reservoir of cells that is not subject to immune surveillance by CTL, and that is not sensitive to anti-retroviral therapies. A similar frequency of latently infected macrophages was shown in the same study, and since these cells are resistant to viral cytopathic effects, and are long-lived, they constitute an equivalent reservoir of "untouchable" cells. Thus, recent detailed information on the molecular characteristics of

the viral life cycle coincides with clinical observations regarding the rapid, but nevertheless incomplete, reduction in viral loads following therapy (Pantaleo, 1997).

3. Immune Dysfunction

Despite an apparent long clinically silent phase during HIV infection, the function of the immune system gradually deteriorates during this time, and the onset of an AIDS-defining illness is only a late manifestation of a progressively failing system (Pantaleo *et al.*, 1993). Loss of $CD4^+$ cells results in lack of cytokine production and co-stimulation for other immune cells, and may partially account for some of the abnormalities observed, however, preceeding the actual decline in $CD4^+$ cell numbers, qualitative defects have been observed (Clerici *et al.*, 1993). Based on extensive studies of asymptomatic HIV-infected patients, Clerici and Shearer proposed a model where T helper (Th) cell function was characterized by sequential unresponsiveness to recall antigens, to allo-MHC, and finally to mitogenic lectins (Clerici and Shearer, 1993). These observations led them to conclude that abnormalities in the function of $CD4^+$ T cells were due to a switch in cytokine production from a predominance of IL-2, IL-12, and IFN-γ (associated with a Th1 response) to increases in IL-4, IL-5, IL-6, IL-10, and/or IL-13 (typical of a Th2 response) (Clerici and Shearer, 1994). Other researchers appeared to disagree with these findings, however, their experimental results were largely in concordance with a lack of Th1 cytokine production by clonal $CD4^+$ and $CD8^+$ cells (Graziosi *et al.*, 1994). Furthermore, analysis of the infectability of Th1, Th2, and Th0 (characterized by producing both types of cytokines) $CD4^+$ T cell clones concluded that Th1 cells were highly resistant to HIV

infection, and that there was a paucity of cytokine production from Th1 clones (Maggi *et al.*, 1994). Recent reports indicated that Th1 cells preferentially undergo activation-induced and Fas-mediated cell death that could be blocked by IL-12 addition, or by antibodies inhibiting Th2 cytokines (Estaquier *et al.*, 1995). Similar results were shown by Clerici *et al.* (1996), however, in their studies lymphotoxin was considered to be the mediator of apoptosis. Thus, these findings and hypotheses provide observational and theoretical evidence of abnormal Th cell function in HIV disease, however, to date no altered signalling pathways that could account for the defects in $CD4^+$ T cells from asymptomatic patients have been identified. Furthermore, cytokine production assays from *in vitro* stimulated cells, or from cells cloned in the presence of exogenous IL-2 and irradiated feeder cells, are prone to artifactual observations as compared to the complex autocrine and paracrine cytokine interplay *in vivo*, and thus it is difficult to attribute functional impairments unequivocally to a cytokine profile switch. Recent developments in intracellular cytokine staining techniques applied to fresh peripheral blood or lymph node samples may yield more objective information on cellular cytokine production (Gallagher, 1997).

HIV infection was associated with decreased natural immunity as mediated by natural killer (NK) and lymphokine-activated killer (LAK) cells in symptomatic and asymptomatic patients, though the defect was largely due to a lack of function, and not quantitative defects (Ullum *al.*, 1995). Further non-specific immune responses, such as complement-mediated lysis of HIV virions and infected cells, was found to be incomplete due to the interaction of the negative regulator of complement activation, complement

factor H, with gp41, and due to the presence of decay accelerating factor (DAF) on the viral surface (Stoiber *et al.*, 1996). B cell hyperactivity, and subsequent hypergammaglobulinemia with unknown specificities, were common features of HIV infection, and have been ascribed in some cases to Th2-type cytokines produced by $CD8^+$ cells (Maggi *et al.*, 1994). Thus, numerous immune abnormalities not directly attributable to the virus itself, are present in HIV infection, and comprise the immune dysfunction observed prior to overt immunodeficiency.

Naturally, much attention has focussed on the role of $CD8^+$ T cells in HIV pathogenesis, and changes in the distribution and function of distinct cell populations have been widely published. Individual reports regarding these findings will not be reviewed here, however, information on altered $CD8^+$ T cell function will be summarized. Expansion of a $CD8^+CD28^-$ T cell subset in HIV infection has been broadly recognized (Hodara *et al.*, 1993), and considering the requirement for CD28 co-stimulation in the induction of cytokine synthesis as well as for lytic activity, lack of expression of this molecule implies impaired antigen responsiveness (Guerder *et al.*, 1995). More recently, $CD8^+CD28^-$ T cells from HIV-infected patients were noted to have significantly shorter telomeres than those from control subjects, or other cells from the same individual (Effros *et al.*, 1996). Telomeres are specialized regions of genetic material at the chromosomal termini, and with each cell division they shorten due to loss of base pairs, thus their length is thought to mirror the mitotic history of a cell. These findings suggested that in HIV infection, $CD8^+CD28^+CD45RA^+$ T cells upon antigen encounter downregulate the CD28 molecule and convert to $CD45RO^+$ expression, divide multiple times, and become poorly

responsive to further antigenic encounters. This would account for the reduced number of $CD8^+CD28^+$ cells in circulation, and for the progressive loss of cytolytic activity against opportunistic microorganisms observed in HIV infection (Borthwick *et al.*, 1994), however, why this phenomenon should occur uniquely with HIV is presently unclear. Surprisingly, $CD4^+$ T cells were not found to have short telomeres (Effros *et al.*, 1996). Further analysis of telomere length and telomerase activity in HIV-patients confirmed these results (Palmer *et al.*, 1997). Therefore, the following hypothesis may be constructed: resting $CD4^+$ T cells become infected, during the ensuing cell activation and cell division the virus integrates into the host cell DNA, transcription is initiated, and viral antigens are expressed on the cell surface. These infected $CD4^+$ cells are destroyed cytolytically prior to further cell divisions, thus, persistent recruitment of new cells results in a peripheral blood $CD4^+$ T cell pool consisting predominantly of "young" cells that have undergone few mitotic events and have relatively uncompromised telomeres. Conversely, $CD8^+$ T cells that have been antigenically exposed, and have clonally expanded, persist in the circulation, although they are poorly functional. In support of this concept, Janossy *et al.* (1993) found proliferative defects in $CD8^+CD45RO^+$ T cells, and $CD8^+$ cells expressing the activation marker DR had much reduced proliferation in response to TCR stimulation, and did not express the IL-2 receptor efficiently (Pantaleo *et al.*, 1990). From these and other studies it may be concluded that cell population dynamics and function are disturbed in HIV infection, however, whether this is unique to lentiviral disease, and what mechanisms are mediating these alterations, is yet to be clearly defined.

The T cell receptor of $CD8^+$ cells has been examined in detail in response to earlier

reports that HIV encodes a superantigen associated with preferential replication in $CD4^+$ cells expressing the Vβ12 gene product (Laurence *et al.*, 1992; Hodara *et al.*, 1993). This theory was refuted upon examination of the TCR proteins expressed on the cell surface as compared to mRNA (Posnett *et al.*, 1993), however, induction of anergy in $CD4^+$ and $CD8^+$ $V\beta8^+$ cells was later again attributed to a superantigen-like effect (Dadaglio *et al.*, 1994). Analysis of mRNA expression and flow cytometric detection of specific Vβ gene products in combination with evaluation of cytolytic function provided convincing evidence for oligoclonal expansion of $CD8^+$ T cells during early infection (Pantaleo *et al.*, 1994). Patients who maintained a broad repertoire of TCR gene usage during the early cytolytic response to HIV fared better than patients with a limited repertoire, and no TCR abnormalities suggestive of a superantigen were detected. Thus, while HIV infection may evoke a strong cytolytic immune response with a unique pattern of TCR-gene usage, unresponsiveness of $CD8^+$ T cells may occur, and may result from inadequate CD28-costimulation, or other pathways resulting in anergy.

4. Immunodeficiency

The final stages of HIV infection are characterized by overwhelming infection with organisms either prevalent in the environment, or persistent, but non-pathogenic, in immunocompetent hosts, by debilitation and cachexia, and by aggressive neoplasms in some patients. A correlation between the $CD4^+$ T cell count and occurrence of an AIDS-defining illness is universally accepted, and statistical analysis has provided probabilities of disease progression associated with specific $CD4^+$ T cell counts (Blatt *et al.*, 1995). For the past three years, quantitative viral RNA determinations in plasma have been

performed, and they correlate well with clinical progression and with $CD4^+$ T cell counts (Bruisten *et al.*, 1997). However, viral load determinations were found to be more accurate at monitoring disease progression, while $CD4^+$ cell quantification best predicted the onset of AIDS (Mellors *et al.*, 1996). Thus, the phenotypic characteristics of the immunodeficient state consist of severely reduced $CD4^+$ T cell numbers, rising viral load in the cell-free compartment, increasing HIV RNA/DNA ratio, and, in the majority of patients, conversion to SI viral phenotypes. However, mechanistically, consensus on the reasons for failure of the immune system, and the inevitable progression of HIV infection to AIDS and death, is lacking. Landmark studies of viral dynamics in response to potent antiretroviral drugs indicated that $CD4^+$ cells turnover at a rate of $2x10^9$ per day, and that virus-producing cells and free virions had a half-life of 2 days (Wei *et al.*, 1995; Ho *et al.*, 1995). This translated to very high replication rates for the virus, and suggested that >99% of plasma virus was derived from actively replicating cells as opposed to chronically low-replicating cells, or latently infected cells.Therefore, the 'set point' of a relatively stable viral load that is achieved within approximately six months after seroconversion, and which has been shown to predict the time to AIDS (Phillips, 1996), is not steady at all, but rather consists of a relative balance of high viral production and viral clearance rates. The dramatic reduction in viremia that ensues with potent anti-retroviral therapy precludes viral detection in the majority of patients adhering to therapy (Perelson *et al.*, 1996), but does not succeed in eradicating infection (Pantaleo, 1997). Rather, patients who have stopped therapy uniformly had a rapid rebound in viral RNA copies (Pantaleo, 1997). Thus, the question remains why the virus causes death of the host in spite of an apparent active and persistent immune response. The surprising observation that profound

reduction of infected cells and free viral particles during anti-retroviral therapy does not impart an advantage to the cellular immune response sufficient to eradicate the virus has evoked questions regarding the overall importance of CTL in HIV infection (Feinberg and McLean, 1997). According to another school of thought, HIV infection is similar to other conventional viral infections in that $CD8^+$ T cells mediate much of the damage, and the virus alone in the absence of an immune response would cause little harm (Zinkernagel and Hengartner, 1994). Albeit, the progressive pathogenesis induced by HIV-1 infection is still largely unexplained, and the beneficial or deleterious contributions from the immune system remain to be elucidated unequivocally.

5. Resistance to Infection

Individuals apparently resistant to infection by HIV-1 have been studied in great detail in order to devise protocols for inducing protective immunity through vaccination, and to better understand the correlates of resistance. Assays to evaluate cellular responses to HIV peptides in individuals exposed to the virus through sexual contact (Clerici *et al.*, 1992; Langlade-Demoyen *et al.*, 1994), intravenous drug use (Beretta *et al.*,1996), or as an occupational hazard (Pinto *et al.*, 1995), indicated that detectable sensitization of the cellular immune system preceded seroconversion, and could occur in the absence of subsequent seroconversion. The delay in antibody detection was remarkable in certain patients at high risk of infection, and illustrated the dichotomy of the humoral and cellular immune system (Langlade-Demoyen *et al.*, 1994). In addition, several reports of children apparently infected at birth, but converting to HIV-negative status associated with

detectable CTL activity provoked hopes of achieving cures for HIV infection (Cheynier *et al.*, 1992; Rowland-Jones *et al.*, 1993; De Maria *et al.*, 1994). These studies were based on *in vivo* observations, and *in vitro* evaluation involved proliferation assays, cytokine production, or cytolysis in response to specific recognition of virally-derived peptides, but no indication of inherent cellular resistance to infection was obtained. Studies of a similar nature on Gambian and Kenyan prostitutes highly exposed to HIV-1, but remarkably resistant, suggested that specific CTL responses (Rowland-Jones *et al.*, 1995), or natural immunity (Fowke *et al.*, 1996) may account for the lack of infection.

The most striking discovery of a mechanism of resistance succeeded the description of β-chemokines as mediators of HIV-suppression (Cocchi *et al.*, 1995). A homozygous 32-base pair deletion in the chemokine receptor CCR5 resulting in a frame shift, and a non-functional receptor, was described in individuals highly exposed to HIV-1, but uninfected (Samson *et al.*, 1996; Liu *et al.*, 1996). This mutation (CCR5Δ32) was associated with near complete resistance to infection by NSI viral isolates, thus confirming the functional importance of the molecule. The heterozygous state has been associated with slower disease progression, but equivalent *in vitro* infectability (Huang *et al.*, 1996; Dean *et al.*, 1996). Individuals homozygously bearing this mutation are phenotypically normal, have no apparent immune defects, and thus this unusual state of "natural" resistance revealed many potential therapeutic venues. As shown in chapter 5 of this dissertation, the 32 base pair deletion in CCR5 was not detectable in members of the Kenyan cohort, accordingly, different mechanisms of resistance are likely present there.

IL-16, a chemokine secreted by $CD8^+$ cells and binding to the CD4 receptor, has been described as mediating cellular resistance to transcription of integrated SI and NSI viral strains by interfering with expression of *tat* and *rev* genes (Zhou *et al.*, 1997). The chemokine does not appear to impede infection, and has to date only been described in cell lines, therefore the *in vivo* relevance is unknown. Another virus-resistant state has been induced in $CD4^+$ cells that were co-stimulated via the TCR and CD28, and lack of CCR5 transcription was considered responsible for this phenomenon (Carroll *et al.*, 1997). However, more recent detailed analyis of the induction and expression of CCR5 in different cell types indicated that this receptor is only very gradually expressed on T cells during activation (Wu *et al.*, 1997), thus the observations by Carroll *et al.* may represent the normal regulation of CCR5, and not a unique state following stimulation with CD3 and CD28 antibodies.

Debate regarding the exact identity of the originally described $CD8^+$-cell-derived factor continues, and Levy *et al.* recently argued that the β chemokines do not account for all the suppressive effects attributable to $CD8^+$ cells (Mackewicz *et al.*, 1997). In support of multiple suppressive factors being elaborated by $CD8^+$ cells were observations from other groups showing that β chemokines did not mediate transcriptional suppression of LTR-mediated gene expression (Leith *et al.*, 1997), nor were they responsible for inhibiting replication in dendritic cell-$CD4^+$ cell co-cultures (Rubbert *et al.*, 1997). Co-culture inhibition of viral replication appeared to correlate with clinical status (Blackbourn *et al.*, 1996; Mackewicz *et al.*, 1991), while supernatant-mediated suppression of viral transcription was not associated with improved clinical status (Copeland *et al.*, 1997).

Thus, it is apparent that various factors produced by $CD8^+$ T cells are able to mediate resistance to infection, as well as to modulate viral life cycle dynamics in infected cells.

In summary, several pathways of resistance to HIV-1 infection or replication have been proposed. Mutations in CCR5 appear to convey the most significant and well-described mechanism of resistance, however, factors distinct from $CD4^+$ cell structural features may significantly modulate infection or impart resistance to infection.

Cytotoxic T Lymphocytes in HIV infection

1. Antiviral Responses

CTL are thought to be the main effectors responsible for reducing the viremia during acute infection, and for maintaining a long, clinically silent period prior to the development of AIDS (Koup *et al.*, 1994). These findings are supported by detecting CTL prior to seroconversion (Langlade-Demoyen, 1994), and by the association of strong CTL responses with relative non-progression (Rinaldo *et al.*, 1995). The association of a reduction in viremia with the emergence of HIV-specific CTL is largely circumstantial, as it is difficult to translate the results of *in vitro* assays into plausible *in vivo* scenarios. Factors such as the ratio of CTL to target cells, induction of CTL responses through infected macrophages or $CD4^+$ T lymphocytes, the presence of modulating cytokines, and, lastly, the virus that is assayed itself, are likely to differ between *in vivo* and *in vitro*

situations. Several studies have attempted to address these deficiencies, and a brief summary of the limitations of commonly used assays is provided here.

Target cells used for CTL assays commonly are MHC-matched, Epstein-Barr-Virus (EBV)-immortalized B lymphoblastoid cell lines (BLCL) that are infected with recombinant vaccinia vectors expressing inserted HIV genes (Gritz *et al.*, 1990). These genes are derived from fully sequenced and cloned laboratory isolates, and the vectors may have single, full-length, or partially deleted HIV gene inserts, or a combination of genes. The advantages of this system are that BLCL are easy to generate and handle, are highly infectable by the vaccinia vectors, and express high levels of the inserted gene product. However, considering the high prevalence of infection with EBV in the general population, background levels of cell lysis may be substantial. As well, due to the limited number of inserts, only cytotoxic responses directed against one segment of the virus are assayed at a time, therefore, several different target cells need to be evaluated to attempt acquiring information on a CTL response directed against a whole virus. Furthermore, derivation of sequences from laboratory HIV-1 isolates frequently entails limited cross-reactivity with primary isolates since substantial variability in epitopes is common, and thus CTL responses relevant *in vivo* may be missed. This phenomenon was well-illustrated by the lack of recognition of HIV_{MN}-derived peptides by CTL clones reactive with autologous virus that were isolated during acute seroconversion (Safrit *et al.*, 1994). Finally, detailed studies on the mechanisms of lysis are hindered by evaluating a transformed B cell while the "natural" target cells are primary T cells or macrophages. Nevertheless, this is the most widely employed system, and many of the conclusions

regarding CTL function have been based on this assay.

The limitations intrinsic to employing vaccinia vectors were addressed by transducing BLCL with the CD4 gene, and subsequent infection with whole HIV-1 (McElrath *et al.*, 1994). The authors provided evidence of high expression of CD4 on the cell surface of the transduced cell lines, and virus infection was achieved, however, replication of NSI strains occurred only to a limited degree. These findings may be explained in terms of recent knowledge about co-receptors: B cell lines expressed CXCR4, the co-receptor for SI strains, but did not express CCR5 (Feng *et al.*, 1996; Wu *et al.* 1997). Therefore, this target cell line can be infected with viral strains using CXCR4 as co-receptor, however, most primary strains bind to CCR5. As described in chapter 4 of this dissertation, BLCL transfected with the genes for CD4 and CCR5 become permissive to infection with any SI or NSI primary viral strain, and thus constitute a useful target cell line for approximating cytolysis against all *in vivo* viruses. Though integrated proviruses in B cells are likely subject to different transcriptional influences than those present in T cells, viral replication does occur, and was sufficient to induce antigenic recognition (McElrath *et al.*, 1994).

The closest approximation of the *in vivo* interaction is achieved with $CD4^+$ T cells or macrophages as target cells for CTL. The limitations of this approach are increased labour-intensity as primary $CD4^+$ cells have to be isolated, activated, and infected if not derived from a naturally infected individual, and the cells are subject to the cytopathic effects of SI viruses. In addition, paucity of $CD4^+$ cells during advanced HIV disease, and

poor mitogen-responsiveness may limit the availability of potential target cells. In lymph nodes from asymptomatic patients the ratio of lymphocytes to infected target cells has been estimated at 15 to 30:1, depending on the patient's viral load, thus commonly used effector to target ratios of 10 to 50:1 should adequately simulate this aspect of the *in vivo* scenario (Klenerman *et al.*, 1996).

CTL may be tested freshly isolated from peripheral blood, in which case they are assumed to be active, antigenically-primed effector cells, or they may be re-stimulated *in vitro* with infected autologous $CD4^+$ cells, or with infected BLCL (McElrath *et al.*, 1994), indicating memory CTL function. The latter method will result in generation of EBV-specific killer cells from individuals infected with EBV, and thus may significantly interfere with interpretation of HIV-specific lytic activity. In summary, interpretation of assays of CTL function requires consideration of the potential experimental limits that may result from the effector or target cell preparation. Improved sensitivity and specificity may be achieved with direct identification and quantitation of antigen-specific cells freshly isolated from peripheral blood or lymph node samples (Altman *et al.*, 1996).

Based on the above discussion, direct evidence for the effectiveness of CTL in reducing viremia is lacking. However, the temporal association of CTL with control of early viremia, and the consistent detection of anti-HIV lytic activity in long-term non-progressing (LTNP) patients strongly supports their role in delaying disease progression (Harrer *et al*, 1995). Albeit, different conclusions were drawn from detailed analysis of viral dynamics in response to antiviral drugs (Wei *et al.*, 1995; Ho *et al.*, 1995): from a

mathematical model based on the rate of viral decline it was concluded that the half-life of infected cells in asymptomatic patients varied from 1.2 to 2.4 days, and that there was no variation according to disease stage as indicated by $CD4^+$ cell counts (Klenerman *et al.*, 1996). Thus, the question arose whether infected $CD4^+$ cells die due to viral cytopathic effects, or due to lysis by CTL. Considering the lack of variability between patients, and the constant slope of viral decay, the authors concluded that the virus is cytopathic, and that CTL contribute relatively little to the elimination of virally infected cells (Klenerman *et al.*, 1996). This view has been shared by other investigators who found a poor correlation between $CD8^+$ cell lytic or suppressive activity and high $CD4^+$ cell counts in a cohort of LTNP patients (Ferbas *et al.*, 1995). High frequencies of Gag-specific CTL precursors were detected in slow and rapid progressors in early disease, while clinical progression was associated with loss of detectable CTL activity (Klein *et al.*, 1995). A theory regarding the importance of immunodominant epitopes has been put forward by Nowak et al. (1995) who compared CTL responses in a slow and a fast progressor. Lytic activity in the slow progressor was characterized by recognition of a single epitope, while three epitope variants were recognized in a fluctuating manner in the fast progressor. The authors argued that strong CTL responses against a single immunodominant epitope will prevent a shift to less recognized epitopes, and thus will allow for prolonged control of a relatively homogenous viral population. This theory did not consider reasons for the eventual failure of the CTL response (Nowak *et al.*, 1995). Others have argued that HIV disease is slowly progressive due to limited availability of target cells in the form of activated $CD4^+$ cells, and that immune responses contribute little to the asymptomatic phase of infection (Coffin, 1995).

Efforts directed at producing an effective vaccine against HIV-1 have failed to this date. The correlates of immune protection are not clearly established, and as such many questions regarding vaccine design remain unanswered. Transfer of *in vitro* expanded CTL has been attempted as a therapeutic modality, and has yielded potentially useful information for vaccine formulation (Riddell *et al.*, 1993). Surprisingly, the first attempt to adoptively transfer CTL reactive with a conserved epitope in Nef resulted in dramatic deterioration of the patient (Koenig *et al.*, 1995). Subsequent to the transfusion, Nef-deleted viral species were recovered, and despite rapid deterioration of the patient strong CTL responses against a number of HIV proteins were detectable. The results of this unique study seemed to support concepts put forward by Zinkernagel and Hengartner (1994) who argued that at high viral loads enhancement of an immune response would be deleterious to the host due to CTL-mediated immunopathology. In the former report the patient had a compromised $CD4^+$ cell count and a high viral load prior to therapy, and both parameters rapidly worsened after transfusion, a scenario compatible with Zinkernagel and Hengartner's predictions. In the patient described, addition of a large number of CTL specific for a single epitope may have altered an already poorly maintained balance by exerting selective pressure on the virus to mutate, and thus escape from a "single track"immune response was promoted.

2. Viral Escape from Immune Surveillance

Assuming that CTL have a protective role in HIV infection, it nevertheless remains unsolved why the host eventually, and inevitably, loses in the battle with the pathogen. If

CTL directed against the appropriate HIV antigens could preserve the life of the host, or at least significantly prolong the disease-free interval, why do they fail to do so, and why are lytic effectors detectable in patients that are near death? In part, the answer to these questions resides in the variability of the virus, and that the virus appears to be able to mutate faster than the host's immune system can follow. A theory put forth suggested that a cellular immune response against conserved and variable epitopes will result in increased viral diversity according to the preponderance of variable epitopes recognized (Nowak *et al.*, 1995). An increase in the quasi-species of HIV would then "dilute" the immune response to a greater number of subdominant epitopes, resulting eventually in overall strong CTL activity directed against inconsequential epitopes expressed by minor viral variants. Close examination of viral evolution over time disputed this aspect of the previous theory, as fast progressors were shown to have relatively homogenous viral populations, while slow progressors had high levels of viral diversity (Wolinsky *et al.*, 1996). Furthermore, mutations resulting in amino acid changes, and therefore influencing binding to MHC and recognition by CTL, were more frequent in slow-progressing patients (Wolinsky *et al.*, 1996). This directly implicates the immune response in "driving" viral selection, and affirms the immunodominant versus variable epitope aspect of the former theory. Thus, if the host immune response could keep viral load reduced by destroying cells displaying viral immunodominant epitopes, viral evolution would progress relatively slow, and CTL responses would be effective for prolonged periods of time. Increased viral quasispecies in patients with more functional immune systems were shown in another study comparing different rates of disease progression (McDonald *et al.*, 1997).

The mechanisms of evading an immune response include mutations within crucial CTL epitopes, affecting MHC-binding, or interaction with the TCR. A further impediment for effective CTL function may be lacking epitope density on the surface of infected cells. Though this appears to be an unlikely *in vivo* scenario with naturally processed peptides, using synthetic peptides it was found that lesser peptide concentrations resulted in poorer recognition by CTL (Tsomides *et al.*, 1994). More recently, TCR antagonistic epitopes have been described that consist of viral amino acid substitutions in the regions contacting the TCR, resulting in proper presentation, but failure to deliver a full stimulatory signal (Klenerman *et al.*, 1994). The consequence was either anergy of the CTL, or, more importantly, competition with wild-type immunogenic peptides and thus inhibition of CTL responses (Klenerman *et al.*, 1994). These findings were substantiated by showing effective CTL inhibition of reverse-transriptase encoded epitope recognition by viable mutants (Meier *et al.*, 1995). Thus, this type of mutation may be more detrimental than simple epitope escape, as inhibition of CTL in addition to competition for recognition by CTL is taking place.

In summary, CTL's in HIV infection face a difficult task: humoral responses have largely been shown to be ineffectual at slowing disease progression (Pantaleo, 1997), thus potential immune modulation of pathogenesis resides within $CD8^+$ lymphocytes. CTL have to attempt lysing infected cells that are displaying ever changing epitopes, and they have to adapt their own TCR specificity for these new epitopes in the absence of appropriate cytokine stimulation, and coincident with peptides that may paralyze instead of stimulate the CTL. It is therefore conceivable that many divergent theories on the beneficial or

deleterious roles of CTL co-exist, however, if the immune response is expected to contribute to achieving a cure for HIV infection, the beneficial functions of CTL will need to be dissected out, and strengthened.

3. Deleterious Lytic Effects

Many syndromes unrelated to immunodeficiency have been reported in HIV-infection. Infiltrates of $CD8^+$ cells into tissues have been associated with localized diseases such as alveolitis in the lung (Agostini *et al.*, 1996a), and neurologic disease (Jassoy *et al.*, 1992). The proposed mechanisms for induction of inflammation include CTL recognition of HIV-derived antigens on infected cells, such as pulmonary macrophages, recruitment of further CTL, and lysis of infected cells. CTL action at this site seemed to evoke inflammatory cell infiltrates and subsequent tissue damage due to unkown inciting stimuli (Agostini *et al.*, 1996a). Lack of IL-2 production resulting from dysfunctional or reduced numbers of $CD4^+$ cells has been shown to be compensated for by non-lymphoid cell production of IL-15, which exerted similar effects as IL-2, and which resulted in activation of pulmonary T cells (Agostini *et al.*, 1996b).

CTL reactive against a variety of uninfected cells have been reported to occur in HIV infection. $CD3^+$ cells from HIV-infected patients, in some cases co-expressing the CD8 molecule, were noted to lyse lectin-activated $CD4^+$ blasts, and rarely $CD8^+$ blasts (Israël-Biet, *et al.*, 1990). The interaction did not involve recognition of HIV-derived antigens, and no MHC-restriction was detectable (Israël-Biet, *et al.*, 1990). Similar

findings were reported by Grant *et al.* (1993), who showed that non-specifically activated $CD8^+$ T cells from patients at various stages of disease lysed uninfected $CD4^+$ mitogen-stimulated blasts, as well as a $CD4^+$, MHC class II negative, T cell line. Mediation of the cytolytic action was through the $\alpha\beta$ TCR, and could not be reduced by MHC class I blocking antibodies (Grant *et al.*, 1993). Clinical significance of this phenomenon was demonstrated by association with decreasing $CD4^+$ cell counts in controlled patient cohorts (Grant *et al.*, 1994). Almost identical findings had previously been observed in infected humans, but not in chimpanzees (Zarling *et al.*, 1990). Thus, these CTL activities differ dramatically from the conventional CTL thought to exclusively depend on recognition of MHC with bound peptide. As the target involved in the majority of these studies was restricted to activated $CD4^+$ cells, and only CTL from HIV-infected individuals lysed such target cells, the phenomenon may result from sensitization of $CD8^+$ cells in the host against altered epitopes expressed on *in vivo* activated $CD4^+$ cells. Cell activation involves *de novo* expression of a large number of surface molecules, however, to account for the findings described above, the target molecule would need to be present between 5 to 7 days after the *in vitro* stimulus, expression would have to be equivalent on cells of disparate MHC phenotypes, and cell death would have to be inducible through recognition of non-MHC linked antigens. These requirements are reminiscent of Fas-FasL interactions, and a plausible scenario might consist of persistent *in vivo* antigenic stimulation with induction of FasL expression on $CD8^+$ T cells that contribute to depletion of uninfected, Fas-expressing, activated $CD4^+$ cells *in vivo*, and are easily detectable with *in vitro* assays. In support of this concept is that relatively few $CD4^+$ cells are actually infected, while unusually vigorous CTL responses unrelated to disease stage are present in

HIV patients, and that adminstration of expanded CTL caused severe deterioration of a patient (Koenig *et al.*, 1995). Direct proof of this theory is currently lacking, however, a large body of literature has implied apoptosis as a main mediator of $CD4^+$ cell depletion in HIV pathogenesis (Li *et al.*, 1995; Westendorp *et al.*, 1995; Estaquier *et al.*, 1995), and ample evidence exists concerning the overexpression of apoptotic mediators in HIV infection (Katsikis *et al.*, 1995; Clerici *et al.*, 1996). Furthermore, a variety of autoimmune syndromes have been associated with HIV disease (Silvestris *et al.*, 1995), and cell-mediated immunopathology in some cases has been sufficiently extensive to prompt comparison with graft-versus-host disease syndromes (Habeshaw *et al.* 1992; Shearer and Clerici, 1993). The induction of autoimmune syndromes in HIV infection might be almost inevitable considering the integration of a substantial number of host molecules into the viral envelope during cell budding (Orentas and Hildreth, 1993). In particular, the presence of large numbers of MHC class II molecules may be responsible for the immunopathology noted in some patients (Arthur *et al.*, 1992), while protection against SIV infection was found to be due to alloimmunization (Stott, 1991). Thus, aberrent interaction of CTL with non-HIV-encoded antigens appears to be common, contributes to non-lymphoid disease, and to lymphocyte depletion. Considering the potential interactions between a virus "studded" with foreign molecules and a new host, a complex interplay of virus-specific and non-specific actions is to be expected, and would be complicated by the resultant cell activation which in turn provides more infectable target cells. In summary, $CD8^+$ lymphocytes in HIV infection have multiple functions, and the effect on the host depends on the particular prevailing cytolytic activity, the stage of the disease, the MHC profile of the host, the genetically-determined use of variable TCR genes, and, lastly, the

type of virus.

Objectives of this Research

Our laboratory had previously described cytolytic activity of $CD8^+$ cells from HIV-infected individuals directed against activated uninfected $CD4^+$ cells (Grant *et al.*, 1993; Grant *et al.*, 1994). Though this phenomenon appeared to be clinically significant, little was known regarding the activation characteristics of the target cells, nor was it clearly established that the interaction is independent of MHC recognition. Thus, the first questions asked were:

1) What mitogenic stimuli confer susceptibility to the target $CD4^+$ cells?
2) What activation indices correlate with lytic susceptibility?
3) Can recognition of HIV-derived antigens be excluded?
4) What is the phenotype of the TCR involved?
5) Is this phenomenon genuinely independent of conventional MHC recognition?

These questions could be answered, and the findings are elaborated in chapter 2. Next, considering that the essential characteristics of the cytolytic interaction were specificity for activated target cells, and lack of MHC class I restriction, the mechanism of lysis was examined in order to determine which mediators were involved. Given the preponderance of HIV-specific CTL in infected patients, it was important to determine whether conventional CTL and $CD4^+$-cell specific CTL had different effector mechanisms.

Thus, the following questions were posed:

1) Are CTL directed against HIV-derived antigens as well as against activated $CD4^+$ cells present within the same effector cell population?
2) Since the two main mediators of cytolysis, perforin and Fas, effect death of the target cell with different kinetics, could physical characteristics in the target cells be indicative of one or the other mediator?
3) Are there differences in susceptibility to biochemical inhibitors, and in dependence on calcium ions?
4) Presuming that lysis of activated $CD4^+$ cells involves induction of apoptosis, can DNA fragmentation be detected in the target cells?

The experiments to answer these questions are described in chapter 3. The results indicated that indeed there were differences in the effector mechanisms employed in killing of HIV-expressing target cells, and in those involved in lysis of activated $CD4^+$ cells. At the time of these experiments, major discoveries in the biology of HIV were publicized. The co-receptors involved in mediating cell entry by different viral strains were identified as chemokine receptors, and views on the pathogenesis of HIV infection, and the role of CTL, changed dramatically. In light of these new findings it was of interest to determine the role of CTL in mediating resistance to infection by HIV-1. The shortcomings of conventional CTL assays (discussed in detail above) constituted major limitations for detecting cytotoxic activity against primary viral isolates. Thus, in order to maximize the sensitivity of detecting CTL responses relevant *in vivo*, a new assay system was devised.

Primary viruses, to which the individual at risk had been exposed to, were isolated, phenotyped, and titered. For cytolytic assays, target cells consisting of autologous BLCL that had been transfected with the genes for CD4 and for CCR5, thus allowing infection with most primary isolates, or autologous infected $CD4^+$ cells were employed. The effector CTL were tested either from fresh peripheral blood samples, or were restimulated with autologous cells infected with primary viruses. Once the details of this assay system were established, the following principal questions were addressed to a cohort of individuals at high risk of infection:

1) Are $CD4^+$ cells from the uninfected persons as susceptible to infection with primary viral isolates as those from control individuals?
2) Are there mutations in the co-receptors, and do they correlate with *in vitro* resistance?
3) Are CTL against primary isolates detectable, and is there cross-reactivity with standard laboratory viral strains?
4) Do phenotypic characteristics of the virus from the infected partner affect *in vivo* resistance?

Results from these investigations are detailed in chapter 4. Briefly, the data indicated that resistance to infection in highly exposed individuals was complex, and contributing factors consisted of inherent $CD4^+$ cell resistance, presence of specific immune responses, attenuation of the virus in the infected individual, and variable risk sexual behaviour.

Similar questions were posed to a different group of resistant individuals. In this case the subjects were at extremely high risk for infection due to prostitution in a geographic region with high prevalence of HIV-1 infection, and due to frequent high risk sexual behaviour. Considering the difficulty of sample acquisition, the following questions were considered to be most relevant:

1) Are $CD4^+$ cells inherently resistant to infection?
2) Are there mutations in the co-receptors that may mediate resistance?
3) Is there excessive production of β-chemokines by $CD4^+$ cells that might account for in vivo resistance?
4) Do $CD8^+$ cells exert suppressive effects in co-culture, or at the transcriptional level, that correlate with resistance or susceptibility?

Results from these experiments indicated that resistance to infection in this cohort likely resided in factors exclusive of $CD4^+$ cells, and that strong suppressive effects of $CD8^+$ cells would be expected to modulate an infection, but not to confer resistance.

Thus, this research was undertaken to explore $CD8^+$ T cell functions in different settings. Initially, a cytolytic activity was characterized in a large number of patient samples, and the mechanism of the interaction was investigated. Next, new assays were developed to customize CTL detection techniques for primary viruses, and these were applied to a group of individuals with potential viral exposure. Lastly, $CD8^+$ T cell suppressive effects were evaluated in a unique patient cohort at high risk of infection.

Chapter 2.

Cytotoxic T-lymphocytes from HIV-infected individuals recognize an activation-dependent, non-polymorphic molecule on uninfected CD4+ lymphocytes.
(Bienzle et al., AIDS 10:247-254, 1996)

In this article the cytolytic interaction of $CD8^+$ T-lymphocytes from HIV-infected individuals with autologous and heterologous activated $CD4^+$ cells are described. The experimental results indicated that this lytic activity was specific for activated $CD4^+$ cells, and that proliferation of the target cells was a good indicator of susceptibility to lysis. Moreover, the lysis was independent of viral replication in naturally infected $CD4^+$ cells, and target cells from MHC-disparate individuals were equally susceptible. Only effector cells from HIV-infected persons lysed the target cells in these assays.

These findings substantiate the specificity of the lysis for activated $CD4^+$ cells, and demonstrate that there is no apparent restriction by MHC class I or class II molecules. The implications from this work are that CTL with unusual specificities exist in HIV-1 disease, and that their action may contribute to the decline of $CD4^+$ cells characteristic of the infection.

The experimental work in this chapter was performed by the author of this dissertation.

Cytotoxic T-lymphocytes from HIV-infected individuals recognize an activation-dependent, non-polymorphic molecule on uninfected CD4+ lymphocytes

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Dorothee Bienzle, Fiona M. Smaill* and Kenneth L. Rosenthal

Objectives: Correlation of lysis of autologous CD4+ target cells by cytotoxic lymphocytes from HIV-seropositive patients to target activation, viral replication, and major histocompatibility complex (MHC) restriction.

Design: Twenty-two HIV-infected patients were evaluated for lysis of activated CD4+ cells, concurrent with measurement of proliferation of the target cells, and with viral replication.

Methods: Titrated standard ^{51}Cr-release assays for specific effector-to-target cell recognition, blocking antibodies and cell depletion for cell characterization, incorporation of [^{3}H]- thymidine for proliferation, and p24 antigen capture assays for viral replication.

Results: HIV-infected patients had cytotoxic lymphocytes capable of recognizing activated CD4+ target cells in a non-MHC-restricted manner. The lysis depended on the degree of target activation, and was independent of viral replication.

Conclusions: This cytolytic activity is unique to HIV-infected patients, and is suggestive of activation-induced cell death that may contribute to the progressive depletion of CD4+ lymphocytes during HIV pathogenesis.

AIDS 1996, **10**:247–254

Keywords: HIV, AIDS, cytotoxic lymphocytes, activation-induced cell death, anti-ergotypic lymphocytes, autoimmune CD4 depletion

Introduction

Clinically, infection with HIV progresses gradually over a period of years eventually culminating in the profound immunoincompetence and susceptibility to opportunistic infections that characterize AIDS. The initial viraemic phase coincides with a strong cytotoxic T-lymphocyte (CTL) response directed against peptides specific for the transmitted virus, whereas neutralizing antibodies are not detectable until several months after seroconversion [1]. During the protracted period of clinical latency strong CTL responses and high levels of antibodies against viral components persist, although gradual erosion of immune function and CD4+ lymphocytopenia develop [2,3]. Abnormalities in T-cell receptor (TCR) gene usage [4,5], subset distribution of lymphocytes [6], loss of memory function [7], and altered B- and T-cell interactions [8] have been described; however, the loss of CD4+ lymphocytes remains largely unexplained. Recently, mathematical models based on quantitative determinations of viral RNA and DNA load in different cellular compartments lead to the conclusion that the progressive loss of CD4+ lymphocytes is likely to result from death of infected cells with exhaustion of the replicative potential of the remaining uninfected pool [9,10]. However, the proportion of infected cells in circulation is low, and although a greater number of viral particles have been detected in the lymph nodes of infected patients [11], destruction of infected lymphocytes due to direct viroly-

From the Molecular Virology and Immunology Program, Department of Pathology, McMaster University, and the *Special Immunology Services Clinic, Chedoke–McMaster Hospitals, Hamilton, Ontario, Canada.

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Requests for reprints to: Dr K.L. Rosenthal, Molecular Virology and Immunology Program, Room 3N26, McMaster University Health Sciences Centre, 1200 Main Street West, Hamilton, Ontario, Canada L8N 3Z5.

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sis or specific recognition of viral peptides by CTL may only account for part of the loss of helper T cells.

A diverse array of functions has been attributed to CTL in HIV infection: classical, major histocompatibility complex (MHC) class I-restricted antiviral responses [12], blockage of viral replication through non-lytic mechanisms [13,14], possible mediation of activation-induced cell death via the Fas/Fas-ligand pathway [15], and non-MHC-restricted lysis of uninfected CD4+ lymphocytes [16,17]. Although it is likely that specific subsets of CD8+ lymphocytes mediate these separate activities, no markers uniquely characterizing the subpopulations have been identified, and firm evidence for beneficial effects of these functions regarding lack of disease progression has only been shown for CD8-mediated viral suppression [13,14]. Indeed, Zinkernagel [18] has argued that virus-specific CTL may cause the immunopathology seen in AIDS by destroying T-helper cells and antigen-presenting cells in a manner analogous to infection with lymphocytic choriomeningitis virus, and that elimination of CTL may lead to a symptom-free virus-carrier state. Although contrary evidence suggests the importance of antiviral CTL responses in exposed, seronegative individuals [19], the role of CTL during the pathogenesis of established infection remains poorly understood.

Viral persistence occurs despite multifaceted antiviral CTL functions, and it may be questioned whether the immunopathology of HIV infection is directly related to the presence of the virus. We have previously demonstrated skewing of the TCR repertoire [20], and the presence of non-MHC-restricted cytotoxic lymphocytes specific for CD4+ lymphocytes in HIV infection [16]. The latter phenomenon is associated with disease progression as measured by a decline in CD4+ lymphocyte numbers [21]. To further address the role of unconventional CTL activities we show here that HIV-infected individuals possess a unique population of CD8+ lymphocytes capable of lysing CD4+ lymphocytes of autologous origin as well as from heterologous HIV-seronegative individuals. This lytic activity is dependent on activation of the target cell, is directed against a non-polymorphic target, and is independent of expression of viral proteins. Lymphocytes directed against activation-associated molecules on other immune cells have been termed anti-ergotypic, and are detectable in cell-mediated autoimmune diseases such as experimental autoimmune encephalomyelitis [22] and myasthenia gravis [23]. Detection of a similar activity in HIV-infected individuals suggests that cell-mediated autoimmunity contributes to the development of CD4+ lymphocytopenia.

Materials and methods

Study subjects

Twenty-two HIV-seropositive patients of the Special Immunology Services Clinic at Chedoke–McMaster Hospitals were studied. The patients' CD4 lymphocyte counts, stage of disease, and antiretroviral therapy are summarized in Table 1. Control blood samples were obtained from HIV-seronegative laboratory personnel. Written informed consent for the studies was obtained from all participants.

Table 1. Patient characteristics.

Patient	CD4 count (×10⁶/l)*	Disease stage†	Therapy
91-06	910	A	-
91-16	320	B	ZDV, ddI
91-93	360	A	-
91-112	1190	A	ZDV, ddI
91-116	790	A	-
91-132	100	C	ZDV, ddI
91-148	210	A	-
91-196	430	A	-
92-217	370	A	-
92-220	590	A	-
92-233	290	B	ZDV
92-242	340	B	ZDV
92-267	410	A	-
92-274	520	C	ZDV
92-276	230	B	ZDV
92-285	530	A	ZDV
92-292	510	A	-
93-331	100	C	ZDV
93-353	910	A	ZDV
93-357	690	A	-
93-367	480	B	ZDV, ddI
93-374	200	B	ZDVT

*As determined routinely by flow cytometry. †According to Centers for Disease Control and Prevention 1993 classification. ZDV, zidovudine; ddI, didanosine.

Cell preparations

Lymphocytes were isolated from heparinized peripheral blood by Ficoll-Hypaque gradient centrifugation. Following two washes with phosphate-buffered saline (PBS), CD4+ lymphocytes were selected by immunomagnetic separation (MiniMACS; Miltenyi Biotec, Inc., Sunnyvale, California, USA), and suspended at 10^6 cells/ml in complete medium [RPMI-1460 supplemented with 10% fetal calf serum, 10 mM HEPES, 2 mM L-glutamine, 1% penicillin and streptomycin (GIBCO, Grand Island, New York, USA), and 2×10^{-5} M 2-mercaptoethanol (Sigma Chemical Co., St Louis, Missouri, USA)]. Separations by this method routinely yielded 96–99% pure CD4+ cell populations as determined by flow cytometry (data not shown). The CD4+ cells were stimulated for 3 days with 5 μg/ml phytohaemagglutinin (PHA; Murex Diagnostics Ltd, Dartford, England), 10 ng/ml phorbol myristate acetate (PMA; Sigma Chemical Co.) in combination with 500 ng/ml ionomycin (Sigma Chemical Co.), or with 1 μg/ml purified plate-bound anti-CD3 antibody (Serotec, Toronto, Ontario, Canada). After 3 days, the cells were washed with PBS and resuspended in complete medium supplemented with 5 U/ml recombinant human interleukin-2 (rhIL-2; Genzyme, Cambridge, Massachu-

setts, USA) for a further 3 days. The timepoint for ^{51}Cr-release assays was chosen in order to optimize TCR-mediated cytotoxicity and maximal activation-dependent susceptibility of target cells.

Effector cells were generated either from the CD4 – fraction by a 7-day expansion with 5 µg/ml concanavalin A (ConA; Boehringer Mannheim Biochemicals, Indianapolis, Indiana, USA) and 5 U/ml rhIL-2 in complete medium or by stimulation with autologous, washed and irradiated (3000 rad) day-3 PHA blasts for 7 days in IL-2-containing medium. In some experiments, CD8+ lymphocytes were positively selected, and stimulation of the CD8 – fraction was undertaken as described above, except that soluble anti-CD3 antibodies were used.

^{51}Cr-release assays

CD4+ target cells stimulated with either PHA, PMA/ionomycin, or anti-CD3 antibody were labelled with 200 µCi $Na_2{}^{51}CrO_4$ (DuPont, Mississauga, Ontario, Canada) for 90 min at 37°C, washed three times, and 5×10^3 cells were added to duplicate or triplicate wells of 96-well round bottom microtitre plates (Nunclon, Roskilde, Denmark). Effector cells were added to targets for effector-to-target (E : T) ratios ranging from 50 : 1 to 10 : 1 in a final volume of 300 µl complete medium. Following a 5 h incubation period, 100 µl supernatant was removed for γ-counting. Spontaneous release was determined from wells with only target cells, and was below 30% in all assays. Maximal release was measured by acid lysis from wells with target cells only. Percentage specific lysis was calculated as follows: [(c.p.m. experimental release — c.p.m. spontaneous release)/(c.p.m. maximal release — c.p.m. spontaneous release)]×100.

Monoclonal antibodies used for blocking in ^{51}Cr-release assays were anti-CD3 (Serotec) and anti-MHC I (W6/32; Serotec; 1/100 dilution), and LA45 (1/100 dilution; generous gift of Dr W. Knapp, University of Vienna, Vienna, Austria), anti-CD4 (1/100 dilution) and anti-TCR αβ (Serotec).

DNA synthesis assays

CD4+ lymphocytes were cultured in complete medium at 10^6 cells/ml with PHA, PMA/ionomycin, anti-CD3 antibodies, or no stimulus. After 3 days, triplicate 100 µl aliquots from each culture were pulsed with 1 µCi of [^{3}H]-thymidine (New England Nuclear Corp., Boston, Massachusetts, USA) for 18 h, and cells were harvested onto glass fibre filter paper with a semi-automatic harvester. [^{3}H]-Thymidine incorporation was determined by liquid scintillation spectroscopy, and the stimulation index was calculated by dividing the c.p.m. incorporated into stimulated cells by the c.p.m. incorporated into unstimulated cells.

p24 assays

At weekly intervals after stimulation, 500 µl aliquots of supernatant from CD4+ lymphocyte cultures exposed to various mitogens were removed and replaced with complete medium and rhIL-2. Triplicate p24 antigen concentrations in supernatant were determined by commercial enzyme-linked immunosorbent assay (Organon Teknika, Durham, North Carolina, USA), and inter-assay control values were normalized to a minimum of 5 pg/ml.

Results

CTL-mediated lysis of CD4+ lymphocytes is dependent on activation

Peripheral blood mononuclear cells from 20 HIV-infected individuals were separated into CD4+ and CD4 – fractions and expanded with PHA and ConA, respectively, or expanded through stimulation with autologous PHA-blasts. Their proliferative capacity was measured by incorporation of [^{3}H]-thymidine, and lysis of autologous targets was assessed in a standard 5 h ^{51}Cr release assay. Results from five representative patients are shown in Fig. 1. Lysis of the target cells was dependent on cellular activation, because non-stimulated autologous cells were not susceptible. Proliferation as evaluated by DNA synthesis was required and correlated with the amount of ^{51}Cr released. These findings were representa-

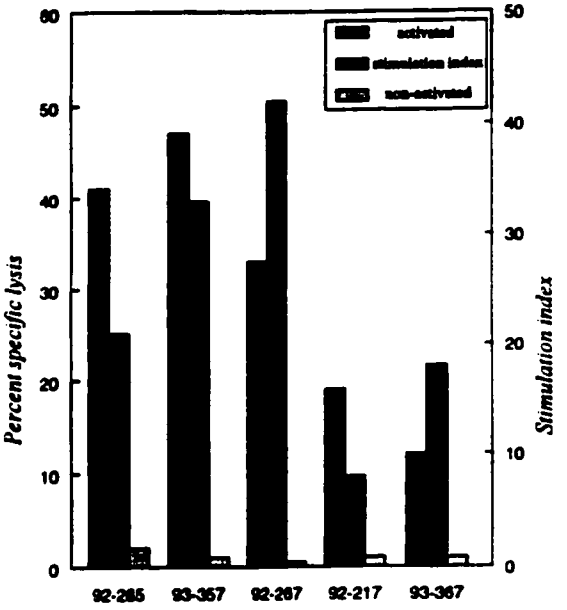

Fig 1. Activated and proliferating CD4+ lymphocytes are susceptible to lysis by autologous cytotoxic lymphocytes from HIV-seropositive patients (92-285, 93-357, 92-267, 92-217, 93-367); non-activated cells are not lysed. Replicate ^{51}Cr-release assays (effector-to-target cell ratio of 50 : 1) of patients' CD4 – cells against their autologous phytohaemagglutinin-stimulated CD4+ lymphoblasts correlated with the stimulation index determined by incorporation of [^{3}H]-thymidine.

Table 2. Lysis of autologous CD4+ lymphocytes by cytotoxic T lymphocytes from HIV-seropositive individuals is not inhibited by antibodies to major histocompatibility complex class I (W6/32, LA45) or CD4, but is reduced by antibodies directed against CD3 (OKT 3) or the αβ T-cell receptor (TCR).

Patient	E:T ratio							
	50:1	25:1	12.5:1	OKT3*	W6/32	LA45	OKT4	αTCR
93-374	31	29	-	8	33	28	28	0
92-267	33	25	17	10	42	30	-	-
91-196	35	32	28	6	43	-	33	4
94-412	20	17	7	0	20	-	22	2
92-276	37	36	23	18	39	34	-	-
91-155	48	37	31	10	51	44	-	-

*All antibodies were tested at effector-to-target cell (E : T) ratios of 50 : 1.

tive for all patients examined; however, the activity could not be detected with effector cells from control subjects. HIV-specific cytotoxic T lymphocytes did not lyse CD4+ blasts, indicating that distinct effector populations mediate lysis of activated CD4+ targets versus infected lymphocytes expressing HIV-derived peptides. Antibodies to the CD3 molecule or αβTCR inhibited the activity, indicating specific involvement of the TCR (Fig. 2; Tables 2 and 3). However, CTL-mediated lysis of autologous CD4+ targets could not be reduced by antibodies directed against MHC class I molecules (Table 2), although this antibody blocks HIV-specific CTL (Table 4). Neither W6/32, a conformation-dependent antibody recognizing class IA, B and C molecules, nor LA45, specific for β_2-microglobulin-free heavy chain expression [24], nor anti-CD4 antibodies inhibited lysis of the targets. Effector cells tested against autologous or heterologous Epstein–Barr virus (EBV)-transformed B- lymphoblastoid cell lines, Jurkat T-cell lines, or CEM.NK^r cell lines yielded specific lysis of less than 5% which was considered insignificant. However, lysis of 25–30% (E : T ratio of 100 : 1) that was not reducible with anti-CD3 antibodies, and therefore presumably mediated by natural killer cells and not TCR+ cells, occurred in experiments using Jurkat cells as targets.

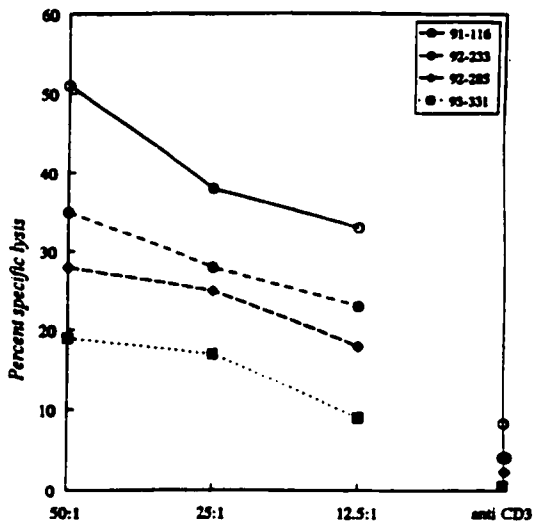

Fig 2. Lysis of autologous CD4+ cells in HIV-seropositive patients (91-116, 92-233, 92-285, 93-331) is inhibited by anti-CD3 antibodies. Phytohemagglutinin-activated lymphoblasts were lysed at different effector-to-target cell (E : T) ratios, and markedly inhibited by blocking antibodies in the assay (E : T ratio for anti-CD3 antibody assay = 50 : 1).

Different mitogens render target cells susceptible to lysis

In order to examine the effect of stimulating cells via different activation pathways, three different mitogens (PHA, PMA/ionomycin and CD3-crosslinking antibodies) were evaluated. The response to the mitogens was

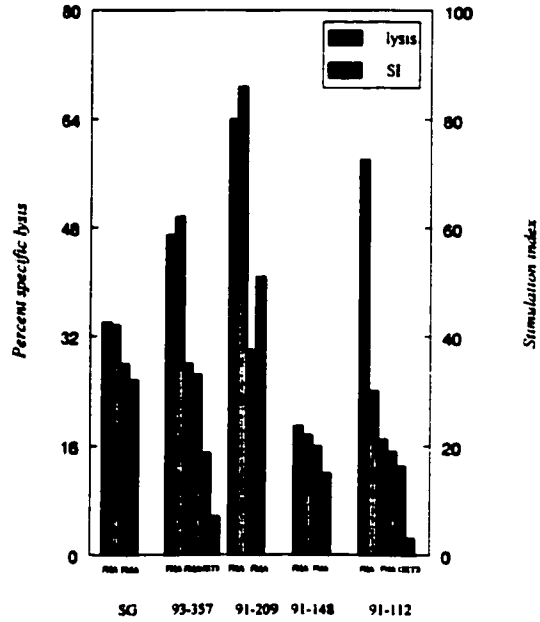

Fig. 3. Different mitogens render CD4+ lymphocytes susceptible to lysis by cytotoxic lymphocytes from HIV-seropositive patients (93-357, 91-209, 91-148, 91-112). Target cells were stimulated with phytohaemagglutinin (PHA; 5 μg/ml), phorbol myristate acetate (PMA; 10 ng/ml) and ionomycin (500 ng/ml), or anti-CD3 antibodies (1 μg/ml), and assessed for susceptibility to lysis after 5 days. Percentage specific release correlated with stimulation index (SI), and CD4+ lymphocytes from an HIV-seronegative individual (SG) are lysed to a similar degree as autologous CD4+ lymphoblasts by patient 93-357.

Table 3. Titration of OKT3 (patient 91-196) and anti-TCR αβ antibodies (patient 91-16) in autologous lysis of CD4+ lymphocytes at an effector-to-target cell ratio of 50 : 1.

Dilution	% Lysis
OKT3	
-	35
1 : 900	21
1 : 300	20
1 : 100	6
1 : 30	12
Anti-TCRαβ	
-	53
1 : 300	21
1 : 100	18
1 : 30	22

Table 4. Inhibition of HIV-specific cytotoxic T lymphocytes (CTL)* by W6/32 (patient 91-155) at an effector-to-target cell ratio of 50 : 1.

BLCL	% Lysis	W6/32 (1:100 dilution)
vac-pol	39	16
vac-env	29	15
vac-lacZ	9	10

*HIV-specific CTL were generated and tested against autologous, B lymphoblastoid cell lines (BLCL), as previously described [21].

measured by [^{3}H]-thymidine incorporation, and correlated with the degree of lysis by effector cells from HIV-seropositive individuals. CD4+ target cells from a seronegative individual (DB) as well as autologous CD4+ lymphocytes were tested against effector cells from an HIV-infected patient (91-148), and patient CD4+ target lymphocytes were tested against their autologous effectors (Fig. 3). Evaluation of proliferation after 3 days showed PHA consistently as the most effective mitogen, although significant cell activation was also achieved with the other mitogens. All activated cells were susceptible to lysis, although the degree of lysis correlated with the amount of DNA synthesis. Within the time-frame examined, stimulation via the CD3 molecule invariably resulted in the least amount of proliferation, and accordingly, only moderate recognition by effector cells. This may be partly due to a longer time requirement for blast transformation following stimulation via the TCR versus binding of a lectin or exposure to phorbol ester/ionophore. The lack of MHC restriction was confirmed by the equivalent degree of lysis of heterologous targets as well as allogeneic targets by CTL from patient 91-148 without prior *in vitro* allostimulation.

Lysis of activated CD4+ cells is independent of viral replication

Concurrent with testing in ^{51}Cr-release assays the CD4+ T-cell populations from patients were stimulated with PHA, PMA/ionomycin, or anti-CD3 antibodies, cultured for 3 weeks, and weekly supernatant aliquots were evaluated for release of soluble p24 antigen. Sur-

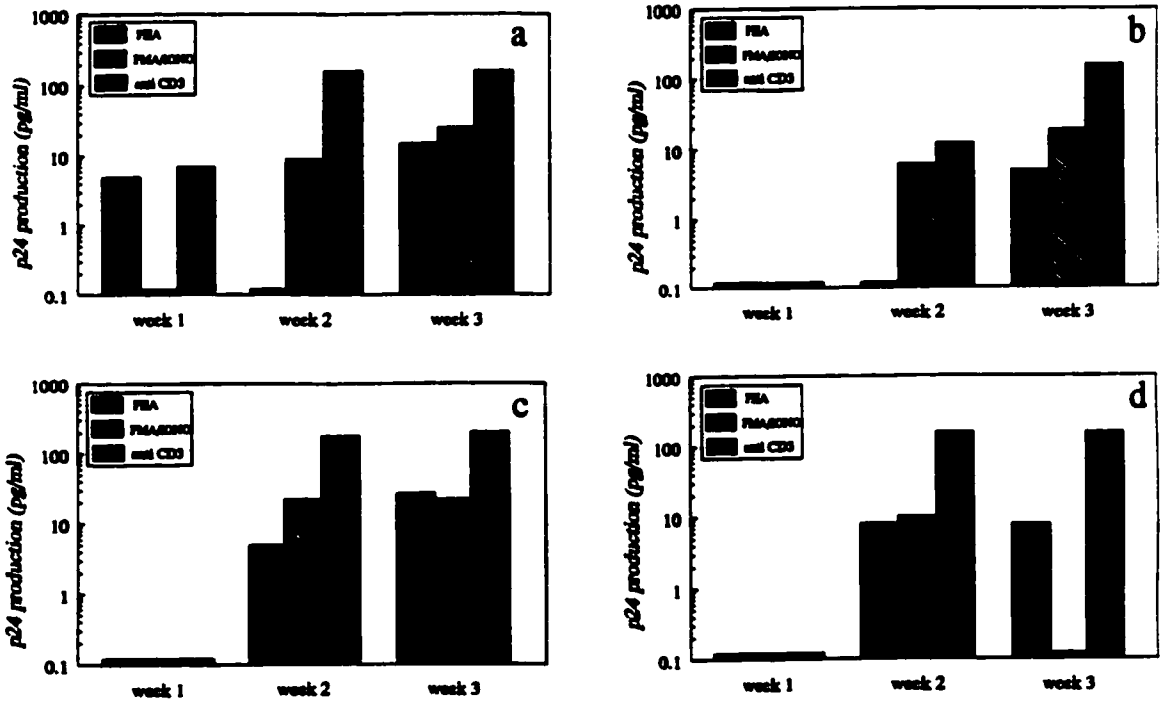

Fig. 4. Stimulation of CD4+ lymphoblasts from four HIV-infected individuals — (a) 91-16, (b) 91-06, (c) 93-353, (d) 92-242 — with different mitogens [phytohaemagglutinin (PHA) 5 μg/ml; phorbol myristate acetate (PMA) 10 ng/ml and ionomycin 500 ng/ml; anti-CD3 1 μg/ml] results in variable amounts of soluble p24 antigen release into the supernatant. Stimulation via the T-cell receptor consistently results in the greatest viral replication.

prisingly, for all patients examined, stimulation with antibodies to the CD3 molecule consistently resulted in the greatest amount of viral protein production (Fig. 4), although PHA activation was most effective at inducing proliferation (Fig. 3). The amount of viral p24 detected correlated with the stage of disease, patients at CDC stage A (1993 classification) commonly had no detectable antigen in supernatants during the first week, and occasionally not during the second week, although all patients' lymphocytes released large amounts of p24 antigen with anti-CD3 stimulation during the third week of culture. These results indicate that incorporation of [^{3}H]-thymidine and susceptibility to lysis by autologous CTL did not correlate with the degree of viral replication.

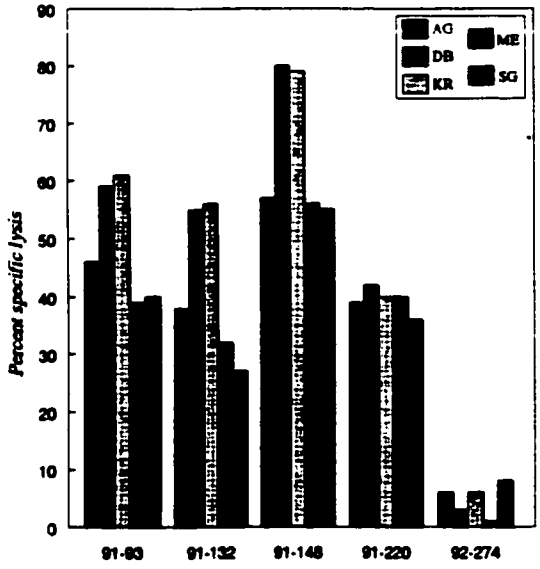

Fig. 5. The target molecule recognized by cytotoxic lymphocytes from HIV-seropositive individuals (91-93, 91-132, 91-148, 91-220, 92-274) on phytohaemagglutinin-lymphoblasts from a panel of HIV-seronegative individuals (AG, DB, KR, ME, SG) is non-polymorphic. The percentage specific release of ^{51}Cr is similar among different targets at an effector-to-target cell ratio of 40 : 1.

The target molecule is non-polymorphic

Effector cells from different HIV-infected patients were tested against a panel of CD4+ target lymphocytes from five HIV-seronegative individuals. The target cells were activated with PHA, and all experiments compared were performed simultaneously. Data presented in Fig. 5 demonstrate that effector CTL from HIV-infected individuals were capable of lysing panels of heterologous uninfected lymphocytes to an equivalent degree. Each CTL population from a patient either lysed a panel of targets at a relatively high level (patient 91-148), or at an insignificant level (patient 92-274), although within each CTL population this was consistent. These findings suggest that a non-polymorphic molecule expressed in a similar manner on activated CD4+ T cells from a diverse group of individuals was recognized by CTL from HIV-seropositive individuals. This effector activity resided within the CD8+ population and was directed against activated CD4+ cells as confirmed by immunomagnetic depletion of the respective cell populations (data not shown)

Discussion

In the present study we show that HIV-infected individuals possess a unique population of cytotoxic lymphocytes that are capable of lysing autologous or heterologous activated CD4+ lymphoblasts, but do not lyse EBV-transformed B-lymphoblastoid cell lines, Jurkat T-cell lines, or CEM cells. The effect is mediated by CD8 CTL, is not restricted by classical MHC molecules, and is distinct from anti-HIV CTL. The target molecule is unique to CD4+ lymphocytes, and is expressed in a non-polymorphic manner dependent on cellular activation as measured by DNA synthesis. Although this activity was determined in HIV-infected individuals, viral replication is independent of CTL-mediated lysis of CD4+ lymphocytes. Thus, these findings may not be unique to this viral infection but may be part of immunoregulatory processes active in a variety of disease states.

The occurrence of CTL-mediated lysis of activated CD4+ lymphocytes is reminiscent of the process of activation-induced cell death whereby the marked proliferation of antigen-specific lymphocytes is terminated or 'shut-off' by a process of apoptosis mediated among lymphocytes [15]. This process is executed by cells expressing the Fas ligand, and those lymphocytes with the Fas receptor are susceptible [25]. Examples of the malfunction of this apparent system of limiting antigen-driven lymphocyte expansion are illustrated in mice homozygous for the *lpr* mutation resulting in a defective Fas molecule and mice with an inactive Fas ligand (*gld* mutation). In both types of mice massive accumulations of terminally differentiated lymphocytes with an unusual phenotype occur in the lymphoid organs, and they die prematurely with profound organomegaly [26]. Cytotoxic lymphocytes specific for proliferating cells with subsequent DNA fragmentation in the targets have as well been described following mitogen stimulation, transformation with c-*myc*, and infection with herpes simplex virus-1 [27], and it is conceivable that aberrant activation of such a phenomenon may contribute to the development of cytopenia in HIV patients. Because it has been difficult to discern effectors from target cells in the above described system of activation-induced cell death among various types of lymphocytes, it remains unclear at present as to whether this pathway of CTL-mediated cell killing is MHC-restricted.

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From the data presented here it is apparent that activation of CD4+ lymphocytes results in expression of a molecule that is recognized by CTL from HIV patients, that specific recognition involves signal transduction via the TCR, and that expression of the molecule ensues from stimulation of several different pathways. The lytic activity is independent of viral replication and involves recognition of a non-polymorphic molecule. The possibility of assaying non-specific cytotoxic activity has been eliminated through the use of α-methyl mannoside, the natural ligand for ConA (data not shown), and through washing and resting of the targets for 2 days prior to the assay. Therefore, the lytic process appears specific for a proliferation-associated molecule and not restricted by conventional MHC class I antigens. There are several examples of microbial infections evoking the generation of non-restricted CTL [28–30], and recently restriction of αβ+ T cells to CD1, a non-polymorphic molecule, has been described [31]. The latter interaction involved presentation of a non-protein antigen of *Mycobacterium tuberculosis* to T cells in the context of the non-classical human MHC molecule CD1. Even more recently, non-MHC-restricted CD8+ T-cell-mediated cytolysis directed against specific TCR Vβ peptides expressed on activated, autologous CD4+ lymphocytes has been described [32]. Although the nature of the antigen recognized in the activity described here has yet to be elucidated, the lack of association with HIV viral protein expression and independence from MHC restriction suggest the target is a unique molecule not specific for a microbial infection.

The preferential induction of viral replication following stimulation via the TCR has been described previously [33], although the authors of that study described a marked difference between stimulation via soluble or immobilized anti-CD3 antibodies. In the present experiments stimulation of positively purified CD4+ cells with immobilized antibodies, or of negatively selected CD8 – populations with soluble antibodies both resulted in p24 production approximately 100-fold higher than stimulation with a lectin or with phorbol ester/ionophore (Fig. 4). Part of the difference of these findings may be accounted for by the inconsistent depletion of CD8+ lymphocytes in the experiments of Moran *et al.* [34], as even low numbers of CD8+ lymphocytes may transcriptionally suppress HIV replication. However, enhancing interaction with other cells in the mononuclear fraction of blood cells may also occur, or the sensitivity of our experiments to detect small differences in p24 production may not have been sufficient.

The differential effect of the different mitogens regarding induction of viral replication reflects unique stimulation of the viral long terminal repeat (LTR). Although similar signals may be transduced via the CD3-ζ chain-associated tyrosine kinase *ZAP 70*, or through direct activation of protein kinase C and release of intracellular calcium by PMA and ionophore, respectively, modulating intracellular messenger molecules may result in preferential induction of transcriptional factors acting on the HIV LTR. Examination of protein phosphorylation patterns following stimulation of T cells with the above mitogens has shown pronounced differences between anti-CD3 stimulation, and exposure to PMA/ionomycin or lectins [35], lending support to the observations in this study that signals via the TCR induce a uniquely potent signal for viral transcription.

In summary, the findings of the experiments described here suggest that CTL directed against activation markers on CD4+ lymphocytes contribute to the pathogenesis of HIV infection. Such anti-ergotypic cells have been described in the pathogenesis of several autoimmune diseases [22,23], and experimental transfer of effector cells in model systems has reproduced the cell-mediated immunopathology [36]. Therefore, we hypothesize that in HIV infection excessive downregulation of activated lymphocytes occurs and that development of CD4+ lymphocytopenia is only partially due to direct viral effects. Virally induced abnormalities in the immunoregulatory system are self-perpetuating and result in the abnormal destruction of lymphocytes by CTL specific for activation molecules. Further work will be directed at identifying the target molecule, and the mechanism of the interaction.

Acknowledgement

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Chapter 3.

CD8$^+$ cytotoxic T-lymphocytes induce apoptosis of CD4$^+$ cells in human immunodeficiency virus-infected individuals.

(Bienzle et al., manuscript in preparation)

In this manuscript the mechanism whereby HIV-expressing target cells, or uninfected CD4$^+$ T cells, are lysed by CTL from HIV-infected individuals, was investigated. Effector cell function was assessed in parallel against target BLCL infected with various recombinant vaccinia virus vectors expressing HIV gene products, and against autologous and heterologous activated CD4$^+$ cells. Biochemical inhibitors of perforin-mediated pathways and Fas-mediated pathways were employed, and the target cell cytoplasm and nuclei were labelled specifically in order to detect differential lytic effects. Finally, DNA fragmentation into nucleosome-sized segments was assessed by flow cytometric analysis and by gel electrophoresis.

The results of the experiments indicate that CTL from HIV-infected individuals were able to lyse different target cells by different mechanisms. Recognition of HIV-derived peptides expressed on BLCL resulted in lysis by mechanisms characteristic of perforin, while interaction with CD4$^+$ cells induced apoptosis in the target cells in a manner consistent with Fas-FasL pathways. These findings suggest that CTL with different specificities co-exist in HIV infection, and may mediate presumably beneficial

effects by lysing infected target cells, as well as deleterious effects by destroying uninfected, but activated, $CD4^+$ T cells.

The experiments described in this chapter were performed by the author of this dissertation. For flow cytometric analysis, the samples were prepared and stained, and the results analysed, by the author, however, the fluorescence activated cell sorter with ultraviolet illumination capacity was operated by David Tinney in the McMaster hospital laboratory.

Summary

Cytotoxic T lymphocytes (CTL) in HIV infection mediate variable effects. Maintenance of the long asymptomatic phase is ascribed to efficient lysis of infected cells by CTL, and to suppressive factors elaborated by noncytolytic $CD8^+$ T cells. On the contrary, adverse effector functions consisting of lysis of uninfected $CD4^+$ cells and non-lymphoid cells have been described, and functionally relate to increased immunopathogenesis. In the experiments described here it was attempted to discern differential cytolytic activities of CTL against uninfected $CD4^+$ cells, and against target cells expressing HIV-derived antigens. Functional, biochemical and morphologic examination of the different interactions indicated that CTL lysis of uninfected, activated $CD4^+$ cells involved apoptosis, while recognition of HIV-antigens in the context of MHC class I molecules resulted in cell death by preformed mediators. These findings were substantiated by evaluating cytoplasmic versus nuclear damage in different target cells, by blocking the availability of calcium, by sensitivity to endonuclease and protease inhibition, and finally, by assessing DNA fragmentation. The results suggested that indeed CTL with differential lytic ability were present in HIV-infected individuals, and lysis of uninfected cells may thus contribute to accelerated loss of $CD4^+$ cells.

Introduction

Cytotoxic T lymphocytes (CTL) are thought to account for the control of plasma viremia and for the long clinically silent period characteristic of HIV infection. *In vitro* measurements of CTL function indicated that HIV infection results in unusually high frequencies of CTL precursors, and that strong CTL responses correlate with prolonged disease-free intervals (1). Early expansion of $CD8^+$ T cells with oligoclonal T cell receptors (TCR) has been associated with more complete viral reduction and improved survival (2). Nevertheless, the persistent presence of strong CTL responses during the terminal phase of HIV infection illustrates the eventual ineffectiveness of CTL to curtail viral spread and disease progression. Substantial doubt regarding the universally beneficial aspects of CTL in HIV infection ensued from the *in vivo* infusion of Nef-specific, *in vitro* expanded CTL to an HIV-infected patient which resulted in profound deterioration of the patient's condition (3). Thus, CTL in HIV infection have variable effects: destruction of virally infected cells with eventual escape of the virus from immune surveillance resulting in an immune response that is "lagging" in specificity and terminally fails, or, induction of $CD4^+$ cell death due to indirect viral effects. Measurements of apoptosis, CTL responses directed against non-virally derived components, and "immune exhaustion" manifested by suppression of $CD4^+$ precursor production correlated with disease progression (4,5,6,7) but were not attributable to conventional antigen recognition in the context of MHC class I molecules, or with target cell lysis by CTL. Daily virus production has been estimated at 10^8 to 10^9 per day, and viral dynamics in response to protease therapy of infected individuals correlated with a turnover rate of $CD4^+$ cells of 1 to $70x10^8$ per day (8,9).

Though these calculations suggested that destruction of infected cells by cytotoxic T lymphocytes (CTL) accounts for the high rate of $CD4^+$ lymphocyte turnover, the persistent net loss of 2 to $20x10^7$ $CD4^+$ cells per day is not reconcilable with these figures, nor with the limited number of cells actually infected (10). Thus, indirect mechanisms are likely to contribute to the ongoing net loss of $CD4^+$ cells that distinguishes HIV infection from other viral infections of the immune system that are terminated within short order.

Apoptosis is a physiological process of cell death that may be initiated by extracellular molecules binding to cell surface receptors, or by interference with intracellular pathways activating a cascade of specific proteases (11). In HIV infection, apoptosis has been identified in infected as well as uninfected cells of the immune system, and inducing ability has been ascribed to various cellular and viral components. The HIV Tat protein by itself, or in combination with gp120, induced cell death in T-cell lines and uninfected PBMC (12,13), while co-expression of CD4 with gp160 resulted in apoptosis in a monocytoid cell line (14). Cross-linking of the CD4 molecule on uninfected PBMC alone (15), or superantigen or mitogen activation of $CD4^+$ cells from HIV-infected individuals, have been reported as sufficient to induce programmed cell death (16). The Fas molecule, TNF, and lymphotoxin have all been described as mediators of apoptotic cell death in HIV infection (17,18,19,20). Though some findings on the occurrence of apoptosis in HIV infection appeared contradictory, consensus prevailed regarding the involvement of uninfected as well as infected lymphocytes, and the requirement for cell activation (21,22). Strong support for the relevance of apoptosis to disease pathogenesis has been demonstrated by *in vivo* occurrence of programmed cell death in the lymph nodes

of HIV-infected persons (23), and by the absence of such cell death in nonpathogenic lentiviral infections (24).

HIV-infected individuals have vigorous CTL responses against virally-derived peptides, but as well have CTL that react with structural proteins such as vinculin, which is not associated with HIV-1 (25), and CTL that recognize uninfected activated $CD4^+$ cells (26,27). Cytolytic responses directed against uninfected $CD4^+$ cells were unique to HIV infection of humans and were not present in chimpanzees (28). Target cell activation was a prerequisite, and the target molecule recognized by CTL was non-polymorphic (29). In this study we sought to examine whether $CD8^+$ T cell-mediated lysis of activated $CD4^+$ cells had features consistent with apoptosis, and whether this was distinct from the recognition and lysis of cells expressing HIV-derived antigens. We found that the interaction of CTL derived from HIV-infected individuals with activated $CD4^+$ cells was distinct from the recognition of HIV-derived peptides in the context of MHC class I molecules, was associated with DNA fragmentation within a 5 hour assay period, and was sensitive to endonuclease inhibition. Morphologic and biochemical features characteristic of apoptosis were present in the target cells. These findings represent a novel aspect of $CD8^+$ T cell function in HIV infection, and may account for the activation-dependent loss of $CD4^+$ cells observed in the disease.

Materials and Methods

Cell preparations

Heparinized blood samples from 19 HIV-seropositive patients were obtained with informed written consent. HIV^- control blood samples originated from laboratory personnel and from an individual during resolution of acute infectious mononucleosis. HIV-patients' $CD4^+$ lymphocyte counts ranged from 0 to 200 (n= 2), 200 to 500 (n= 12), and over 500 (n= 5) per µl. PBMC's were isolated by gradient centrifugation and washed twice with PBS. Cells were separated immunomagnetically (MiniMACS; Miltenyi Biotec, Inc., Sunnyvale, California, USA) into $CD4^+$ and $CD4^-$ fractions yielding routinely 97 to 99% pure $CD4^+$ populations (data not shown). In some cases, $CD8^+$ cells were positively purified. $CD4^+$ cells were cultured with PHA (5 µg/ml; Murex Diagnostics Ltd., Dartford, England) and rhIL-2 (10 U/ml; Genzyme, Cambridge, Massachusetts, USA) in RPMI supplemented with 10% FCS, 10mM Hepes, 2mM L-glutamine, 1% penicillin and streptomycin. After 3 days, the $CD4^+$ PHA blasts were washed, one fraction was irradiated at 2500 rads, and mixed with the $CD4^-$ fraction at a 1 to 10 ratio, the other fraction was cultured in rhIL-2-supplemented medium. BLCL were generated by standard transformation with Epstein-Bahr virus (EBV), and for cytotoxicity assays were infected overnight at a multiplicity of 15 to 1 with recombinant vaccinia virus vectors expressing the HIV_{IIIB} or HIV_{MN}-derived *env, gag,* or *pol* genes, or a *β-galactosidase* gene (all obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. B. Moss). Effector cells from HIV-infected individuals were generated during 7 days of culture with autologous irradiated $CD4^+$ PHA blasts, and then

were tested against heterologous and/or autologous $CD4^+$ target cells, and against autologous BLCL infected with vaccinia vectors. For allocytotoxicity assays the effector cells were generated from 8 to 9-day cultures of 10^7 responder PBMC with 10^6 irradiated stimulator PBMC. Anti-EBV CTL were detected directly from freshly isolated PBMC against autologous BLCL.

Cytotoxicity assays

Release of ^{51}Cr into the culture supernatant was used to assess cytoplasmic damage, and ^3H-thymidine activity was counted to indicate nuclear disintegration. Target $CD4^+$ PHA blasts, or autologous BLCL, were doubly labelled with 1μCi ^3H-thymidine (New England Nuclear Corp., Boston, Massachusetts, USA) for 18 hours, and with 200 μCi $Na_2{}^{51}CrO_4$ (DuPont, Mississauga, Ontario, Canada) for 90 minutes. Stimulator BLCL and autologous BLCL were used as target cells for allocytotoxicity assays and EBV-specific assays, respectively. The target cells were washed 3 times, and incubated with effector cells for 5 hours in duplicate or triplicate wells (depending on the number of cells available) in 300 μl of medium. Effector to target cell ratios ranged from 50:1 to 10:1. Control wells consisted of target cells alone, target cells and reagants, and target and effector cells with the following blocking antibodies: anti-CD3 (OKT3), anti-MHC class I (W6/32) or anti-CD8 (OKT8; all from Serotec, Toronto, Ontario, Canada) . The radioactivity in 100 μl aliquots of supernatant was determined by β and γ counting. Percent specific lysis of maximum lysis was calculated by subtracting spontaneous release from experimental and acid-treated wells. Only experiments with spontaneous ^{51}Cr and ^3H-thymidine release of less than 25% and less than 15%, respectively, were included.

Chromium release from control BLCL infected with *βgal*-expressing vaccinia virus vectors was deducted from experimental values when assessing HIV-specific lysis.

Reagants

In order to characterize the biochemical pathways involved the following reagants were tested during the 5 hour assay at the indicated concentrations: aurintricarboxylic acid (ATA, dissolved in absolute ethanol), $ZnSO_4$ and EGTA (diluted in water) (Sigma Chemical Co., St. Louis, Montana, USA). Stock solutions (10 mM) of the protease inhibitors PMSF and leupeptin (LP; Sigma) were prepared in DMSO and diluted with complete medium for final test concentrations ranging from 0 to 1 mM. 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA-AM, Molecular Probes, Inc., Eugene, Oregon, USA) dissolved in DMSO at a final concentration of 0.5 μM was added to the target cells for 90 minutes prior to the assay, and during the 5 hour incubation period. Addition of the reagants to target cells alone did not result in significant ^{51}Cr or ^{3}H-thymidine release.

Flow cytometry

For flow cytometric detection of apoptosis the cytotoxicity assay conditions were approximated by incubating 10^7 effector cells with 10^6 $CD4^+$ PHA blasts or BLCL for 5 hours at 37° C. The cells were then washed once with PBS/FCS and stained with 1 μg/ml of the fluorescent dye Hoechst 33342 (H33342, Molecular Probes) for 15 minutes at 37°C and for 15 minutes on ice. A CD4-FITC antibody (Serotec) was added for 15 minutes on ice, and propidium iodide (PI) at 2.5 μg/ml for 5 minutes on ice. For detection of BLCL

an antibody to CD19 and a secondary FITC-goat anti-mouse antibody (Serotec) were used. After a final wash and fixation in 1% paraformaldehyde 50,000 cells were analyzed using a FACSTAR® (Becton Dickinson and Co., Mountainview, California, USA) instrument equipped with uv and argon laser beams, with gating on live lymphocytes by forward and side scatter. Apoptotic cells were enumerated by measuring H33342 staining intensity in CD4-FITC or CD19-FITC positive and PI negative cells. The apoptotic state of H33342 bright-staining cells was confirmed in some experiments by measuring the proportion of hypodiploid DNA with hypotonic PI staining (30). Control samples analyzed in each experiment consisted of $CD4^+$ target cells or BLCL alone, effector cells alone, and effector-target cell mixtures prior to incubation.

DNA fragmentation analysis

10^6 cells were pelleted and lysed in 0.5% Triton X-100, 10mM Tris (pH 7.5), and 1mM EDTA (pH 8.0). High and low molecular weight DNA was separated by centrifugation (12,000 x g) for 10 minutes, and precipitated with NaCl (final concentration 0.5M) and 1 volume of isopropanol at -20°C. Following two ethanol washes the samples were dissolved in Tris/EDTA buffer (pH 8.0), and analysed by electrophoresis in 1.5% agarose gels stained with ethidium bromide. Samples consisted of $CD4^+$ cells and BLCL alone, effector and target cell mixtures that had been incubated for 5 hours at 10:1 ratios, $CD4^+$ cells immunomagnetically isolated at the end of the 5 hour assay, and effector cells alone. Immunomagnetic selection of $CD4^+$ cells in itself did not result in detectable DNA fragmentation.

Statistical Analysis

Differences in the sample means were compared with Student's t-test.

Results

Cytolysis directed against activated CD4⁺ cells is distinct from HIV-specific reactivity

We first sought to determine whether effector cells generated by stimulation with autologous CD4⁺ PHA blasts were able to lyse CD4⁺ cells as well as cells expressing HIV-derived antigens. Parallel ^{51}Cr release assays using uninfected activated CD4⁺ target cells, as well as autologous BLCL infected with recombinant vaccinia vectors expressing *env*, *gag* or *pol*, indicated that two distinct CD8⁺ effector mechanisms were present. In 10 of 19 patients examined CTL were detected that killed activated CD4⁺ target cells but were not reactive against BLCL expressing *env*, *gag*, or *pol*-derived products (Fig. 1a and b). The converse was noted as well, in several individuals responses directed specifically against HIV-derived antigens were not accompanied by lysis of activated CD4⁺ cells (Fig. 1c), or, in one individual, both effects were seen concurrently (Fig. 1d). A similar degree of specific lysis was observed with autologous or heterologous CD4⁺ target cells (Fig. 1a and 1d). Both types of cytotoxicity could be abolished if blocking antibodies to the CD3 or CD8 molecules were included in the assay, however, only lysis of BLCL was blocked by

W6/32 (data not shown). These findings indicated that CD8⁺ T cells in HIV infection were capable of recognizing a target specific for activated CD4 cells, as well as conventional HIV-derived antigens in the context of MHC class I antigens. Equivalent lysis of autologous and heterologous CD4⁺ PHA blasts confirmed the lack of MHC restriction previously reported (29). Thus, cytotoxic effector cells with multiple specificities reside within the CD8⁺ cell fraction from HIV-infected individuals.

Cytoloysis of activated CD4⁺ cells is characterized by nuclear damage

In order to examine the mechanisms of the cytotoxic interactions, the target cells were labelled with ^{51}Cr and ^{3}H thymidine, and incubated with effector cells for a 5 hour assay period. Release of ^{51}Cr and ^{3}H thymidine into the supernatant was measured to indicate cytoplasmic and nuclear disintegration, respectively. In all experiments, killing of BLCL expressing HIV antigens was associated with release of ^{51}Cr into the supernatant, however, little ^{3}H-thymidine was detected (Table 1). On the contrary, lysis of activated CD4⁺ cells consistently resulted in specific release of ^{51}Cr as well as ^{3}H-thymidine (Table 1). On some occasions, the relative release of ^{3}H thymidine exceeded the amount of ^{51}Cr, indicating that nuclear disintegration may have predominated in the lytic interaction.These results suggest that CD8⁺ T cells specifically lyse activated CD4⁺ cells by mechanisms involving early nuclear and cytoplasmic damage, while within the time frame of these experiments absence of nuclear disintegration characterizes the class I MHC-restricted killing of autologous BLCL expressing HIV-derived antigens. Therefore, lysis of different target cells appeared to be associated with distinct effector mechanisms.

Selective inhibition of CTL killing

BAPTA-AM is a compound that specifically chelates intracellular Ca, while EGTA chelates divalent cations predominant in the extracellular environment (31). Preincubation of target cells with BAPTA-AM, or addition of EGTA to the assay, nearly completely abrogated cell lysis of activated CD4⁺ cells as well as of HIV-expressing BLCL (Fig. 2a). Thus, in these experiments, both types of CTL effector mechanisms depended on the availability of Ca.

The effector phase of cytolysis is mediated by proteases acting on cytoplasmic and nuclear components. Selective inhibition of protease interaction with specific substrates has been shown to block programmed cell death, as well as to restore immune function in cells from HIV-infected individuals (32). The effect of two classes of protease inhibitors on the different cytotoxic interactions were examined by adding leupeptin, a broadly acting cysteine protease inhibitor, and PMSF, a serine protease inhibitor, to the assays at various concentrations. A dose-dependent effect was observed with both compounds, however, greater inhibition occurred with leupeptin in both types of assay (Fig. 2b). For all experiments combined, inhibition of cytolyis of activated $CD4^+$ cells was significantly greater than inhibition of autologous BLCL-killing ($p<0.1$). These findings suggest that different proteases are involved in mediating the different effector functions of CTL from HIV-infected individuals, and that cysteine proteases are of greater importance in the interaction with activated $CD4^+$ cells.

To further dissect the pathways involved in the cytoxic interactions of CTL from HIV patients with different targets, the effects of ATA, a broadly acting endonuclease inhibitor (33), and of Zn^{++}, an effective inhibitor of DNA disintegration in certain cell types and with specific stimuli were examined (34). ATA markedly reduced the interaction between CTL and activated $CD4^+$ cells, while only a moderate effect on release of specific label was observed with BLCL as target cells ($p<0.01$) (Fig. 3). ATA was tested at concentrations ranging from 0.1 to 5 mM in each assay, and near maximal inhibition of cytoplasmic as well as nuclear damage was noted at 0.5 mM, while even 5 mM did not significantly affect lysis of BLCL. Similarly, Zn^{++} was tested at concentrations ranging

from 0 to 5 mM.The most profound inhibition was noted on killing of $CD4^+$ cells, however, a moderate reduction of lysis was as well observed in HIV-expressing cells, particularly with ^{51}Cr release ($p<0.02$ for ^{51}Cr, $p<0.01$ for ^{3}H) (Fig. 3a). Control experiments involving EBV-specific cytolysis indicated that this interaction was insensitive to inhibition by ATA and Zn^{++}, while allocytotoxicity was nearly completely inhibited (data not shown). The differential sensitivity of cytolysis of activated $CD4^+$ cells and HIV-expressing BLCL to endonuclease inhibition is suggestive of cell death being induced by different mediators.

Cytolysis of activated $CD4^+$ cells results in apoptotic nuclear changes

In order to confirm that the interactions of CTL with activated $CD4^+$ cells and HIV-BLCL result in distinct cellular changes the cytotoxicity assay was approximated by incubation of effector cells with target cells for 5 hours, and by subsequent flow cytometric evaluation of the degree of Hoechst 33342 fluorescence in subpopulations of cells identified by specific cell surface markers (35). Figure 4 shows a representative experiment of CTL incubated with CD4-FITC positive cells or CD19-FITC positive BLCL at time 0 and at the end of the 5 hour assay period. At the beginning of the assay (0 hours) the CD4 positive cell population was H33342 dull, while at 5 hours a marked shift in staining intensity was evident (Fig 4a, upper panel). Specific gating on the CD4 positive cells more clearly demonstrated the shift in H33342 staining (Fig. 4a, lower panel). These findings were confirmed in 10 separate experiments, and correlated with specific lysis in ^{51}Cr release assays. On the contrary, incubating CTL with CD19 positive BLCL as target cells revealed little change in the H33342 staining intensity (Fig. 4b). Thus, CTL lysis of

activated $CD4^+$ cells produced nuclear changes consistent with apoptosis in the target cells.

Electrophoretic analysis of DNA samples from the effector and target cell populations of both types of cytotoxicity assays substantiated the flow cytometric and chromium release assays: characteristic DNA laddering was seen specifically in the $CD4^+$ cell population isolated following incubation with CTL from HIV-infected persons (Fig. 5). No DNA cleavage was evident in BLCL isolated by CD19 immunomagnetic affinity. These data indicate that cytolytic interaction of CTL with different target cells may result in apoptosis in the target cells, or, may predominantly induce cytoplasmic damage.

Discussion

In this report we have demonstrated that CTL from HIV-infected individuals have different specificities, and may kill different target cells by apoptotic and non-apoptotic mechanisms. Lysis of activated $CD4^+$ cells was characterized by release of cytoplasmic and nuclear labels, was sensitive to endonuclease inhibition, and resulted in increased staining of fragmented DNA as well as chromatin breakdown into nucleosomal fragments. In contrast, specific lysis of autologous BLCL expressing HIV-derived gene products involved predominantly cytoplasmic damage, and within the time frame of this assay parameters of nuclear damage were minimally affected.

The main mediators of inducing lysis of target cells by CTL are perforin and members of the Fas/TNF family. Both effector mechanisms will result in DNA degradation terminally, however, perforin-induced cell damage is not characterized by the same apoptotic morphologic changes in the nucleus within as short a time frame. The importance of each effector mechanism is highly dependent on the target cells as well as on the timing of the interaction. In HIV infection, apoptotic changes have been identified in infected as well as uninfected $CD4^+$ cells *in vitro* as well as *in vivo* in lymph node biopsies (22,23). Though the importance of apoptosis in the loss of $CD4^+$ cells is evident, it is not clear whether programmed cell death is induced by extracellular Tat or gp160 or gp120 protein, by persistent cellular activation in the absence of sufficient co-stimulation and/or cytokines, or through aberrent expression of Fas or TNF molecules. Fas expression is upregulated in HIV infection on $CD4^+$ and $CD8^+$ lymphocytes, as well as on

macrophages (14,16). Killing of activated uninfected $CD4^+$ lymphocytes (36) via the Fas-FasL-dependent pathway has been shown for HIV-infected macrophages (37). The latter phenomenon appears to be akin to the lysis directed against uninfected activated $CD4^+$ lymphocytes observed in our experiments in that there is no apparent restriction to self-MHC molecules. Such reactivity may be mediated by Fas or by TNF, however, within the time frame of our experiments Fas is a more likely mediator (38,39). Killing of activated $CD4^+$ cells by $CD8^+$ dendritic cells in mice occurred via the Fas-FasL pathway (40), further suggesting that downregulation of proliferating $CD4^+$ cells may involve several effector cells utilizing a similar mechanism for inducing programmed cell death.

Generally, $CD4^+$ CTL are thought to kill predominantly through Fas, while $CD8^+$ CTL may use either Fas, TNF, or perforin (37). Fas-dependent killing is thought to be independent of extracellular Ca (41), however, in our experiments release of ^{51}Cr was dependent on the presence of Ca. This may reflect a requirement of Ca for cell movement, as direct cell-cell contact optimizes Fas-FasL interaction. Differential dependence on intracellular Ca availability for activation of granule exocytosis and for expression of FasL has been demonstrated following TCR stimulation, and correlated with distinct functional acitivity (41). Accordingly, our results were compatible with CTL from HIV-infected persons being able to use either an apoptosis-inducing molecule, or a preformed mediator such as perforin, depending on the target cell. Anti-$CD4^+$ cell lysis has all the features of activation-induced cell death, while killing of BLCL expressing HIV antigens is characteristic of predominantly cytoplasmic damage induced by preformed mediators, but lacking rapid intracellular signals for DNA disintegration.

The system of infecting autologous BLCL with recombinant vaccinia vectors expressing HIV-derived gene products is the most commonly utilized procedure for evaluating CTL responses in HIV infection. However, there are potential shortcomings of the assay leading to lack of detection of CTL: the HIV genes are derived from the cloned laboratory strains HIV_{IIIb} or HIV_{MN} that may have limited cross-reactivity with the epitopes predominant during the primary patient *in vivo* immune responses. Therefore, only responses directed against well-conserved epitopes would be detected, possibly explaining the lack of specific lysis in several of our assays. In addition, the cytolytic process following antigen recognition in the context of the appropriate MHC molecules on a B cell may be different than those of a T cell or a monocyte/macrophage, and the events observed here may not exactly mimic the *in vivo* recognition of HIV infected cells. Nevertheless, BLCL are susceptible to Fas-mediated killing (42), suggesting that the differences observed in CTL effector mechanisms in this study are representative of differently activated pathways depending on the antigen recognized.

In summary: in this report we show that CTL from HIV-infected individuals lysed different target cells through distinct effector mechanisms. Death in activated $CD4^+$ cells was characterized by the nucleosomal DNA fragmentation typical of apoptosis, and therefore likely involved the apoptosis-mediating FasL molecule. On the contrary, HIV-specific cell lysis was associated with predominantly cytoplasmic damage, and consistent with exocytosis of preformed mediators. These findings may account for the early impairment of T cell function, and the more gradual loss of $CD4^+$ T cell numbers characteristic of HIV infection, in that CTL eradicating virally-infected cells switch to

CTL inducing apoptosis of activated $CD4^+$ cells.

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Table 1. Differential cellular damage induced by CTL from HIV patients

Lysis of HIV-expressing target cells involves predominantly cytoplasmic damage, while lysis of $CD4^+$ cells is associated with nuclear disintegration. CTL from 4 HIV patients were incubated with doubly-labelled autologous and/or heterologous PHA-activated $CD4^+$ cells, and with autologous BLCL infected with vaccinia vectors. Release of radioactive label into the supernatant was counted, and specific lysis calculated.

	^{51}Cr release*					^{3}H-thymidine release *				
	target					target				
	autolog.	heterol.	BLCL§			autolog.	heterol.	BLCL§		
patient	$CD4^+$	$CD4^+$	env	gag	pol	$CD4^+$	$CD4^+$	env	gag	pol
94-391	39	ND	21	17	8	31	29	5	3	4
93-354	15	ND	37	24	19	26	ND	3	4	3
94-436	34	48	27	29	13	42	36	0	1	1
94-419	6	5	26	21	18	10	12	2	0	0

* Percent specific release at effector to target ratios of 50:1.

§ Percent specific lysis for control infections with *β gal* vectors has been deducted.

Legend

Figure 1. Anti-CD4$^+$ cell lysis can occur independent of HIV-specific cytotoxicity.

CD8$^+$ effector cells from four different patients (a=91-155, b=94-391, c=SC, d=94-436) were tested against autologous activated CD4$^+$ cells (-●-), heterologous activated CD4$^+$ cells (-○-), and BLCL infected with recombinant vaccinia vectors expressing *env* (-▲-), *gag* (-▼-), or *pol* (-◆-).

Figure 2. CTL lysis is Ca-dependent and differentially sensitive to protease inhibition.

EGTA and BAPTA-AM inhibit *env*-specific and anti-CD4$^+$ cell lysis (a), while both interactions are partially sensitive to the protease inhibitors PMSF and leupeptin. The target cells were incubated with 5 μM BAPTA-AM overnight, and during the assay, EGTA was added during the assay at a final concentration of 3 mM, PMSF and leupeptin at 300 μM and 100 μM, respectively. Mean percent inhibition of specific lysis for 8 patients at effector to target ratios of 50:1 is shown.

Figure 3. Endonuclease inhibition reduces lysis of activated CD4$^+$ cells.

The endonuclease inhibitors ATA and Zn^{++} affect HIV-specific killing minimally (a), while lysis of activated CD4$^+$ cells is reduced (b). ATA and Zn^{++} were added to the assays at 0.5 mM and 2.5 mM, respectively. Inhibition of specific lysis for 5 different patients at effector to target ratios of 50:1 are shown.

Figure 4. Activated CD4⁺ cells undergo apoptosis during incubation with CTL.

Incubation of CTL from HIV-infected individuals induces apoptosis in CD4⁺ target cells, but not in autologous *vac-env* infected BLCL. CTL were cultured for 0 or 5 hours with activated CD4⁺ target cells (a), or with BLCL (b), stained with Hoechst 33342 and anti-CD4FITC or anti-CD19FITC, respectively, and analyzed for apoptosis by multiparameter flow cytometry.

Figure 5. CD4⁺ cells, but not CD19⁺ BLCL, have fragmented DNA following incubation with CTL from HIV-infected patients.

CD4⁺ or CD19⁺ cells were incubated with CTL for 0 hours, and for 5 hours, were immunomagnetically re-isolated from the cell mixture, and genomic DNA from 10^6 cells was extracted. The samples were analysed by gel electophoresis and examined for DNA fragmentation.

Figure 1.

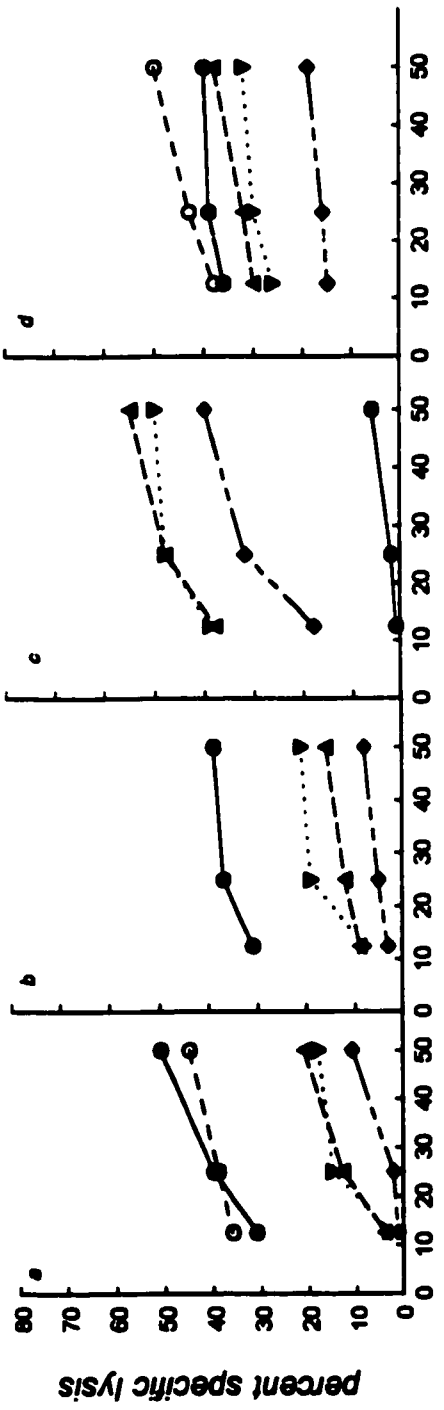


Figure 2.

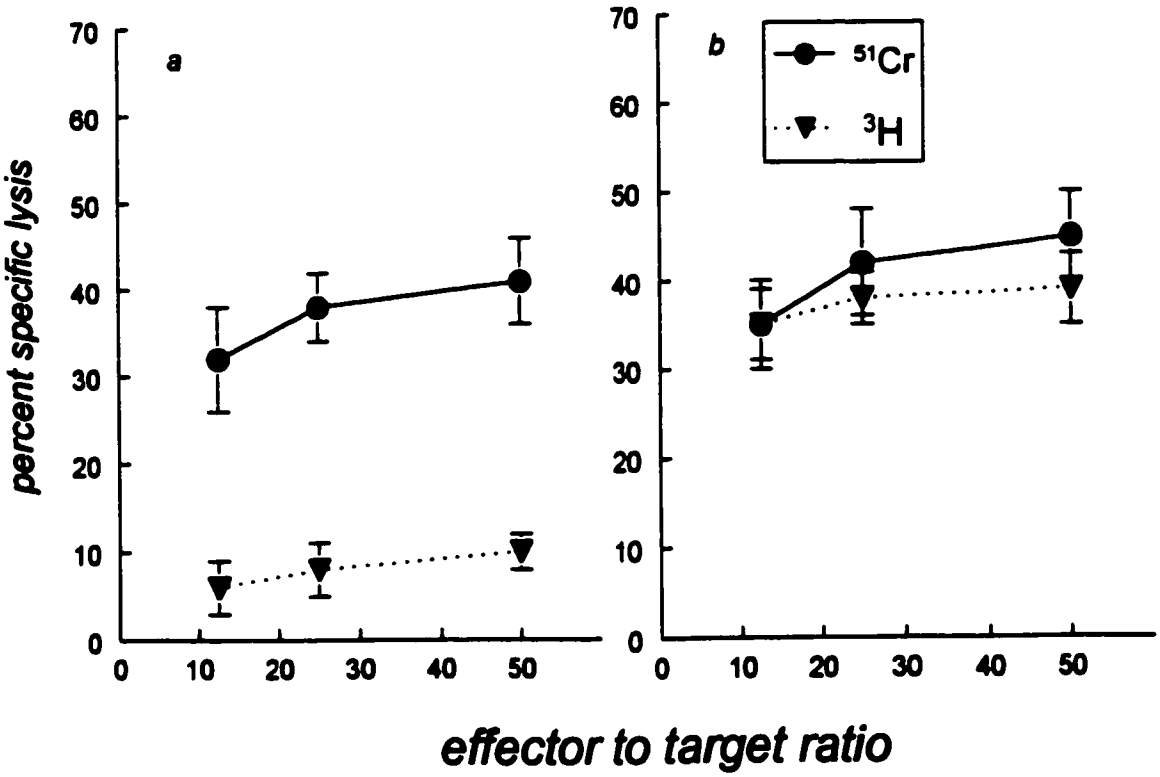

a
b
^{51}Cr
^{3}H
percent specific lysis
effector to target ratio
0
10
20
30
40
50
60
70

Figure 3.

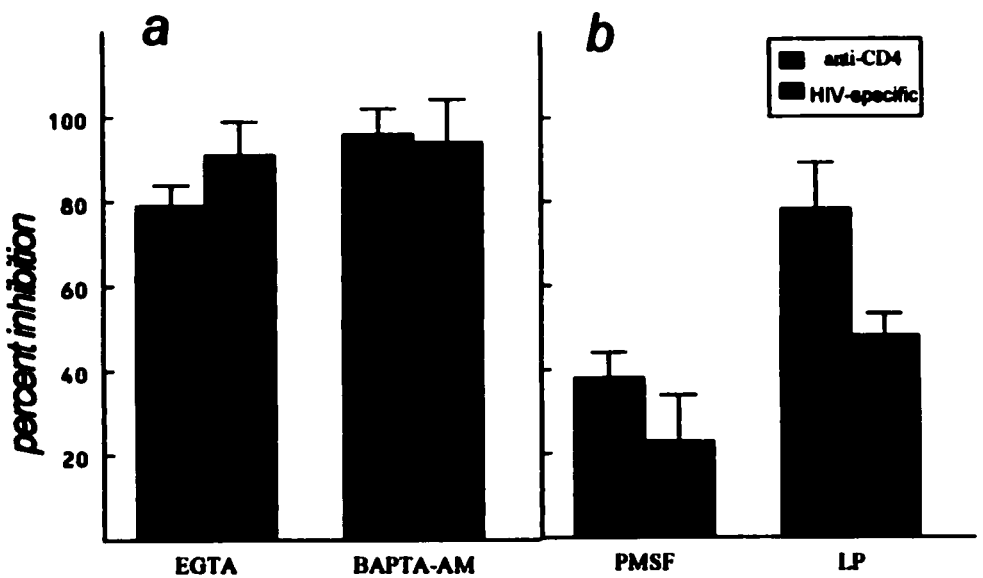

a
b
anti-CD4
HIV-specific
percent inhibition
100
80
60
40
20
EGTA
BAPTA-AM
PMSF
LP

Figure 4.

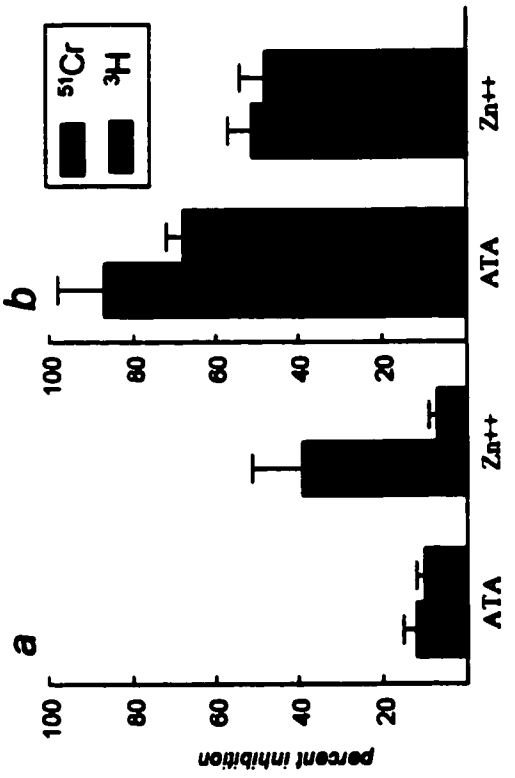
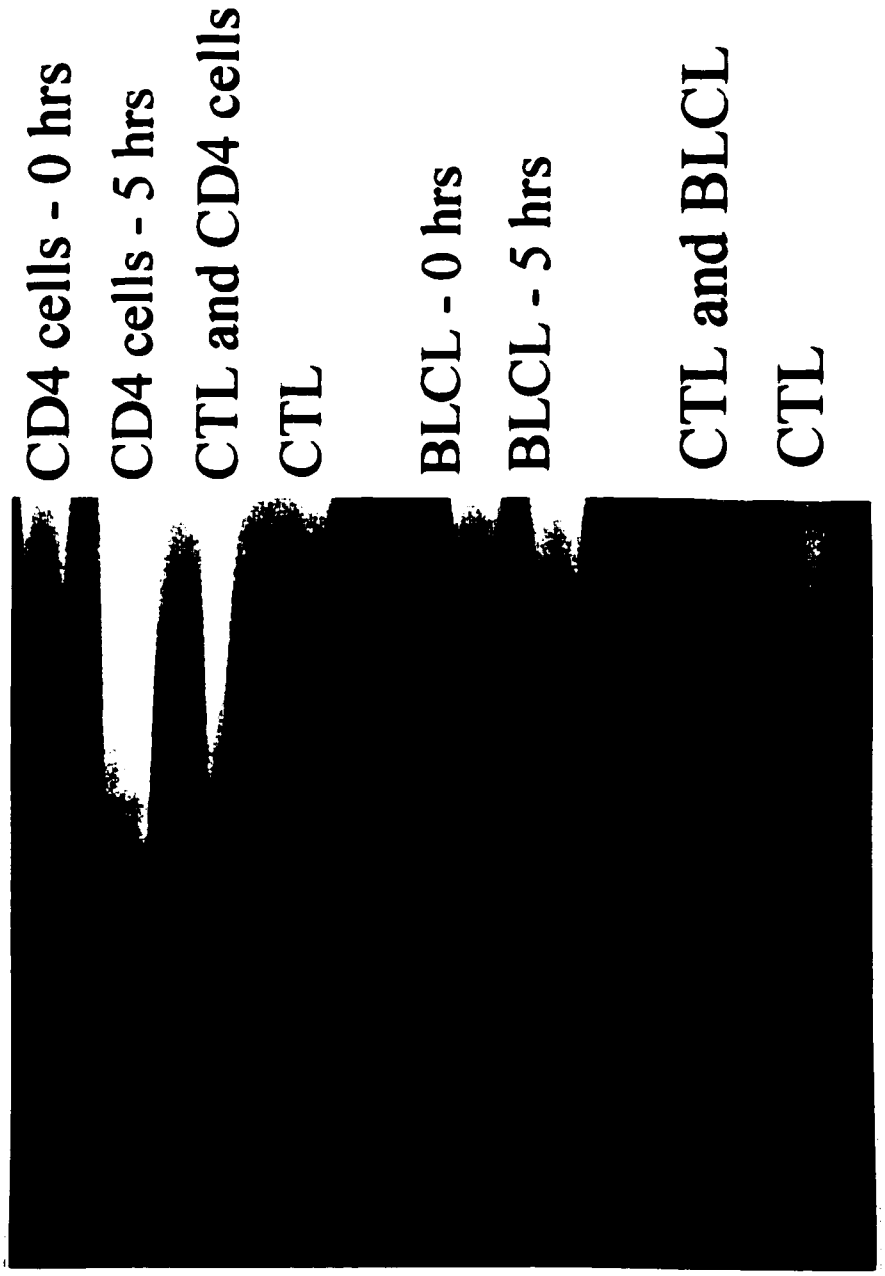

a
b
100
80
60
40
20
percent inhibition
ATA
Zn++
ATA
Zn++
51Cr
3H
HIV-specific
anti-CD4

Figure 5.


CD4 cells - 0 hrs
CD4 cells - 5 hrs
CTL and CD4 cells
CTL
BLCL - 0 hrs
BLCL - 5 hrs
CTL and BLCL
CTL

Chapter 4.

Multiple factors contribute to lack of infection by HIV-1 in exposed-uninfected individuals: the role of CCR5 genotype and primary isolate-specific cytotoxic T-lymphocyte responses.

(Bienzle et al., manuscript in preparation)

In this work it was attempted to identify correlates of protection in a cohort of individuals exposed to HIV-1 and not infected. Sexual exposure within and prior to the current relationship was calculated, or estimated, respectively, and a transmission probability was established. Virologic factors inherent to the infected partner's isolate, including the viral phenotype and replicative capacity, were determined, and *in vitro* infectivity of the HIV-negative partner's $CD4^+$ cells was assessed. Furthermore, the CCR5 genotype was determined, and a novel assay for detecting cytotoxic responses against primary isolates was designed. In this assay transformed BLCL were established for each subject, the cell lines were transfected with the genes for CD4 and CCR5, and subsequently infected with primary viral isolates.

The results indicated that all individuals had a high probability of infection which was modulated by protective effects of CCR5 genotype, viral attenuation, and cytolytic immune responses. CTL were detected in five of eleven subjects, including individuals homozygous or heterozygous for CCR5Δ32. This implied that limited infection sufficient

to prime cellular immune responses had occurred in these subjects, and hence CTL likely constituted a significant component of the *in vivo* resistance.

The implications of this work are that CTL constitute a significant component of resistance to infection by HIV-1. Thus, immune responses elicited by potential vaccines should include cellular lytic responses, and potential administration of cytokines important for enhancing CTL function should be considered.

One of the questionnaires administered to the participating couples in this study was designed by Dr. Mahin Baqi at the University of Toronto, the other questionaire was designed by Dr. Colin Kovacs at 235 Danforth Ave, Toronto. In addition, Dr. Baqi interviewed several of the patients regarding their sexual behaviour, and compiled the transmission probability figures for the current relationship of the participants. The author of this dissertation interviewed the remainder of the patients, and performed all the experimental work.

Summary

Correlates of resistance to infection by human immunodeficiency virus type 1 (HIV-1) are important for defining potential therapeutic interventions, and for prophylactic vaccination. In this study, a cohort of eleven couples discordant in their HIV-infection status were analyzed regarding their risk of infection. Characteristics assessed were the route and frequency of exposure to HIV-1, the CCR5 genotype, the viral phenotype and replicative capacity, and the presence of cytotoxic T lymphocytes (CTL) specific for the partner's primary isolate. A sensitive, novel assay for detecting CTL specific for primary isolates was devised. Behavioural characteristics in subjects of this cohort all entailed a high risk of transmission that appeared to be modulated by CCR5 genotype, limited viral replicative ability in the infected partner, and CTL able to lyse infected target cells. CTL were detectable in five of eleven HIV-negative individuals including persons with homozygous, heterozygous, and wild-type genotypes for a 32 base pair deletion in CCR5, suggesting that exposure sufficient to generate CTL may occur in the absence of an intact co-receptor. Hence, resistance to HIV-1 infection was not invariable, and a complex interplay of multiple factors modulated the risk inherent to exposure.

Introduction

Understanding the factors that confer resistance to HIV-1 infection is important for the development of preventative and therapeutic modalities. Our current understanding of the biology of HIV-1 has expanded drastically with the recent identification of chemokine receptors as co-receptors for viral entry (1-6). Briefly, macrophage-tropic viral isolates, predominant during the asymptomatic phase of infection, are characterized by failure to induce syncytia in indicator cell lines (NSI), and enter target cells through interaction with the CD4 molecule and a CC-chemokine co-receptor, most commonly CCR5 (1-3). Conversely, T cell-tropic viral strains emerge during the later stages of infection, induce syncytium formation (SI) in susceptible T cell lines, and rely on CXCR4, a member of the CXC family of chemokine receptors (4). Most primary SI viruses, isolated from patients directly and not passaged in cell lines, retain the ability to bind to CCR5, or use CXCR4 (7).

An individual's capacity to resist exposure to HIV-1 is likely dependent on several factors: the route of exposure, the quantity of the inoculum, the phenotype of the virus, the capacity of the immune system to respond effectively, and pre-existant protective factors. In the HIV-1 epidemic, a large population of exposed and infected persons exists, and currently infection almost invariably results in death of the host. However, if "natural" resistance does occur, the probability of identifying the essential features of such a state would be highest in uninfected members of populations with the greatest exposure. Using this approach, several groups of HIV-negative individuals with variable risks of infection

were identified. Study of two seronegative persons whose sexual behaviour entailed a high probability of transmission revealed that their cells resisted *in vitro* infection (8). Subsequently, discovery of a homozygous deletion of 32 base pairs in their CCR5 (CCR5Δ32) molecule provided a structural explanation for this unique resistance (8). Henceforth, epidemiologic studies indicated that this homozygous mutation occurs in approximately 1% of the general population, with a heterozygote frequency of 25% (9), and that there are no associated phenotypic abnormalities (10). Thus, the homozygous genotype was found to be highly protective for HIV infection, while one mutant allele imparted a slower disease progression (11). Though CCR5Δ32 individuals are highly resistant, rare cases of infection have been reported, and were characterized by a rapid disease course (12-14).

Immune responses were thought to confer protection from HIV infection in individuals at increased risk due to sexual behaviour (15,16), occupational exposure (17), intravenous drug use (18), exposure to infected mothers (19-21), and prostitution (22,23). Though in these reports the CCR5 genotype of the individuals was not known, T cell responses, as indicated by proliferation to HIV peptides, interleukin (IL-2) production, or by cytolysis of cells expressing HIV-derived peptides, were interpreted as mediating the resistant state. A theory evolved from these studies suggesting that strong cell-mediated immune responses induced by a predominance of T helper (T_h) type 1 cytokines will enhance protective immunity, or favorably modulate established infections (24).

Specific cellular factors affecting transcription or replication may influence

established infections. Among these, the CC chemokines macrophage inflammatory protein (MIP)-1α, MIP-1β, and RANTES appeared to account for much of the original $CD8^+$ cell-derived suppressive factor (24), while high concentrations of IL-16 (25), and as yet unidentified factors distinct from chemokines (26, 27) exerted transcriptional control of viral expression. Activation of $CD4^+$ cells by CD3 and CD28 co-stimulation resulted in a refractory state for further viral binding, presumed to be due to lack of CCR5 transcripts (28). Thus, structural features of $CD4^+$ cells, and specific T cell immune responses, may mediate resistance to infection, while excessive chemokine production, or transcriptionally active inhibitory factors, may modulate viral replication.

In this study we explored several factors that may contribute to resistance in a cohort of highly exposed, but uninfected, individuals. We were particularly interested in examining immune responses specific for primary viral isolates, and in correlating these with the CCR5 genotype. To this end, we designed a new sensitive assay for cytolytic T cell (CTL) function. Autologous B cell lines (BLC) transfected with genes coding for CD4 and CCR5 were infected with primary viral isolates, and subsequently served as target cells for cytolytic T cells. The results obtained indicate that resistance in this cohort is due to complex mechanisms, and that the viral phenotype in the transmitter, as well as the immune response and receptor integrity in the recipient, contribute to lack of infection.

Materials and Methods

Patient population

Eleven couples were recruited through primary care physicians, or referred from the Special Immunology Service at McMaster University, or the Immunodeficiency Clinic at The Toronto Hospital. Eligible couples consisted of a seronegative partner who had been exposed to HIV-1 through unsafe sexual behaviour for more than 2 years, and an infected partner. Informed consent was obtained from all participants. Demographic information on the study population is summarized in Table 1. As part of the interview process, all couples were counselled on prevention of HIV transmission.

Confirmation of HIV status

HIV-1 antibodies were evaluated by standard ELISA technique, and peripheral blood samples from all seronegative individuals were subjected to PCR amplification (Roche HIV Amplicor). The infected participants were unequivocally HIV-antibody and PCR positive.

Virus isolation

Peripheral blood lymphocytes from the HIV-positive subjects were prepared by Ficoll-Hypaque (Pharmacia) density centrifugation of heparinized blood samples, and immunomagnetically (MiniMACS, Miltenyi Biotec, Sunnyvale, CA) depleted of $CD8^+$ cells. This cell separation technique routinely removes >96% of the $CD8^+$ cells (data not shown). The $CD8^-$ fraction was co-cultured with PHA-stimulated $CD4^+$ T cell blasts from

a healthy control individual at 1:4 ratios in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (250 U/ml), streptomycin (250 µg/ml), 10 mM HEPES buffer, and 10 U/ml of recombinant human interleukin-2 (IL-2). The same donor $CD4^+$ blasts were used for all viral isolations. The cultures were maintained for two to three weeks, fresh donor $CD4^+$ PHA blasts were added weekly, and part of the supernatant was replenished and tested for p24 antigen concentration (Organon Teknika, Durham, NC) twice weekly. If no virus was recovered in three separate attempts the procedure was repeated with PHA-stimulated cord blood lymphocytes that had been pretreated with 2 µg/ml polybrene (Sigma). The resulting viral stocks were titered, aliquoted and stored in liquid N_2. The syncytial phenotype of the viral isolates was determined by infection of MT-2 cells and evaluation of syncytia at 3 and 5 days. The laboratory viral strains HIV_{BAL} and HIV_{IIIB} were obtained from the AIDS Research and Reference Reagent Program, and were propagated in peripheral blood mononuclear cells.

Infections with primary isolates

$CD4^+$ peripheral blood lymphocytes from the seronegative partners were immunomagnetically isolated, stimulated with PHA (5µg/ml) and 10 U/ml IL-2 for 3 days, and pretreated with polybrene (2µg/ml, Sigma, St. Louis, Missouri) for one hour. The cells were then washed, and 10 ng of p24 from the respective HIV-seropositive partner's primary isolate were added to 10^6 $CD4^+$ blasts in a final volume of 1 ml of medium. In separate experiments $CD4^+$ cells were infected with 10 ng of p24 from HIV_{BAL} and/or HIV_{IIIB}. After 2 hours of incubation at 37°C the cells were washed three times with 1% fetal bovine serum in PBS, and cultured in triplicate in 96-well plates at 10^6 cells/ml of

medium. Half the supernatant was replenished weekly, and the p24 concentration at 7 and 11 days was determined in duplicate.

CD4 and CCR5 transfection of BCL

For all HIV-negative subjects, two HIV-positive subjects, and a control individual, a BCL was established by transforming $CD8^+$ cell-depleted PBMC with supernatant from B95.8 cell lines constitutively producing Epstein Barr virus. For transfections, pcDNA3.1/zeo-CD4 (kindly provided by Dr. Mark Luescher, University of Toronto) and pcDNAI/amp-CCR5 (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. Nathaniel Landau (1, 30)) plasmid DNA was purified from bacteria (QUIAGEN, Chatsworth, California), and 5 µg of each were added to $5x10^6$ exponentially growing BCL in the presence of 400 µg/ml DEAE-dextran. The cells were agitated for 1 hour at 37°, were washed carefully, and cultured in 20% RPMI for 48 hours. Thereafter, the transfected BCL were pretreated with 2 µg/ml polybrene for 1 hour, and infected as described above with primary HIV isolates, and with laboratory isolates. Infections were evaluated by measuring p24 concentration in the supernatants after 48 hours, and by intracellular staining with an anti-p24-FITC antibody (Virostat, Portland, Maine) and flow cytometric analysis.

CTL assays

Effector CTL were generated by infecting $CD4^+$ PHA-blasts from the seronegative individuals with primary isolates, as described above. After three days of culture, an aliquot of supernatant was retained for p24 evaluation, and the infected $CD4^+$ cells were

irradiated (3000 rads), washed twice, and mixed with autologous PBMC at 1:10 ratios. This cell mixture was incubated for 7 days at 37° in medium lacking IL-2, and assayed for effector cytolytic activity. Target cells in the experiments consisted of transfected BCL and of $CD4^+$ PHA blasts that had been infected 48 hours prior to the assays. In each experiment transfected, but uninfected BCL, and uninfected $CD4^+$ PHA blasts were assayed as control targets. The cells were labelled with 100 μCi of ^{51}Cr (NEN, Mississauga, Ontario) for 90 minutes, washed thrice, and incubated in triplicate with effector cells for 5 hours at various effector to target cell ratios. Blocking antibodies to the CD3 molecule, and to MHC class I antigens, were included in each assay. Spontaneous release was measured by incubating target cells alone, and maximal release by lysing target cells with 1N HCl. Release of radioactive label into the supernatant was counted in a gamma counter, and percent specific lysis was calculated as (mean test cpm-mean spontaneous cpm)/(mean maximum cpm-mean spontaneous cpm) x 100. Only assays with less than 30% spontaneous release were considered valid.

CCR5 genotyping

Genomic DNA was isolated from all study participants by a standard guanidinium thiocyanate procedure (DNAzol, Life Technologies, Gaithersburg, Maryland), and 0.5 μg of DNA was subjected to PCR amplification with primers spanning the described 32-base pair deletion in CCR5 (11). Using the primers 5'-CAA AAA GAA GGT CTT CAT TAC ACC-3' and 5'-CCT GTG CCT CTT CTT CTC ATT TCG-3' a wild-type fragment of 189 bp, and a 157 bp-fragment from the mutant allele were discernible on electrophoretic analysis in 3% Metaphor (FMC Bioproducts, Rockland, Maine) gels.

Statistical analysis

Each study participant filled out an extensive questionnaire regarding sexual history, sexual practices with the study partner and with other contacts, condom use, behavioral motivation for practicing safe sex, concurrent medical conditions, and medication use. In addition, ten couples were interviewed for further information on the above factors. A transmission probability based on the exposure within the current relationship, and on an estimate of previous exposure, was calculated as detailed in Table 1. Differences between in vitro parameters were compared with Student's T test.

Results

Study population

Eleven HIV-1 discordant couples consisting of 8 homosexual and 3 heterosexual couples were recruited for this study. The seropositive individuals varied widely in disease stage, $CD4^+$ cell count, and viral load (Table 1). The risk of infection for the HIV-negative partners was variable, and sexual practices in the current relationship were not necessarily reflective of those in previous encounters (Table 2). For example, individuals 201 and 206, whose probability of transmission with their current partner was relatively low, had practiced high-risk sexual behaviour previously with more than 100 encounters, and therefore had a higher risk score than initially apparent. In contrast, the risk for acquiring infection in individual 208 resided almost exclusively in her current relationship.

Viral isolation

From the 11 HIV-infected partners 7 NSI viruses and 2 SI viruses were isolated (Table 1). From two individuals (103 and 109) no virus could be cultured despite at least four attempts, and use of more permissive cord blood lymphocytes. Subject 103 had been infected for only 3 years, and had a normal $CD4^+$ cell count and repeatedly undetectable viral load. Subject 109 first tested seropositive 9 years ago, but had an exceptionally high $CD4^+$ cell count and undetectable viral load, suggesting she may have harboured an attenuated virus. Both individuals were on triple antiretroviral therapy. In general, the viruses isolated corresponded to the disease stage and the $CD4^+$ cell count of the patients (Table 1), and SI viruses replicated faster and to a higher titer than NSI viruses.

In vitro infection of cells from HIV-negative individuals

$CD4^+$ PHA blasts from each HIV-negative individual were infected with 10 ng of p24 from their partner's primary isolate, and from the laboratory strains HIV_{BAL} and HIV_{IIIB}. In those cases where the partner's virus could not be isolated (103 and 109), another primary NSI isolate was used in addition to the laboratory strains. The results from the infections indicated that individual 201 was highly resistant to infection with the primary and the laboratory NSI strains (Fig.1). Subject 204 was poorly infectable with his partner's isolate, but susceptible to both laboratory strains. In addition, cells from subjects 206 and 212 sustained only reduced replication by NSI strains, though the difference was not signficant ($p<0.2$). All individuals were similarly susceptible to infection with the laboratory SI strain HIV_{IIIB}. $CD4^+$ cells from a control unexposed individual were as susceptible to infection as those from members of the cohort (data not shown).

CCR5 genotype

Among the HIV-negative participants two individuals heterozygous for CCR5Δ32 were identified (206 and 212), and one person homozygous for the CCR5Δ32 deletion (201) (Fig. 2). No deletion in the amplified segment of CCR5 in the seropositive subjects or the control individuals was apparent.

CTL assays

In order to assess potential immune responses against primary viruses in individuals exposed to HIV-1, but not infected, it was important to devise a sensitive assay

for detection of cytolytic activity. To encompass all potentially in vivo relevant CTL epitopes (35), effector cells in these assays were generated against primary isolates, and tested against CD4/CCR5 transfected target BCL infected with a panel of viruses consisting of the primary isolate and laboratory isolates. In addition, autologous infected $CD4^+$ cells were used as targets where feasible in order to simulate the in vivo immune environment as closely as possible. Transfection of BCL with CD4 and CCR5 genes allowed for subsequent infection with primary HIV-1 isolates, and p24 antigen was detectable intracellularly as well as in the cell supernatants (Fig. 3). Time course experiments indicated that the maximal infectability was at approximately 48 hours post-transfection, and that p24 antigen production peaked between 48 and 72 hours, and did not continue to rise thereafter. p24 production from infected BCL was of lesser magnitude than from infected autologous $CD4^+$ PHA blasts (Fig. 3), but sufficiently high to allow for antigen processing and presentation. Thus, this system allowed for detection of CTL against primary isolates, and in the absence of a functional CCR5 co-receptor.

The results from the CTL assays directed against patient HIV isolates are summarized in Table 3. Three individuals (201, 205, 209) had high cytolytic activity against infected BCL and infected autologous target cells, two individuals (203, 212) had moderately high killing, and in six assays either low or only nonspecific lysis was observed. Surprisingly, the CCR5Δ32 homozygous individual, 201, had CTL that efficiently lysed BCL infected with the NSI isolate from his partner, 101, and his own $CD4^+$ cells infected with the primary SI isolate 108. In addition, his CTL lysed target cells infected with both laboratory viral strains (Fig. 3, and Table 4). For subject 205, high specific lysis was

detected against the primary isolate, as well as against HIV_{BAL}, but not against HIV_{IIIB}. This individual, similar to subjects 202, 212, and 216, had significant lysis of control non-infected BCL suggesting the presence of circulating anti-EBV CTL in a substantial proportion of this HIV-negative cohort. Subject 209 had high killing of his autologous $CD4^+$ cells infected with primary NSI isolate 116, and moderate killing of the infected BCL, and of both laboratory strain-infected target cells (Table 4). Cytolytic activity against HIV-infected target cells was sensitive to inhibition by anti-CD3 and anti-MHC class I blocking antibodies in all cases. No cytolytic activity could be induced in control individuals not exposed to HIV-1 in vivo. Thus, CTL activity against primary viruses was detected in five of eleven exposed-uninfected individuals, and there was good concordance between lysis of transfected BCL and autologous $CD4^+$ cells.

Discussion

In this study, mechanisms mediating apparent resistance to infection in a cohort of individuals at high risk for acquiring HIV-1 were examined. The participants were in a stable relationship, their partner's viral isolate could be examined, and immune reactions specific for that isolate could be assessed. Individuals resisting infection by HIV-1 are invaluable for identifying correlates of protection, and for effectively designing potential vaccines.

The assay for detection of CTL described in these experiments was useful for evaluating virus-specific responses that may be undetected with conventional procedures. Commonly, CTL are detected against MHC-matched target cells infected with recombinant vaccinia virus vectors with HIV-gene inserts. The genes are derived from cloned laboratory strains, most commonly HIV_{MN} or HIV_{IIIB} (35). These assays result in efficient expression of the vaccinia-encoded gene, however, necessarily only a limited range of potential epitopes are expressed, and EBV-directed lysis can interfere with interpretation. Using primary $CD4^+$ cells as infected target cells is the closest proximation to in vivo interactions, however, only a limited number of $CD4^+$ cells are available from patients in advanced stages of disease. In addition, isolation of fresh PBMC is labor-intensive, and, in individuals homozygous for CCR5Δ32, PBMC are not infectable with primary isolates. Thus, the assay utilized in the experiments described here could be adapted for primary viral strains in individuals regardless of the integrity of the co-receptors, and should be sensitive to detecting CTL with specificity to all potential HIV-1

epitopes. It was not possible from the results here to conclude whether the predominant immune responses were directed to Env or Nef, or other proteins, however, the overall sensitivity of detecting HIV-CTL was maximized.

Several factors potentially contributing to resistance in the cohort examined here were identified. Foremost, absence of a functional CCR5 molecule has been associated with highly significant protection against infection (8-11). The CCR5Δ32 homozygous man in this study had a high risk of transmission due to sexual contacts prior to the current relationship, but had a relatively low risk at the time of this study. His partner's virus was of NSI phenotype, but replicated fast-high, and he had detectable CTL against all viruses examined. These findings suggest that his encounter with HIV-1 was sufficient to prime an immune response, implicating that viral entry into cells was potentially achieved using an alternate CC-chemokine co-receptor, or CXCR4. It is currently unknown why primary SI viruses only infrequently appear to infect CCR5Δ32 individuals, as presumably expression of CD4 and CXCR4 should be sufficient. It has been hypothesized that sexual transmission of HIV-1 may involve an obligatory macrophage-tropic phase, though two CCR5Δ32 individuals were reported to have become infected by sexual transmission (12,14). Albeit, subject 201 had a previous partner who died of AIDS, and thus may have been sensitized to SI viruses in the past, or sustained a limited infection with a NSI isolate able to interact with CCR2b or CCR3.

Heterozygosity for CCR5Δ32 has been associated with limited disease progression, and no apparent in vivo protection from infection (11). In vitro infection

experiments in this study indicated reduced infectability of heterozygous cells with NSI isolates, however, the difference was not statistically significant. Interestingly, though several HIV-positive partners of this cohort would be considered slow progressors, and heterozygous individuals may comprise 24% of this subgroup of HIV-infected individuals (37), this phenotype was not identified among the infected participants. The frequency of the heterozygous phenotype among the HIV-negative individuals was consistent with that in the caucasian population (9).

Factors inherent to the potential transmitter that influence the risk of infection in the recipient include the quantity of virus transmitted, and the genotype and the replicative capacity of the virus. Transmission has been considered a highly complex process involving a minor variant from a swarm of quasispecies from infected individuals with selective amplification and penetration in the new host (38). Viral load in plasma was highly correlated with viral load in semen and seminal plasma, suggesting that individuals with viremia undetectable by currently available methods are less likely to transmit infectious virions during sexual contact (39). Regarding the subjects in this study, the viral load was undetectable in four individuals with normal $CD4^+$ cell counts, and this likely contributed to the protective effect for their partners. Since in this study viral characterization was achieved by standardized co-culture with the same donor cells for all individuals, differential replicative capacity was considered to be an effect of the viral genotype, and not of the origin of the donor cells (40). Hence, different viral concentrations resulting during co-culture presumably reflect genuinely different properties of the viral isolates. As such, a significant protective effect for the partner was

attributed to lack of in vitro replication of the viruses from subjects 203 and 209. The reasons for the limited replicative ability of these viruses were not explored in this study, however, deletions in *nef* and accessory genes have been demonstrated in long-term non-progressing patients (41,42).

A protective effect from HIV-infection has been attributed to CTL in a number of individuals exposed, but not subsequently infected (15-17,19-21). While it is apparent that sensitization of T cells precedes production of antigen-specific antibody, and the period between T cell reactivity and seroconversion may involve several months (15), it is difficult to convincingly demonstrate limited infection in vivo including viral internalization, and proteasomal processing for MHC class I presentation. The findings in this study, and those cited above, indicated that CTL may be detected in persons exposed to HIV-1 but not infected. This strongly suggests that CTL are integral to protective immunity, and therefore should be part of an immune response induced by potential vaccines. Furthermore, despite divergent opinions regarding the beneficial aspects of CTL during pathogenesis, elicitation of cytotoxic function against HIV may be the only correlate of effective mucosal protection (43).

In summary, participants in this study had variable risks of infection due to exposure within their current relationship and during previous sexual encounters, CCR5 genotype, transmitter viral characteristics, and presence or absence of immune responses. The probability of infection in some subjects was reduced due to contributions from the above factors, however, invariable resistance was not identified.

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Table 1. **Demographic features of 11 HIV-positive individuals.**

		HIV+						
couple id.	age, sex	HIV+ (yrs)*	CD4+ count	viral load (copies/ml)	viral phenotype	virus replication in donor cells[β]	disease stage[#]	therapy
101	52, ♂	6	640	<500[‖]	NSI	126,540	A1	triple[$], acyclovir
102	37, ♀	[3][‡]	80	7,000[¶]	NSI	71,420	B3	triple
103	39, ♂	3	670	<500[‖]	NVI[£]	NVI	A1	triple
104	38, ♂	[13][§]	200	2,784[‖]	NSI	12,300	C2	none
105	25, ♂	6	260	NA[¥]	NSI	27,490	B2	triple, acyclovir
106	61, ♂	12	180	137,500[‖]	NSI	4,760	B3	none
107	36, ♂	13	273	23,070[‖]	SI	187,400	C2	triple, acyclovir
108	30, ♂	13	84	87,070[‖]	SI	635,200	C3	none
109	45, ♀	10	920	<500[‖]	NVI	NVI	A1	triple, acyclovir
112	32, ♂	4	540	<500[‖]	NSI	24,590	A1	triple
116	56, ♂	3	140	6,219[‡‡]	NSI	170,500	B3	triple

* Number of years since the first seropositive test.
‡ Seropositive test one year ago, probable exposure 3 to 5 years prior.
** Seropositive test 5 years prior, seroconversion illness and probably exposure 13 years prior.
♬ p24 concentration at 14 days of co-culture with donor $CD4^+$ cells
♯ CDC disease stage (*ref* 31)
❙ Chiron 2.0 assay, sensitivity 500 copies/ml.
¶ Nasba assay, sensitivity 400 copies/ml.
‡‡ Roche HIV Monitor Test, sensitivity 500 copies/ml.
¥ Not available
£ No virus isolated.
$ Triple therapy consisting of AZT, ritonavir or saquinavir or indinavir, and 3TC.

Table 2. Risk factors for infection in 11 HIV-negative individuals

identification	sex, age	transmission probability current relationship (%)*	transmission probability prior to current relationship (%)‡	CCR5 genotype§
201	41, ♂	13	80	-/-
202	34, ♂	68	20	+/+
203	38, ♂	97	80	+/+
204	48, ♂	100	100	+/+
205	60, ♂	100	100	+/+
206	25, ♂	13	80	+/-
207	30, ♂	98	20	+/+
208	29, ♀	100	10	+/+
209	41, ♂	30	80	+/+
212	34, ♂	78	80	+/-
216†	31, ♂	98	10	+/+

* Calculated according to n = probability of evading infection = $(1-p_{anal})^a(1-p_{vaginal})^v(1-p_{oral})^o(1-p_{seroconv.})^s$ (Ref. 32,33)

with p_{anal} = probability of transmission per anal sex act; ^{a} = total number of anal sex act

$p_{vaginal}$ = probability of transmission per vaginal sex act; ^{v} = total number of vaginal sex act

p_{oral} = probability of transmission per oral sex act; ^{o} = total number of oral sex act

$p_{seroconv.}$ = probability of transmission per sex act during seroconversion; ^{s} = total number of sex acts during seroconversion

‡ Estimated according to the number of unprotected sexual contacts reported

† Deceased

§ CCR5 genotype was determined by amplification of a gene segment including the 32 bp deletion (Ref. 11)

Table 3. CTL from HIV-negative individuals specifically lyse target cells infected with primary HIV isolates

subject	target*	% specific lysis	target‡	% specific lysis effector:target ratio			target§	% specific lysis effector:target ratio		
		50:1		50:1	25:1	12:1		50:1	25:1	12.5:1
201	BCL-tf	5	BCL-1^0	42	33	22	CD4-1^{0¶}	45	38	32
202	BCL-tf	14	BCL-1^0	14	12	11	CD4-1^0	15	13	14
203	BCL-tf	2	BCL-1^{0**}	14	14	12	CD4-1^{0**}	21	22	20
204	BCL-tf	3	BCL-1^0	3	2	0	CD4-1^0	0	1	4
205	BCL-tf	14	BCL-1^0	39	37	36	CD4-1^0	46	43	42
206	BCL-tf	0	BCL-1^0	6	1	4	CD4-1^0	12	9	6
207	BCL-tf	0	BCL-1^0	0	0	0	CD4-1^0	0	0	0
208	BCL-tf	5	BCL-1^0	5	4	3	CD4-1^0	ND‖	ND	ND
209	BCL-tf	3	BCL-1^{0**}	28	25	22	CD4-1^{0**}	55	45	37
212	BCL-tf	17	BCL-1^0	30	25	18	CD4-1^0	48	41	34
216	BCL-tf	19	BCL-1^0	18	14	15	CD4-1^0	10	7	5

* BCL were transfected with CD4 and CCR5 plasmid DNA, and not infected

‡ BCL were transfected with CD4 and CCR5 plasmid DNA, and infected with partner's primary HIV isolate.

§ CD4+ cells were infected with partner's primary HIV isolate.

‖ Not done

¶ CD4+ cells were infected with primary SI virus 108. ** NSI HIV isolate 116 was used.

Table 4. CTL from HIV-negative individuals specifically lyse cells infected with HIV laboratory strains

subject	target cell	% specific lysis			target cell	% specific lysis		
		50:1	25:1	12:1		50:1	25:1	12:1
201	$BCL\text{-}_{IIIB}$*	25	18	13	$BCL\text{-}_{BAL}$‡	29	26	20
203	$BCL\text{-}_{IIIB}$	ND‖	ND	ND	$BCL\text{-}_{BAL}$	6	5	3
205	$BCL\text{-}_{IIIB}$	31	28	27	$BCL\text{-}_{BAL}$	30	29	29
209	$BCL\text{-}_{IIIB}$	19	17	16	$BCL\text{-}_{BAL}$	21	20	21
212	$BCL\text{-}_{IIIB}$	ND	ND	ND	$BCL\text{-}_{BAL}$	16	14	13

* Target BCL were infected with HIV_{IIIB}
‡ Target BCL were infected with HIV_{BAL}
‖ Not done

Legend.

Fig. 1

10^6 $CD4^+$ PHA blasts from HIV-1 negative individuals were infected with 10 ng of p24 antigen from primary viral isolates, or from the laboratory strains HIV_{BAL} and HIV_{IIIB}. Viral replication was assessed by measuring p24 production in culture supernatants on day 11.The SI strain HIV_{IIIB} replicated equally well in all cells, while there was some variability in replication of HIV_{BAL}. The primary isolates replicated poorly in $CD4^+$ cells from subjects 201, 204, 206, and 209.

Fig. 2

A segment of CCR5 flanking the described 32 bp deletion was PCR-amplified from genomic DNA from HIV-1 negative individuals. The products were analysed by gel electrophoresis. Subjects 202, 203, 204, and 205 have the CCR5 wild-type genotype, 206 is a heterozygote, and 201 has a homozygous deletion in CCR5.

Fig. 3

$CD4^+$ cells and transfected BCL from a CCR5Δ32 individual (201) are infectable, and are lysed by autologous CTL. Intracellular p24 staining of $CD4^+$ cells infected with a 1° SI HIV isolate (*a*), and of transfected BCL infected with a primary NSI HIV isolate (*b*). Day 3 p24 production (*c*) by infected $CD4^+$ cells and by transfected BCL. Autologous $CD4^+$ cells infected with a primary SI HIV isolate, and transfected BCL's infected with a primary, or a laboratory, NSI HIV isolate are lysed by CTL from individual 201 (*d*).

Figure 1.

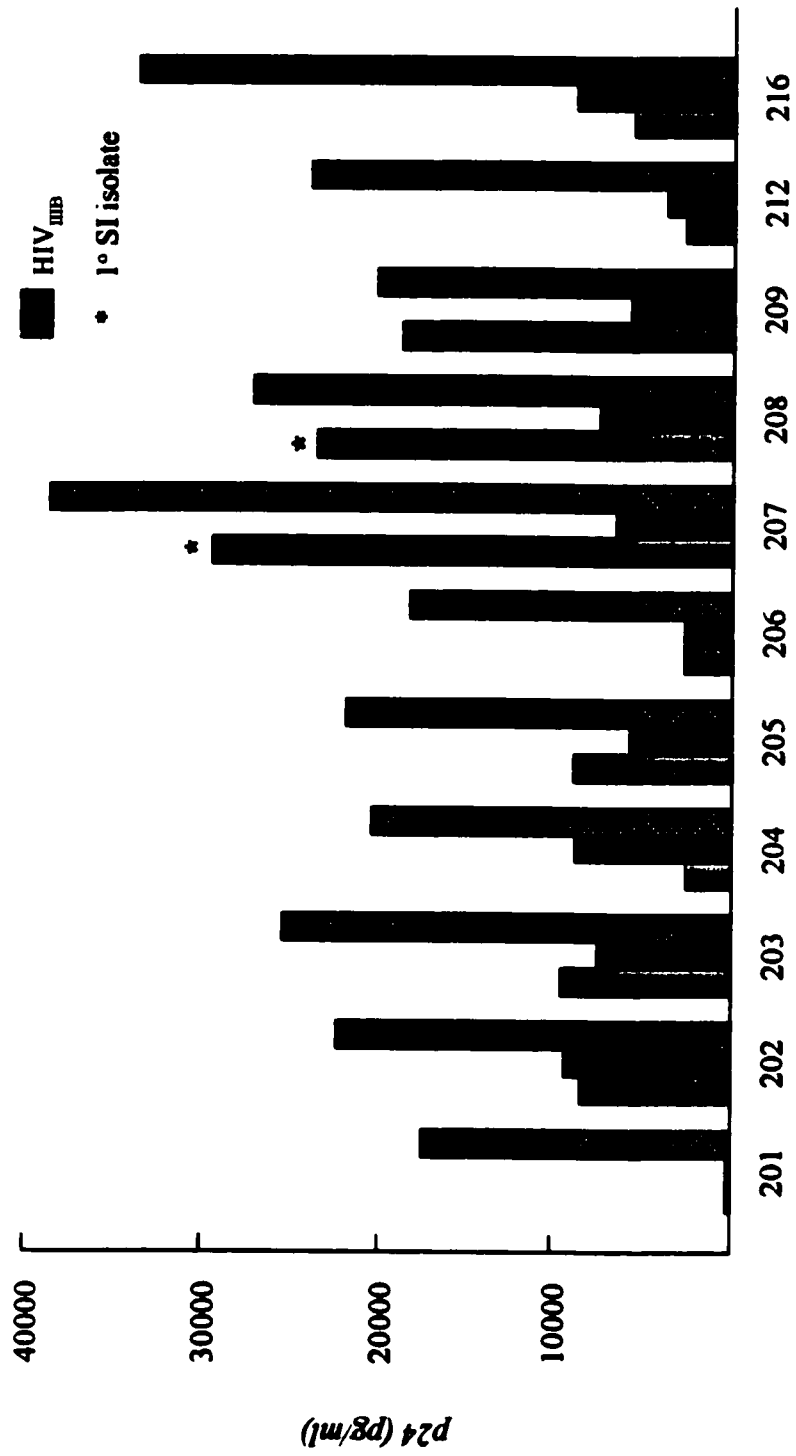

1° HIV isolate
HIV$_{BAL}$
HIV$_{IIIB}$
1° SI isolate
p24 (pg/ml)
40000
30000
20000
10000
201
202
203
204
205
206
207
208
209
212
216

Figure 2.

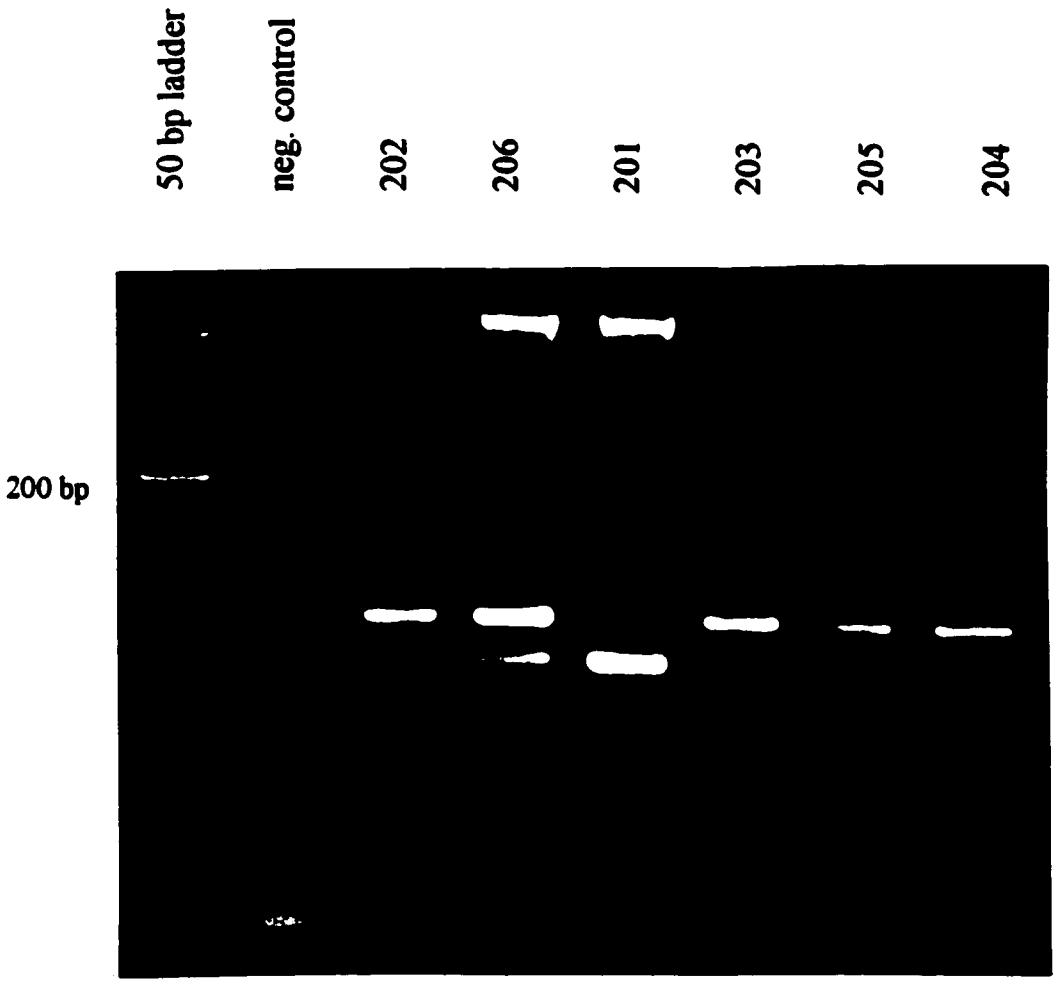
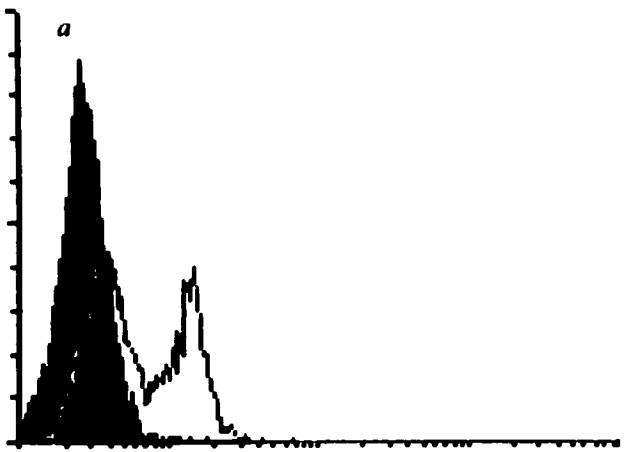
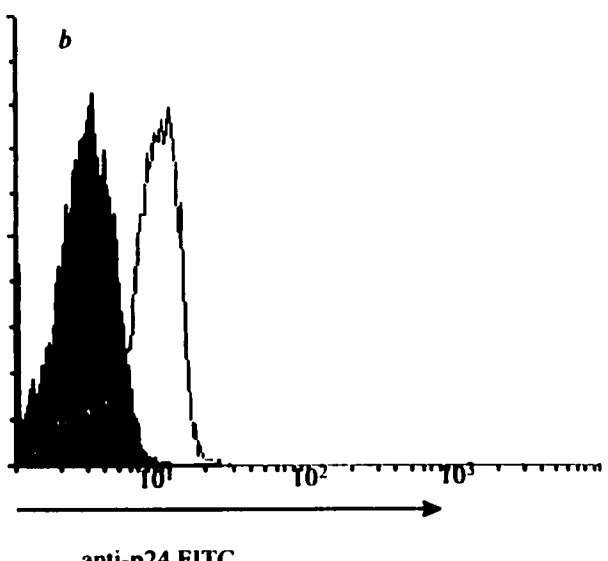
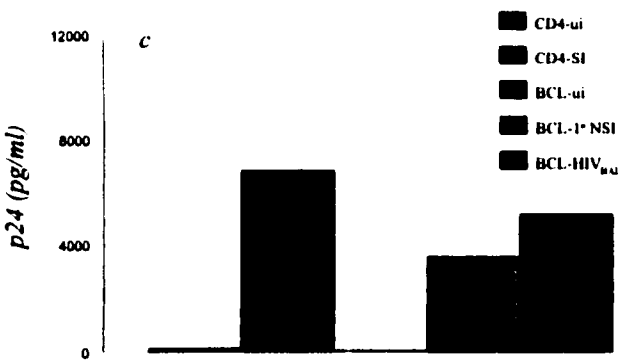
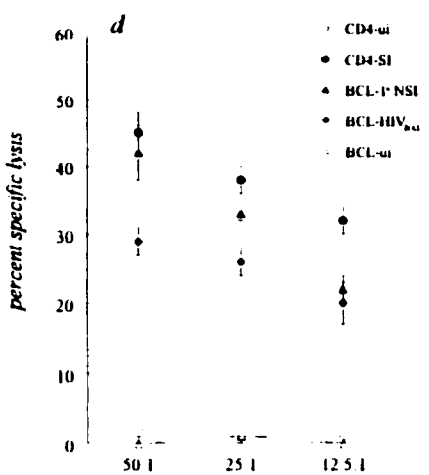


Figure 3.


a


b
10^1
10^2
10^3
anti-p24 FITC


c
12000
8000
4000
0
p24 (pg/ml)
CD4-ui
CD4-SI
BCL-ui
BCL-1* NSI
BCL-HIV$_{ui}$


d
60
50
40
30
20
10
0
percent specific lysis
CD4-ui
CD4-SI
BCL-1* NSI
BCL-HIV$_{ui}$
BCL-ui
50:1
25:1
12.5:1

Chapter 5.

In vitro correlates of infectivity and resistance to HIV-1 infection in persistently seronegative prostitutes from Nairobi, Kenya.
(*Bienzle et al., manuscript in preparation*)

For this work, samples acquired from Kenyan prostitutes who have remained seronegative for HIV-1 infection for more than 3 years despite high exposure rates, and high risk sexual behaviour, were analyzed. Considering the *in vivo* resistance despite exceptionally high risk of infection, *in vitro* susceptibility to infection by primary local isolates was determined first. Further, potential factors of mediating resistance such as high production of chemokines were evaluated. $CD8^+$ T cell suppressive functions were investigated by assaying reduction in viral replication in $CD4^+$ and $CD8^+$ cell co-cultures, and reduction in transcriptional activation by $CD8^+$ cell-derived supernatants. Finally, the genetic integrity of the co-receptors for HIV-1 infection was assessed.

Results from these studies indicated that there was no *in vitro* resistance to infection, and co-receptor genotypic abnormalities were not detected. However, cell-mediated suppression of viral replication was high, suggestive of potential ability to moderate an infection. Overall, this study suggested that the remarkable resistance to infection observed in the study population likely resides in extra-lymphoid tissue.

The experiments described in this chapter were performed by the author of this

dissertation, except for the transcriptional inhibition assays that were undertaken by Dr. Karen Copeland.

Summary

Samples from Kenyan prostitutes that have resisted infection by HIV-1 for more than 3 years despite frequent exposure were examined in vitro. $CD4^+$ PHA blasts were infected with primary Kenyan HIV isolates, and cellular and transcriptionally active factors from $CD8^+$ cells were assessed. No in vitro resistance to infection by NSI or SI HIV isolates was detected, and no excess production of β-chemokines. Co-culture of $CD8^+$ cells profoundly reduced the replication of primary NSI isolates. Transcriptional inhibition was detected in most of the study participants, however, did not correlate with in vivo seropositive or seronegative status. The 32-base pair deletion in CCR5 associated with resistance to infection in Caucasian individuals was not identified in these subjects. Thus, lack of infection in this cohort is not attributable to factors inherent to $CD4^+$ cells, but may be associated with strong inhibitory activity by $CD8^+$ cells and unique features of mucosal sites.

Introduction

Several studies have reported on individuals that remain persistently seronegative despite repeated exposure to the human immunodeficiency virus type 1 (HIV-1). These have included health care workers with accidental parenteral exposure to infected blood or body fluids [1], infants born to infected mothers [2, 3, 4], needle-sharing intravenous drug users [5], individuals engaged in unprotected sexual intercourse with HIV-seropositive partners [6, 7], and prostitutes [8, 9]. The risk for infection among these cohorts varied greatly, and several potential mechanisms of resistance have been identified.

In several studies, T-cell mediated immunity has been considered the essential element of resistance to infection. T-helper cell function was evaluated by measuring interleukin 2 (IL-2) production or lymphocyte proliferation in response to HIV-derived peptides [6,10], and in some reports the presence of HIV-specific cytotoxic T lymphocytes (CTL) was assayed[1-4,7,9]. Strong cellular responses to HIV antigens preceded actual seroconversion in some individuals at high risk of infection [6,7], while a single parenteral exposure to a contaminated fluid was sufficient to induce detectable *env*-specific CTL in health care workers that remained uninfected [1].Thus, it has been postulated [10] that the occurrence of HIV-specific T cell responses in the absence of antibody production may be due to a low antigenic dose sufficient to induce limited virus replication and presentation by major histocompatability class I (MHC I) molecules. Subsequently, a cytokine-mediated Th1 immune response would upregulate cellular effector functions, and downregulate T cell help for B cells [11]. Confounding this theory

is the long lag period that has been observed between PCR-positive status and seroconversion in some individuals at risk for infection [7,10].

Antiviral factors produced by $CD8^+$ cells that inhibit viral replication were originally described in 1986 [12]. $CD4^+$ cells were fully infectable, however, in the presesnce of $CD8^+$ cells there was limited replication of the virus. Recently, factors produced by $CD8^+$ lymphocytes that are able to suppress viral replication have been identified as the β-chemokines macrophage inflammatory protein (MIP)-1α, MIP-1β, and RANTES (Regulated on Activation, Normal T cell Expressed and Secreted) [13]. Subsequently, the chemokine receptor CCR5, to which all three chemokines bind, was found to be the main co-receptor for primary HIV isolates of the macrophage-tropic (M-tropic) phenotype [14]. M-tropic isolates were characterized by lack of syncytium-induction (NSI) in cell culture, while many T-cell tropic isolates will induce syncytia in permissible cells [14]. Furthermore, a mutation resulting in a 32 base pair deletion (CCR5Δ32) and lack of expression of CCR5 was identified in a small group of individuals at high risk for HIV infection [15]. Since individuals with the homozygous mutation in CCR5 had $CD4^+$ lymphocytes that were highly refractory to infection with all NSI isolates, this phenotype appeared to impart a high degree of resistance to *in vivo* infection. However, the protective effect was not absolute, as recent cases of infection in men with the CCR5 homozygous deletion illustrated (16,17,18). Infection in these individuals was attributed to the potential use of the chemokine receptors CCR3 or CCR2b by some primary isolates, or due to intravenous infection with syncytium-inducing (SI) isolates utilizing the chemokine receptor CXCR4. Thus, a structural basis for resistance to

infection by HIV has been identified. Whether T cell-mediated immune responses as described above contribute to resistance in CCR5Δ32 persons is presently unknown.

Inherent cellular resistance to infection with NSI isolates in one study had been found to be due to overproduction of the β chemokines [19], and has recently been described in $CD4^+$ cells stimulated with anti-CD3 and anti-CD28 antibodies [20, 21]. This stimulatory pathway mimicked antigen presentation and co-stimulation by antigen-presenting cells, and resulted in decreased detection of CCR5 transcripts compared to CXCR4 transcripts, and profound resistance to infection with NSI isolates [20]. Overexpression of the CD8 T cell-derived cytokine IL-16 in Jurkat cells reduced transcription of HIV following infection, thus limiting viral replication after initial integration [22]. Furthermore, as yet undefined factors distinct from chemokines affect transcriptional control of virus expression [23].

Thus, resistance to infection, or factors limiting viral replication, in individuals exposed to HIV has been attributed to specific cellular immune responses, to structural changes in the viral receptor, to intrinsic overproduction of anti-viral factors, and to unique stimulation states of the target cells. However, many cases of resistance to infection are not accounted for by these factors, and additional means of evading infection despite repeated exposure remain to be discovered.

In this study we examined the in vitro susceptibility to HIV infection of $CD4^+$ lymphocytes from a group of highly exposed sex workers from Nairobi, Kenya. These

women had a significantly increased risk of seroconverting, but have remained uninfected [8]. Resistance to infection in this cohort could not be accounted for by in vitro resistance, nor by overproduction of chemokines, or by the described 32 base pair deletion in CCR5. Factors elaborated by $CD8^+$ cells effectively reduced replication of NSI isolates, however, did not act at the level of transcriptional control.

Materials and Methods

Study subjects

Thirteen cellular samples from a cohort of female sex workers who were persistently seronegative and remained seronegative after three or more years of follow-up were available for study. The risk of infection in this cohort has increased steadily over time, however, the individual women have remained uninfected as evaluated by serology, immunoblot, and PCR amplification of *env*, *nef*, and *vif* sequences, as described in detail previously [8]. Two samples from infected members of this study cohort were available for virus isolation, one sample from an unexposed Kenyan individual, and one sample from an unexposed Caucasian person.

Virus isolation

In order to assess *in vitro* characteristics of samples from Kenyan resistant individuals it was important to employ an isolate from the same geographic region. Therefore, HIV was isolated from samples from the two infected cohort members, and from a Kenyan man who reported acquiring his infection in Kenya. The virus stocks were produced by co-culture of $CD8^+$ cell-depleted PBMC's from patients with donor HIV-negative $CD4^+$ PHA blasts. The cultures were maintained for two to three weeks, fresh HIV^--$CD4^+$ PHA blasts were added weekly, and part of the supernatant was replenished and tested for p24 concentration (Organon Teknika, Durham, North Carolina, USA) twice weekly. The viral phenotype was determined on MT-2 cells, and the stocks were aliquoted and stored in liquid N_2. From these Kenyan individuals two NSI strains of HIV (K293 and

K1075) and one SI strain were isolated (K-po).

Cell preparations and infections

$CD4^+$ and $CD4^-$ cell fractions were immunomagnetically (MiniMACS, Miltenyi Biotec, Sunnyvale, California) isolated from cryopreserved PBMC's from the persistently seronegative cohort. On some occasions, the $CD8^+$ fraction was further purified from the $CD4^-$ cell population. This purification method routinely yielded >97% pure cell populations (data not shown). For infectivity studies, the $CD4^+$ cells were stimulated with 5 µg/ml PHA (Murex Diagnostics Ltd., Dartford, England) and 10 U/ml IL-2 (Genzyme, Cambridge, MA, USA) in 10% RPMI 1640 supplemented with antibiotics for 5 days. The cells were then pretreated with 2 µg/ml polybrene (Sigma, St. Louis, Missouri, USA) for one hour, washed, and exposed to a standardardized infection with 10 ng of p24 from the respective primary isolate per 10^6 $CD4^+$ PHA blasts for 2 hours. Following extensive washing, the samples were cultured in 10% RPMI supplemented with 10 U/ml IL-2 in triplicate 96-well plates. p24 levels and chemokine concentrations (R&D Systems, Minneapolis, MN, USA) in supernatants of uninfected and infected cultures were determined on days 8 and 11. At that time, the remaining cellular fractions were collected, and genomic DNA extracted.

CCR5 genotyping

A portion of the CCR5 gene spanning the 32-base pair-deletion was amplified from genomic DNA with the sense primer 5'-CAAAAAGAAGGTCTTCATTACACC-3', and the anti-sense primer 5'-CCTGTGCCTCTTCTTCTCATTTCG-3' as described [24]. This

amplification yielded 189 bp and 157 bp fragments from the wild-type and deleted CCR5 gene, respectively. The amplified products were resolved by electrophoresis in 2% Metaphor (FMC BioProducts, Rockland, ME, USA)/1% agarose gels.

$CD8^+$ cell inhibition studies

To assess cellular suppression of viral replication, $CD8^+$ cells were added directly to autologous $CD4^+$ cells at a 1:4 ratio immediately after infection. The supernatants were replenished biweekly, and the p24 levels in uninfected cell cultures, infected $CD4^+$ cell cultures, and in $CD4^+$-$CD8^+$ co-cultures were determined on day 11. Transcriptional inhibition by $CD8^+$ cells was assessed by culturing $CD8^+$ cells with PHA (5 µg/ml) and IL-2 (20 U/ml) for 3 days, followed by extensive washing and re-culture in RPMI 1640 and IL-2 for 3 further days. Supernatants were collected, and tested for suppression of chloramphenicol-acetyl-transferase (CAT)-linked LTR expression in Jurkat cells transfected by the DEAE-dextran method [25].

Statistical analysis

Differences between the control infections and the sample population were evaluated with the Wilcoxon Signed-Rank test for nonparametric samples [26]. Inhibitions of viral replication were compared by the paired t-test [27].

Results

Susceptibility of CD4⁺cells from Kenyan resistant prostitutes to in vitro infection

Two NSI isolates (K293 and K1075), and one strongly syncytium-inducing virus (K-po) were isolated from the infected Kenyan samples. Purified $CD4^+$ cells from thirteen persistently seronegative women and two control individuals were infected with the primary isolates in two separate experiments. p24 levels in supernatants were measured on days 8 and 11. Although the individual viruses differed in their replicative ability, no significant differences were observed between the the sample population ($\bar{x}$=1,151, SD=242 pg/ml) and the control 8404 (1040 pg/ml) infected with K293 ($p<0.02$) in experiment 1. Similarly, in the second experiment, $CD4^+$ cells from the persistently seronegative cohort ($\bar{x}$=14,875 pg/ml, SD=2,750 pg/ml) were not significantly ($p<0.05$) different regarding infectability from an unexposed Caucasian control subject (20,500 pg/ml) (Fig. 1).

Comparably, infection with a rapidly replicating, and highly syncytial-inducing primary isolate (K-po), did not show a significant difference ($p<0.02$) in infectivity between the exposed uninfected cohort ($\bar{x}$=21,627 pg/ml, SD=12,224 pg/ml) and the unexposed Kenyan individual (17,303 pg/ml for 8404) (Fig. 2).

CCR5 genotype

A segment of genomic DNA including the 32 base pair deletion in CCR5 conferring resistance to infection with M-tropic isolates was amplified by PCR and

analysed by gel electorphoresis. No change in electrophoretic mobility was observed in any of the 13 samples from exposed uninfected subjects, nor in the unexposed control (Fig. 3).

β chemokine production by CD4⁺ cells

In order to determine whether β-chemokine production is associated with reduced or enhanced infectivity of purified CD4⁺ cells, the concentrations of MIP-1α, MIP-1β, and RANTES were determined in the supernatant of samples concurrent with measurements of p24 concentration. Chemokine production by CD4⁺ cells was consistent within the same individual, but independent of the infection status of the cell or the type of virus used (Fig. 4). In the majority of the supernatants only low concentrations of RANTES were detected. Although subject 1800 was relatively resistant to infection by the SI isolate, chemokine production was below the detection limits of the assay. On the contrary, the supernatant of CD4⁺ cells from subject 1378 contained relatively high amounts of MIP-1α and MIP-1β, but the cells were highly susceptible to infection with NSI and SI isolates. Thus, no correlation of viral replication with chemokine production by CD4⁺ cells was apparent.

CD8-mediated viral suppression

To assess the ability of CD8⁺ cells to suppress viral replication in the subjects of this study, unstimulated CD8⁺ cells were added to CD4⁺ cells immediately following infection. Production of p24 was monitored in the supernatants of uninfected cultures, CD4⁺ cells alone, and CD4⁺ cells with CD8⁺ cells. Surprisingly, in four of four individuals

examined, profound inhibition of replication of the NSI isolate was observed ($p<0.02$) (Fig. 5). There was no reduction in p24 content in SI-infected culture supernatants from the Kenyan samples. However, the North American control individual markedly suppressed the Kenyan NSI isolate, and the Kenyan SI isolate by approximately 50 per cent. Thus, $CD8^+$ cell-mediated suppression appears to be specific for NSI isolates among the cohort examined here, and efficiently reduces viral replication.

To delineate the mechanism of the suppressive effect by $CD8^+$ cells, transcriptional inhibition of LTR-mediated gene expression by supernatants of lectin-stimulated $CD8^+$ cells from 7 persistently seronegative women, one infected individual, and two control individuals were examined. Variable degrees of inhibition were observed among all groups (Table 1). Within the persistently negative cohort an inhibitory effect on LTR-mediated transcription was seen in several individuals, while enhancement was found in two other subjects (Table 1). Overall, no correlation with HIV seronegative or seropositive status, or with exposure to HIV was found.

Discussion

In this study we have evaluated the *in vitro* susceptibility of $CD4^+$ cells from a group of persistently seronegative, commercial sex workers from Nairobi, Kenya, who are at high risk of infection with HIV-1. This cohort constitutes a subgroup of a large health study of prostitutes in Nairobi [8]. For all participants enrolled in the study, the odds ratio for seroconversion escalated with the duration of prostitution, and the probability of remaining seronegative exponentially decreased over time [8]. However, in the cohort reported here, a protective effect that increased with the duration of prostitution was observed [8].This is a very unusual situation, and since these individuals represent a unique opportunity to study apparent natural resistance to HIV infection, we sought to evaluate in vitro correlates of infectivity and resistance.

Exposure of $CD4^+$ cells from the resistant women to primary isolates of Kenyan-derived HIV-1 of both NSI and SI phenotypes revealed no constitutive barrier to HIV infection or replication in vitro. These findings exclude the presence of a structural defect such as an altered receptor or co-receptor for the virus, and suggest that $CD4^+$ cells from these women are inherently susceptible to infection, and competent to allow for viral replication. No difference regarding the syncytial phenotype was observed indicating that the change in tropism that is commonly associated with a switch from NSI to SI phenotype likely could proceed unimpaired in this cohort.

Primary HIV isolates during the asymptomatic phase of infection predominantly

infect macrophages, and are of NSI phenotype. In addition, recently a panel of primary SI isolates were found to efficiently replicate in macrophages as well, and to depend on CCR5 as coreceptor [28]. In this study, we examined whether production of chemokines by $CD4^+$ cells was associated with resistance to infection with primary NSI and SI viruses. Excessive production of the chemokines MIP-1α, MIP-1β, and RANTES (30,000 to 100,000 pg/ml) had correlated with partial or complete resistance to infection by $CD4^+$ cells, while exogenous addition of 200,000 pg/ml of all three chemokines was required to block viral replication in $CD4^+$ cells [19]. In this study, MIP-1α and MIP-1β levels in $CD4^+$ supernatants exceeded 40,000 pg/ml in only one subject (1378), and this was independent of infection. Variability in the amount of chemokines elaborated was observed between individuals, and there was concordance for the three chemokines produced within the same individual. This suggests polymorphism for induction of chemokine expression, similar as has been shown for expression of CCR5 [29]. Thus, competition for binding to CCR5 appears to require very high amounts of all three chemokines in combination, and autologous production at levels exceeding those observed in this study may be required for a protective effect. These findings further support the lack of resistance of isolated $CD4^+$ cells from persistently seronegative Kenyan sex workers to infection, however, a protective effect mediated by chemokines or other factors elaborated by epithelial cells or stromal cells at the site of initial exposure cannot be excluded. Interestingly, synthesis of the above chemokines has been associated with a Th1 immune response induced by a bacterial antigen [30]. Th1 immune responses correlated with relative protection from HIV infection and from disease progression, while in the later stages of HIV disease cellular defects became more pronounced and antibody production persisted, characteristic

of type 2 immune responses [31,11]. The subjects in this study are exposed to numerous other sexually transmitted organisms [32], and conceivably induction of a type 1 immune response, and local secretion of chemokines, could influence establishment of HIV infection at the mucosal site.

Concurring with previous reports that the CCR5Δ32 phenotype is unique to individuals of Caucasian descent [33], no deletions were identified in the subjects of this study. Interestingly, HIV-1 subtypes A, B, C, D, and E, and group O viruses, all rely on CCR5 for viral entry, and do not replicate in cells from CCR5Δ32 homozygous individuals [34]. Thus, resistance in Kenyan subjects is not due to properties of $CD4^+$ cells. Transcriptional inhibition by lectin-stimulated CD8 cell-supernatants did not distinguish between resistant and susceptible individuals. This was not surprising, since factors acting at the level of gene transcription may be elaborated by uninfected and infected individuals, and no clinical correlation has been identified [35]. The nature of the factors mediating this effect is presently unknown.

Non-cytolytic $CD8^+$ cell-suppression of viral replication has been described in long-term non-progressing patients [36], and correlated with CD4 count and lack of disease progression [37,38]. Although the β chemokines MIP-1α, MIP-1β, and RANTES accounted for most of the reduced infection by NSI isolates [13], discussion continues regarding the complete identity of the $CD8^+$ cell-derived factors [39]. The suppressive effect observed by co-culture of $CD8^+$ cells in this study was specific for NSI isolates, and therefore may have been partially mediated by $CD8^+$ cell production of chemokines.

However, only one CD8 cell per four CD4 cells was present, and the $CD8^+$ cells were not previously in vitro stimulated, suggesting that very high constitutive production of chemokines would be required. Since cell-to-cell contact improved suppression by CD8 cells [12], it is likely that factors other than chemokines contribute to the cumulative effect observed in this study.

The contribution of cytolytic immune responses to HIV resistance in this cohort of persistenly seronegative individuals was not assessed, instead $CD8^+$ cell non-lytic effector functions were evaluated. Previous studies have identified T cell immune reactivity in a small percentage of exposed sex workers from Gambia [9], and cytolytic responses may as well have contributed to the resistance observed in subjects of this study. As there was no inherent interference with infection of isolated $CD4^+$ cells, there are likely factors preventing or limiting infection at the mucosal site of initial exposure. Nonlytic $CD8^+$ cells may exert their most profound effect by limiting an infection to a degree sufficient for generating MHC-class I restricted CTL that in turn are then able to eliminate virally infected cells at a localized site.

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Legend

Figure 1.

$CD4^+$ PHA-blasts from persistently seronegative women (556, 857, 1025, 1250, 1275, 1358, 1362, 1378, 1800) are as susceptible to infection by primary NSI Kenyan HIV isolate K293 as those from a non-exposed control individual (8404) ($p<0.02$). Similarly, there was no significant difference in infectivity by NSI isolate K1075 of $CD4^+$ PHA-blasts from individuals 858, 887, 893 and 1458 ($p<0.05$).

Figure 2.

$CD4^+$ PHA-blasts from persistently seronegative women are susceptible to infection by a primary Kenyan SI isolate (K-po). There was no statistically significant difference between the mean of the uninfected population and a local control individual's cells (8404) ($p<0.02$).

Figure 3.

Gel electrophoresis of a PCR-amplified segment of CCR5 reveals no deletion in the samples of Kenyan origin. Lane 1 is a 50 bp ladder, lanes 2 to 7 are samples from the persistently negative cohort, lane 8 is a sample from Caucasian heterozygote (not included in the cellular studies), and lane 9 is a negative control lane.

Figure 4.

Production of β-chemokines by uninfected and infected $CD4^+$ cells does not correlate with relative resistance to infection. The concentration of φ24 (Fig. 1 and 2) was determined concurrently with the concentrations of MIP-1α, MIP-1β, and RANTES in supernatants of infected and uninfected cells.

Figure 5.

Co-culture of $CD8^+$ cells with infected $CD4^+$ cells markedly ($p<0.01$) reduces the replication of a NSI virus, but not of a SI virus in the cohort of Kenyan women, while the control individual was able to profoundly inhibit the NSI isolate, and partially the SI isolate. $CD4^+$ cells were infected, and cultured alone, or with autologous $CD8^+$ cells, at 4:1 ratio for 11 days.

Figure 1.

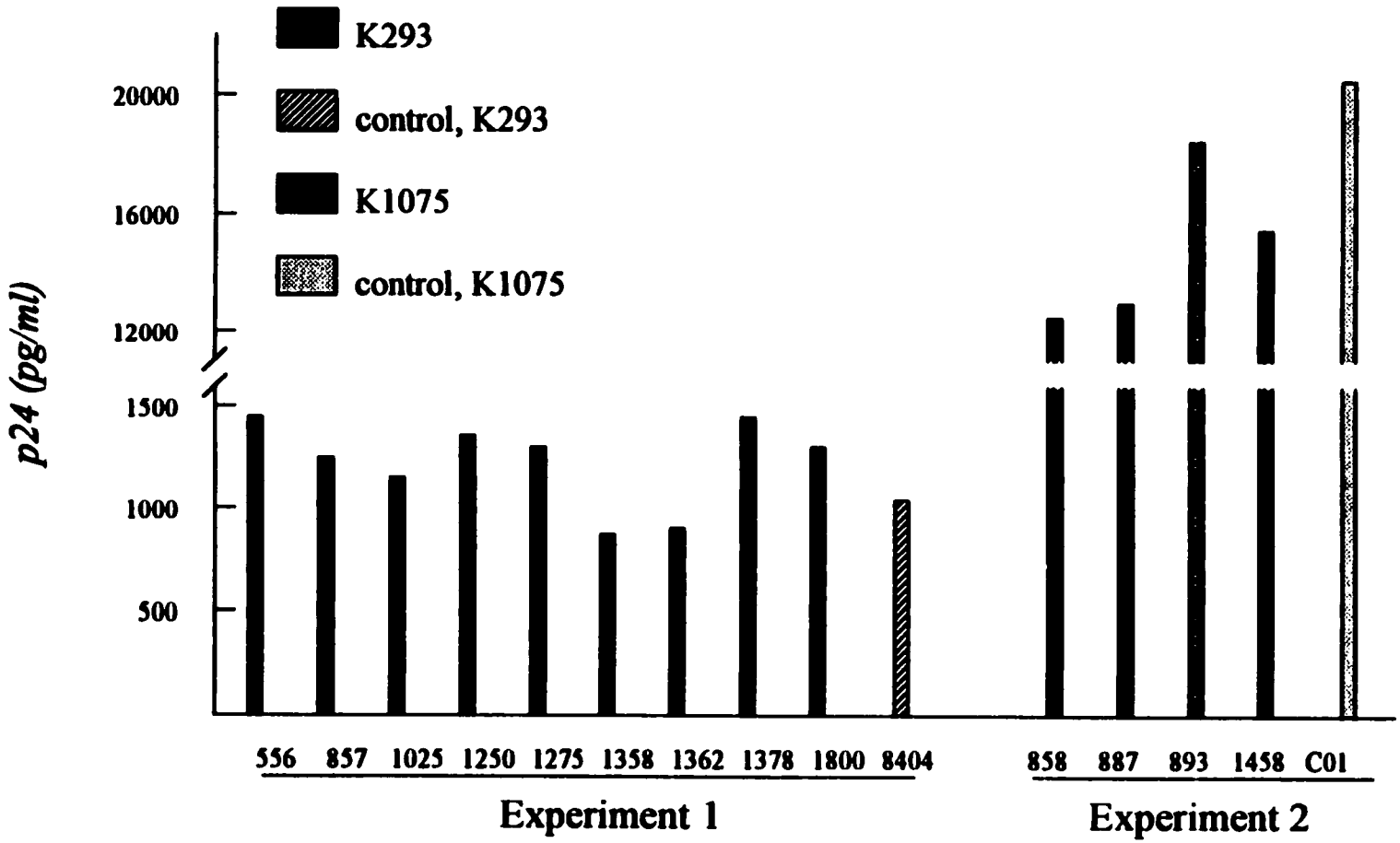

K293
control, K293
K1075
control, K1075
p24 (pg/ml)
20000
16000
12000
1500
1000
500
556 857 1025 1250 1275 1358 1362 1378 1800 8404
858 887 893 1458 C01
Experiment 1
Experiment 2

Figure 2.

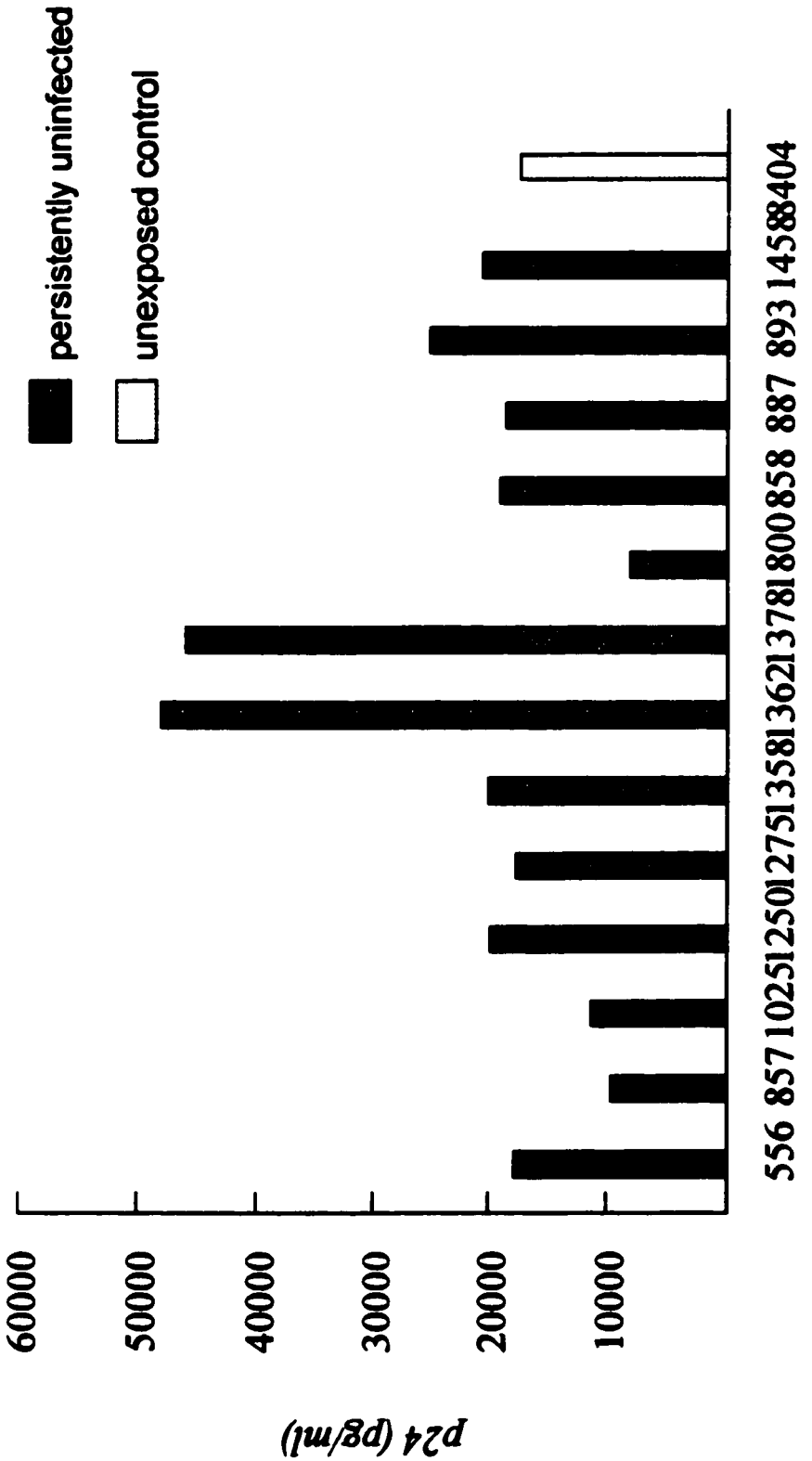

persistently uninfected
unexposed control
p24 (pg/ml)
60000
50000
40000
30000
20000
10000
556 857 1025 1250 1275 1358 1362 1378 1800 858 887 893 1458 8404

Figure 3

50 bp ladder

858

887

893

1358

1378

1458

1800

CCR5+/-

neg. control

Figure 4.

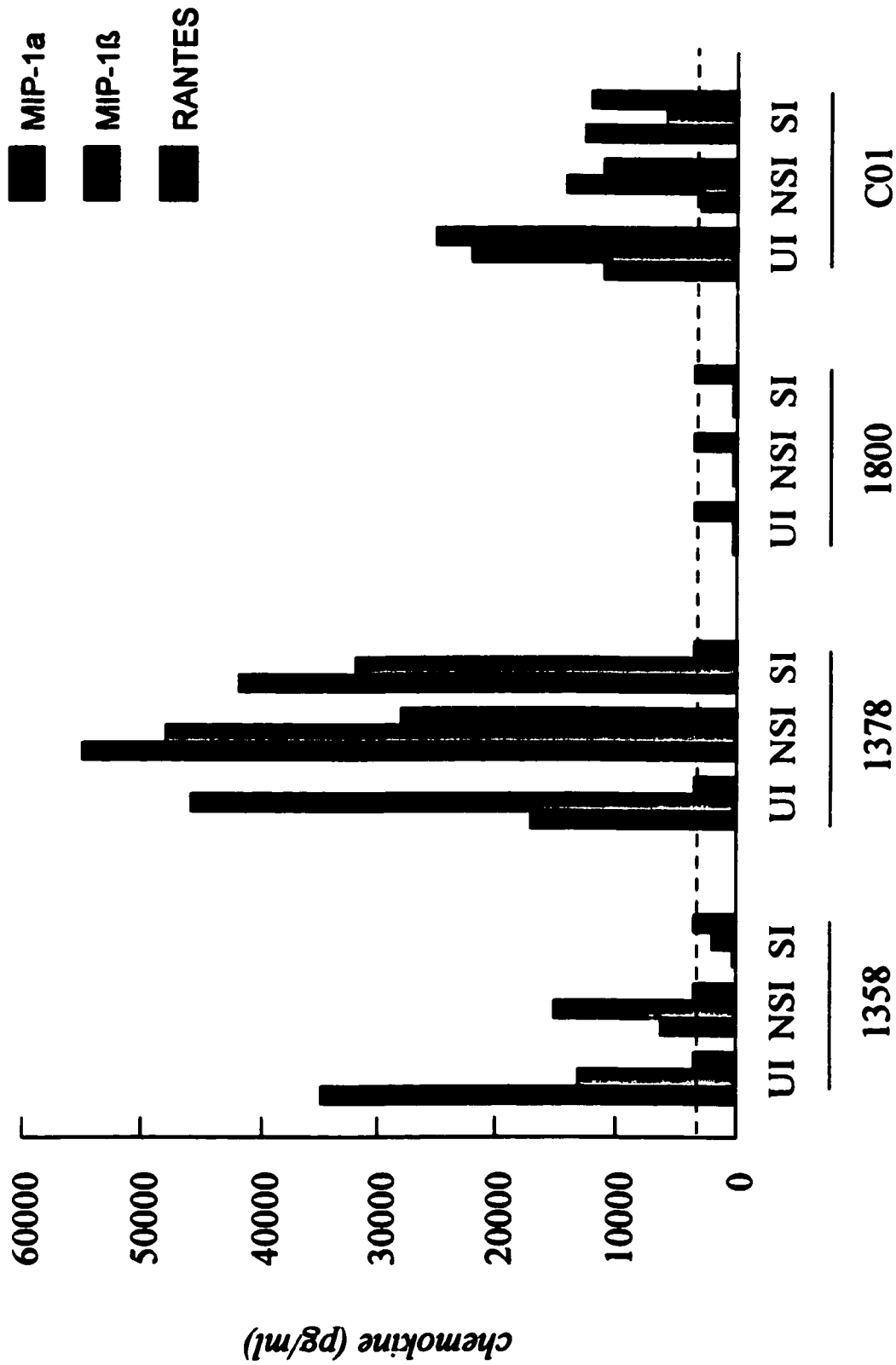
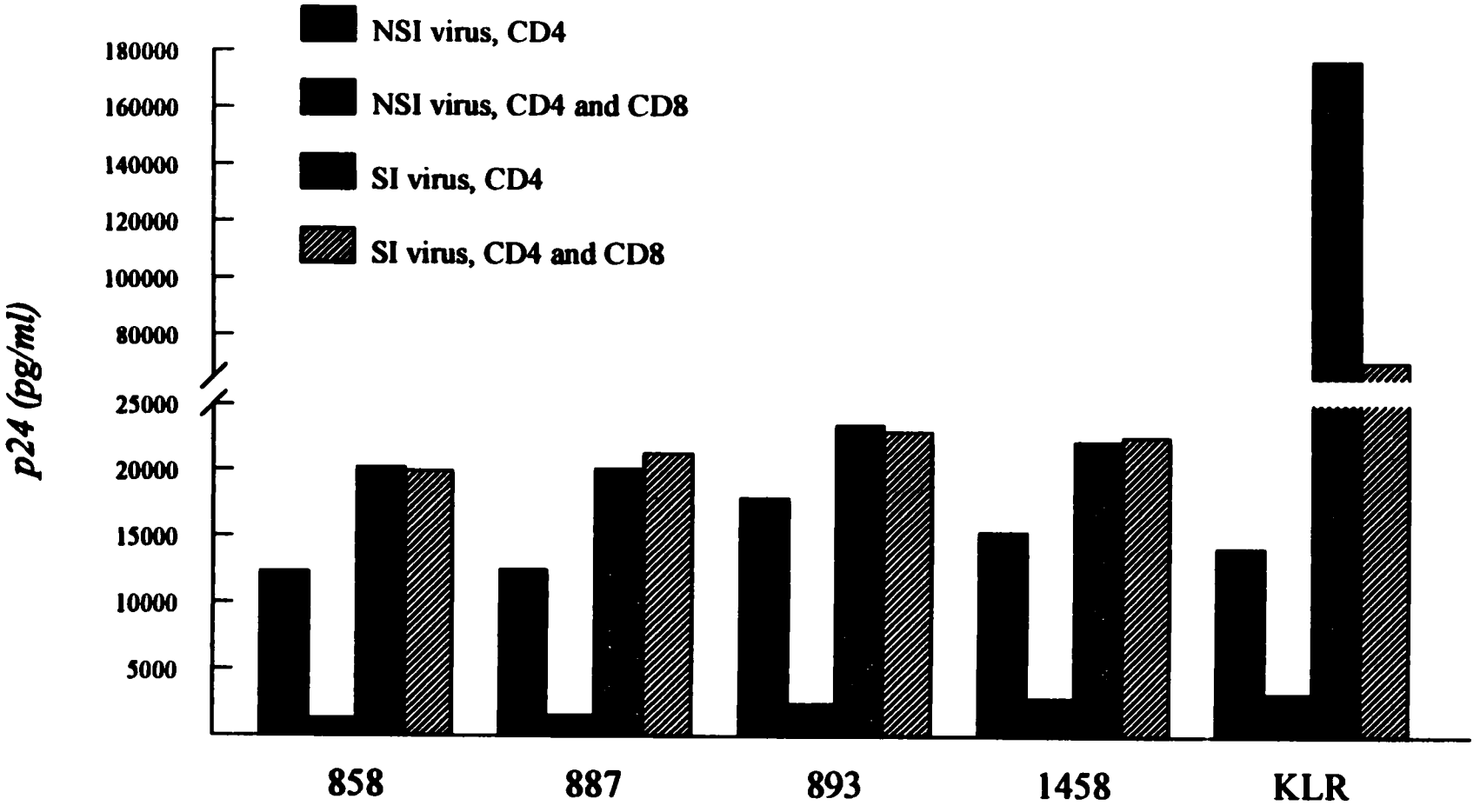

MIP-1a
MIP-1ß
RANTES
chemokine (pg/ml)
60000
50000
40000
30000
20000
10000
0
UI NSI SI
UI NSI SI
UI NSI SI
UI NSI SI
1358
1378
1800
C01

Figure 5.


NSI virus, CD4
NSI virus, CD4 and CD8
SI virus, CD4
SI virus, CD4 and CD8
p24 (pg/ml)
180000
160000
140000
120000
100000
80000
25000
20000
15000
10000
5000
858
887
893
1458
KLR

Chapter 6.

Summary

The experiments described in this dissertation were conducted to investigate the diverse roles of $CD8^+$ T lymphocytes in HIV infection and in resistance to infection. The overall results from the experiments performed are in concordance with the general view that there are beneficial as well as deleterious effects that may be attributed to $CD8^+$ T lymphocytes in HIV pathogenesis. In particular, in the initial chapters of this dissertation, effects of cytotoxic T lymphocytes were demonstrated that exert an overall detrimental effect on the host, namely the destruction of uninfected activated $CD4^+$ T lymphocytes. Though it may be argued that limiting $CD4^+$ T cell activation may provide fewer targets for full viral integration, and thus a beneficial effect of this phenomenon may exist, data on a limited number of patients suggested this aspect of CTL function is adverse to the patient's disease progression (Grant et al., 1994). Intuitively, destruction of activated $CD4^+$ cells regardless of their infective state would appear to be detrimental as impaired ability to respond to antigens of any nature would ensue. Thus, CTL acting to induce apoptosis in activated $CD4^+$ cells likely mediate a deleterious effect.

On the other hand, examining the role of CTL in contributing to resistance to HIV infection in chapter 4, it may be concluded that presence of CTL is associated with lack of infection. Control, unexposed individuals did not have HIV-specific CTL, while five of

eleven exposed uninfected persons did. Thus, at least circumstantially, CTL are implicated in mediating protection possibly by curtailing an early encounter with virus at a mucosal site, or in the draining lymph node. Though it is not possible to quantitate the protective effect of HIV-specific CTL in mediating resistance, cumulative experience suggested that various exposed uninfected individuals are characterized by having such CTL (Shearer and Clerici, 1996). Similarly, induction of immune responses associated with protection from infection were characterized by CTL specific for certain MHC alleles in non-human primates (Heeney et al., 1994). Thus, little doubt regarding the involvement of CTL in mediating resistance exists, however, the specific contribution will be difficult to evaluate in human subjects.

A different aspect of $CD8^+$ cells was examined in the work in chapter 5. Here, suppressive effects of $CD8^+$ cells on HIV replication were evaluated. This facet of $CD8^+$ cell function in HIV infection is unanimously accepted as beneficial, and little doubt regarding the inhibitory effect of co-culture of $CD8^+$ cells with infected $CD4^+$ cells exists. Demonstrating the ability of $CD8^+$ cells from commercial sex workers in Nairobi, Kenya to limit replication of HIV-1 contributed a further piece to the evolving picture of factors that are able to influence transcription or replication of the virus. In particular, the suppressive capacity of $CD8^+$ cells has usually been evaluated in the context of modulating an existing infection, while in the report in this dissertation it was associated with apparent resistance to infection. It is presently unclear whether suppressive factors elaborated by $CD8^+$ cells may be capable of mediating resistance, or whether apparent resistance results from a combination of cytolytic and suppressive activities, as well as other factors.

Thus, in summary, variable aspects of $CD8^+$ T lymphocytes in the context of HIV-1 infection and resistance have been examined in the experimental work comprising this dissertation. The information gained from the work contributes to delineating further the multi-faceted roles of $CD8^+$ T cells in specific circumstances associated with the pathogenesis of HIV-1 infection, or with exposure to HIV-1. While detrimental functions of $CD8^+$ T cells exist, and at times may predominate during disease progression, the beneficial aspects appear to be indispensable for a gradual course of infection, and for possibly creating a state of immune protection.

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