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CHARACTERIZATION OF IL-2 INDUCIBLE CYTOTOXIC LAK FUNCTION IN HIV-1 INFECTED INDIVIDUALS

BY

CHRYSSA GRYLLIS

A thesis submitted to the Faculty of Graduate Studies and Research, McGill University, in partial fulfillment for the degree of Doctor of Philosophy.

Department of Experimental Surgery

McGill University

Montreal, Canada

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IL-2 INDUCIBLE LAK RESPONSES IN HIV-1 INFECTED INDIVIDUALS

ABSTRACT

Inducible LAK cell responses were studied in HIV-seropositive individuals lacking clinical symptoms, and overt AIDS patients. Inducible LAK cell responses have been operationally defined as, non-MHC-restricted and antigen-nonspecific cytotoxic activity observed following IL-2 stimulation. HIV-seropositive asymptomatic individuals exhibited an enhanced LAK cell response against HIV-infected targets while lysis of uninfected targets remained at control levels. LAK activity of AIDS patients however, was significantly diminished when compared to healthy controls. Immunomagnetic negative selection depletion experiments indicated that LAK cell activity is mediated primarily by CD56-expressing lymphocytes, both at the progenitor and effector cell level. Of interest, in HIV-seropositive asymptomatic individuals we observed the emergence of a second CD8-expressing cytotoxic population that mediates IL-2-induced non-MHC-restricted and antigen-nonspecific cytotoxicity. Overall we demonstrated that CD56-expressing LAK cells of HIV-seropositive patients exhibited a decreased ability to mediate cytotoxicity on a per cell basis against a panel of different targets. *In vivo*, this inhibition may be amplified by decreases in absolute numbers of CD56-expressing lymphocytes per ml of blood. HIV-infection therefore results in dramatic changes on the number, function and phenotype of the effector cells mediating IL-2 inducible LAK cell responses.

RESUME

Dans cette étude nous avons examiné les cellules tueuses activées par des lymphokines (Lymphokine Activated Killer cells), sur des individus séropositifs, par le virus VIH, sans symptômes cliniques et sur des patients souffrant du SIDA (Syndrome d'immunodéficience acquise). Les réponses des cellules LAK ont été operationellement définies comme activité non-MHC-restricted (Complexe Majeur d'Histocompatibilité-restreint) et antigène non-specifique, observées suivant une stimulation par IL-2 (Interleukine 2). Des individus VIH-séropositifs ont exhibés une activité augmentée des cellules LAK contre des cibles infectées par le VIH, tandis que la lysis des cibles non-infectées a été resté aux niveaux des contôles. Cependant, l'activité LAK des patients souffrant du SIDA, a diminué d'une manière significative en comparaison avec des populations contrôles. Les expériences par déplétion négative immunomagnétique ont indiqué que l'activité de la cellule LAK est liée premièrement au lymphocytes exprimant CD56, aussi bien au niveau du progeniteur et de l'effecteur .On a constaté avec intérêt, que sur des individus séropositifs-VIH sans symptômes, nous avons observé l'émergence d'une deuxième population cytotoxique exprimant CD8, qui est le médiateur d'activité cytotoxique non-MHC-restricted (Complexe Majeur d'Histocompatibilité-restreint) et antigène non-spécifique, induit par incubation avec IL-2. En tout cas, nous avons démontré que les $CD56^+$ LAK de patients séropositifs-VIH ont exhibé une abilité cytotoxique diminuée par cellule contre un panel des cibles différentes. Cette inhibition *in vivo*, peut être amplifiée par des diminutions en nombre absolut par ml de sang de lymphocytes exprimant CD56. Par conséquent, l'infection par VIH a comme résultat des changements importants sur le nombre, la fonction et le phenotype de l'effecteur LAK cellules induit par IL-2.

To my parents , Constantinos
and the memory of my late cousins Jacob and Jacob.

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PREFACE

This thesis is written in accordance to the particular option 7 described in Guidelines concerning Thesis Preparation:

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Publications presented in chapters 2, 3, 4 and 5 are in the same form that have been published or submitted for publication. Connecting texts between chapters have been

included to insure continuity of the text. Chapter 1 includes a general introduction and an extensive literature survey pertinent to our subject. A general conclusion is presented in chapter 6. Bibliography concerning chapters 1 and 6,as well as connencting texts, is presented at the end of chapter 6. Manuscripts presented in chapters 2, 3, 4 and 5 have been published or submitted for publication in the following periodicals:

Chapter 2: Gryllis C., Wainberg M. A., Gornitsky M., and B. Brenner. 1990. Diminution of inducible lymphokine-activated killer cell activity in individuals with AIDS-related disorders. AIDS 4:1205.

Chapter 3: Brenner B., Gryllis C., Gornitsky M., Cupples W., and M. A. Wainberg. 1991. Differential effects of chemotherapy-induced and HIV-induced immunocompromise on NK and LAK activities using breast cancer and HIV-1 seropositive patient populations. Anticancer Res. 11:969.

Chapter 4: Gryllis C., Wainberg M. A., Bentwich Z., Gornitsky M., and B. G. Brenner. 1992. Increased LAK activity against HIV-infected cell lines in HIV1$^+$ individuals. Clin Exp. Immunol. 89: (in press).

Chapter 5: Gryllis C., Wainberg M. A., and B. G. Brenner. 1992. Novel non-MHC restricted cytotoxic cells in HIV-1 seropositive groups. J. Immunol (submitted for publication).

The candidate was responsible for all the research described in chapters 2 to 5, with the exception of experiments concerning NK and LAK activity of cancer patients in chapter 3. In addition, immunofluoresence analysis and HIV-infection of target cell lines was performed by Hugo Soudeyns. Flow cytometry was performed by Franca Sicilia on a routine basis, while flow cytometric analysis of the depletion experiments was done by the candidate.

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ABBREVIATIONS

ADCC	Antibody dependent cellular cytotoxicity
AIDS	Acquired immunodeficiency syndrome
ARC	AIDS-related complex
AZT	Azidothymidine
CD	Cluster of differentiation (of human leukocyte antigens)
CMV	Cytomegalovirus
CNS	Central nervous system
CSF	Colony stimulating factor
CTL	Cytotoxic T-lymphocytes
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
E:T	Effector : target ratio
GM-CSF	granulocyte-macrophage-colony stimulating factor
HIV	Human immunodeficiency virus
HLA	Major histocompatibility complex of humans
HTLV	Human T-cell leukemia virus
IFN α,β,γ	Interferon alpha, beta, gamma.
Ig	Immunoglobulin
IL-1,2,3,6,12	Interleukin-1,2,3,6,12
kDa	Kilodalton
KS	Kaposi's Sarcoma
LAK	Lymphokine-activated killer
LFA-1	Lymphocyte function-associated -1
LGL	Large granular lymphocytes

LPS	Lipopolysaccharide
LTR	Long terminal repeats
LU	Lytic unit
MHC	Major Histocompatibility Complex
N-CAM	Neural-cell adhesion molecule
nM	nanomolar
NK	Natural killer
NKCF	Natural killer cytotoxic factors
TCR	T-cell receptor
TNF	Tumor necrosis factor
PBL	Peripheral blood lymphocytes
PCP	Pneumocystis pneumonia
PDGF	Platelet-derived growth factor
PFP	Pore-forming protein
PGL	Progressive generalized lymphadenopathy
PHA	Phytohemagglutinin
pM	Picomolar
PMA	Phorbol 12-myristate 13-acetate
PMN	Peripheral mononuclear cells
RNA	Ribonucleic acid
RPMI	Roswell's Park Memorial Institute
RT	Reverse transcriptase
SIV	Simian immunodeficiency virus
TCGF	T-cell growth factor
TIL	Tumor infiltrating lymphocytes.
VIP	Vasoactive intestinal peptide

APPENDIX I

PRINCIPAL FEATURES OF CD MOLECULES

CD Designation	Common Synonyms	Main Cellular Expression	Known or Proposed Function(s)
CD2	T11;LFA-2	T cells,NK cells	Adhesion molecule
	SRBCR		T cell activation
CD3	T3; Leu-4	T cells	Signal Transduction
			after antigen recognition
CD4	T4; Leu-3	Class II MHC	Binds Class II MHC
		restricted T cells	signal transduction
CD8	T8; Leu-2	ClassI MHC	Binds Class I MHC
		restricted T cells	signal transduction
CD11a	LFA-1	Leukocytes	Adhesion
	α chain		binds ICAM-1
CD11b	Mac-1;	Granulocytes	adhesion;phagocytosis
	CR3 α chain	Monocytes,NK	of iC3b-coated particles
CD11c	p150,95;	Granulocytes	adhesion?phagocytosis
	Cr4 α chain	Monocytes,NK	of iC3b-coated particles
CD16	FcRIII	NK,Macrophages	Low affinity
		Granulocytes	Fcγ receptor, ADCC
CD25	Tac;p55	Activated T, B ,NK	Complex with p70 to
	low affin.IL-2R	and macrophage	form high affinity IL-2R
CD56	Leu-19;	NK cells	Homotypic adhesion
	NKH1		isoform of N-CAM
CD57	Leu-7;	NK cells	??
	HNK1	ubsets of T cells	

CHAPTER 1

GENERAL INTRODUCTION
SURVEY OF THE LITERATURE

1. HUMAN IMMUNODEFICIENCY VIRUS AND AIDS

1.1 HISTORY

AIDS was first recognized as a new disease in the early 80's (CDC, 1981), when several doctors noticed an increased frequency of Pneumocystis pneumonia and Kaposi's sarcoma (KS) cases among previously healthy homosexuals (CDC, 1982). The combined immunodeficiencies in these patients who suffered from no genetic immune dysfunctions indicated the emergence of a new syndrome of acquired immunodeficiency (Gottlieb *et al.*, 1982). Subsequent occurrence of AIDS among hemophiliacs, blood transfusion recipients, sex partners of risk-group members, and children born to mothers at risk suggested that this disease was spread by a transmissible agent through genital secretions and blood.

The similarity of AIDS to the disease caused in cats by feline leukemia virus (Hardy, 1985) as well as the discovery and isolation of the first human T-cell leukemia virus (HTLV)-1 from T-cells of patients that suffered from T-cell malignancies (Poiesz *et al.*, 1981) led many investigators to the search of a human retrovirus. Indeed a human retrovirus was isolated as the causative agent of AIDS and named lymphadenopathy virus (LAV) by Montagnier's group (Barre-Sinoussi *et al.*, 1983.) and human T-cell lymphotropic virus type III (HTLV-III) by Gallo's group (Gallo *et al.*, 1984.). The confusion over the nomenclature of the virus was ended when a commission proposed the term Human Immunodeficiency Virus (HIV)-1 to identify the etiologic agent of AIDS (Coffin *et al.*, 1986).

1.2 CLASSIFICATION

HIV is a member of the lentivirus family of animal retroviruses based on genomic sequence homologies, morphology and life cycle (Gonda *et al.*, 1986). Lentiviruses include visna virus of sheep as well as the bovine feline and simian macaque immunodeficiency viruses (Gonda *et al.*, 1986; Letvin, 1990). They all induce slowly progressive fatal diseases. These viruses elicit long latency infections as well as short term cytopathic effects. A persistent viremia associated with weak neutralizing humoral responses is observed. High mutational rates, cytolytic effects upon host cells and central nervous system (CNS) involvement are also shared features among lentiviruses (Letvin, 1990). Of interest SIVmac, a virus isolate from a rhesus monkey lymphoma has been shown to induce an AIDS-like disease on a variety of Asian macaque species (Letvin, 1990).

A second human lentivirus, HIV-2 has been isolated from sera of AIDS patients in West Africa (Clavel *et al.*, 1986). All previous isolates of HIV have been consequently designated HIV-1. HIV-2 is antigenically distinct from the HIV-1 that causes AIDS in Central Africa Europe and Oceania. Antisera from HIV-2 do not recognize the HIV-1 *env* antigen (Clavel *et al.*, 1987) and there is no ADCC crossreactivity observed between these two viruses (Ljunggren *et al.*, 1988). HIV-2 shows the same tropism as HIV-1 for $CD4^+$ cells, and results in a syndrome indistinguishable from HIV-1 (Brun-Vezinet *et al.*, 1987).

Both HIV-1 and HIV-2 have similar structures, resembling that of other retroviruses. The HIV virus consists of two identical single strands of RNA and viral enzymes packaged within a core of viral proteins. These core proteins are surrounded by a phospholipid bilayer envelope derived from the host cell membrane that includes viral glycoproteins (Varmus, 1988). The dense viral core is, as in most other lentiviruses, bar-shaped. Nucleotide sequence comparisons have indicated that HIV shares more homologies with ungulated lentiviruses than with any other retroviruses (Varmus, 1988; Gonda *et al.*, 1986).

1.3 EPIDEMIOLOGY

Most evidence suggests that the HIV virus has evolved in Africa and from there has been transmitted to the rest of the world (Karpas, 1990). Up to date 450,000 AIDS cases have been reported worldwide (WHO and CDC, 1992). This is an underestimate since AIDS cases are estimated to exceed one million, and with this number reflecting only one tenth of the HIV-seropositive population (Anderson and May, 1992).

Of the reported cases, about 200,000 are in USA, 60,000 in Europe and 130,000 in Africa (WHO and CDC, 1992). The majority of AIDS cases in Europe and America fall within the high risk groups, namely, homosexual or bisexual males and intravenous drug abusers. In addition, female exposure represents 10% of the reported AIDS cases (WHO and CDC, 1992). Of interest, heterosexual exposure in USA although less than 1% before 1985 increased to 4% in 1988 (Peterman *et al.*, 1988) and in 1991 represented 6% of the reported AIDS cases (WHO and CDC, 1992).

In Africa, the HIV spread shows an entirely different pattern more indicative of a slowly developing epidemic. HIV virus is spread mainly through heterosexual contact and consequently, similar male and female rates of exposure are observed (Anderson and May, 1992). The AIDS pandemic has reached alarming proportions in sub-Saharan Africa where in most afflicted countries (including Uganda Kenya and Zambia) AIDS is the leading cause of adult mortality, and 20% to 30% of pregnant women are HIV carriers. Statistical and epidemiological models predict, that although it may take 30 years for HIV prevalence to rise from one thousandth of a percent to one percent, it will take only 10 years to reach 20% from 10% (Anderson and May, 1992).

Initial infection is either unnoticed or is accompanied by a flu-like illness with fever, muscle pain, sore throat and rash. During this period, the virus is replicating and can be detected within the blood and cerebrospinal fluid. After this initial phase, virus titers drop significantly. The first HIV specific antibodies appear 3-20 weeks post infection (Inagawa *et al.*, 1989). A long asymptomatic latency period from 2 up to at least 10 years follows,

during which the immune system is functional. The average time of latent infection was reported to be less than 2 years in 1984, 4.5 years in 1986, about 8 years in 87/88, 10 years in 1989 and almost 12 years in 1990 (Rees, 1991). Children under seven years of age and adults above 60 years of age have shorter average latency periods ranging from 2.0 to 5.5 years (Medley *et al.*, 1987). Studies in 1988 estimated that, the probability of an infected person to develop AIDS within 15 years of HIV exposure ranges from 78 to 100% (Lui, 1988).

AIDS is characterized by an overwhelming depletion of $CD4^+$ cells and AIDS-associated opportunistic infections including protozoa (*Pneumocystis carinii*), mycobacteria, fungi (*Candida*) and viruses (herpes simplex virus, cytomegalovirus and varicella-zoster). Malignant neoplasms such as B-cell lymphomas, frequently develop in AIDS patients. Anywhere from 30-60% of AIDS homosexuals develop KS. Up to two thirds of patients may suffer from AIDS-encephalopathy, a form of dementia (Navia *et al.*, 1986; Rosenberg and Fauci, 1989b).

1.4 GENOMIC STRUCTURE

The length of the HIV genome varies from 9.2 to 9.7 kilobases depending on the viral isolate (Alizon *et al.*, 1984; Hahn *et al.*, 1984). Three sequences code respectively for the three structural proteins: a) the ***gag*** gene codes for a p55 precursor polyprotein that can be cleaved into the viral core proteins p24, p17, p9 and p7 (Varmus, 1988), b) the **pol** gene, codes for a protease that cleaves the *gag* precursor protein (Kohl *et al.*, 1988), a reverse transcriptase that makes proviral DNA from viral RNA, and an integrase that is required for proviral insertion into the host DNA (Hansen *et al.*, 1988; Sherman and Fyfe 1990), and c) the ***env*** gene codes for the precursor polyprotein gp160, that is processed intracellulary by endoproteolytic cleavage into the envelope protein gp120 that binds CD4, the HIV viral receptor and the transmembrane gp41 that is necessary for viral fusion to target cell (Veronese *et al.*, 1985; Kozarski *et al.*, 1989).

DNA transcription is regulated by long terminal repeats (LTR) that flank both sides of the viral structure. LTRs contain characteristic regulatory elements such as the TATA promoter, polyadenylation signal sequences, cis-acting elements such as *spi* binding sites, as well as negative regulatory elements (NRE), the NFkB enhancer region and the transactivating responsive (TAR) sequence (Starcich *et al.*, 1985; Vaishnav and Wong-Staal, 1991).

Regulatory genes include a) the ***tat*** gene which codes for a 14-15 kDa protein that binds the TAR region of the LTR (Rosen *et al.*, 1985; Arya *et al.*, 1985); *tat* gene products are necessary for viral replication, upregulating HIV expression by directing an increase in the HIV mRNA accumulation (Rosen *et al.*, 1985; Vaishnav and Wong-Staal, 1991), b) the ***rev*** gene that encodes for a 20 kDa protein that is required for HIV replication and proper postranscriptional splicing of *gag* and *env* proteins (Feinberg *et al.*, 1986; Cochrane *et al.*, 1990), c) the ***nef*** gene which codes for the 27 kDa protein (Arya and Gallo, 1986) that can downregulate HIV transcription by interacting with the NRE domain (Ahmad and Vankatesan, 1988). *Nef* mutations result in an increased viral RNA accumulation that can cause cytopathic effects (Luciw *et al.*, 1987). In addition *nef* downregulates CD4 expression (Garcia and Miller, 1991) and thus may be important in maintaining latent infection, d) the ***vif*** (virion infectivity factor) gene which encodes for a 24 kDa protein (Lee et al., 1986; Arya and Gallo, 1986) that is not required for replication nor for cytopathogenesis (Sodroski *et al.*, 1986). It seems that *vif* is involved in infection by free virus, while unnecessary for cell to cell infection (Strebel *et al.*, 1987; Fisher *et al.*, 1987), e) the ***vpu*** gene that codes for a 16 kDa protein which seems to be required for optimal assembly and packaging of new virions (Strebel *et al.*, 1988), and finally f) the ***vpr*** gene, that codes for a 15kDa protein that accelerates the replication and cytopathic effects of HIV in $CD4^+$ T-cells, (Wong-Staal *et al.*, 1987) monocytes and macrophages (Cohen *et al.*, 1990).

Heterogeneity of HIV isolates from patient to patient (Luciw *et al.*, 1984; Shaw *et al.*, 1984) as well as within the same patient (Hahn *et al.*, 1986) has been observed. Although variations extend throughout the entire HIV genome, *env* sequences display a tenfold higher rate of divergence than the other sequences (Hahn *et al.*, 1986). The low fidelity of HIV reverse transcriptase, being one third to one tenth that of other retroviruses, is associated with the high variability seen in HIV isolates (Preston *et al.*, 1988; Roberts *et al.*, 1988).

HIV-2 shares the same molecular organization and reproductive biology as HIV-1 (Clavel *et al.*, 1987). However HIV-2 lacks the *vpu* gene present in HIV-1 (Cohen *et al.*, 1988), and expresses a novel gene *vpx* that encodes for a 14 kDa immunogenic core protein absent in HIV-1 (Henderson *et al.*, 1988). *Vpx* is not required for viral replication, however, *vpx*$^-$ mutants are less efficient in infecting primary lymphocytes (Guyader *et al.*, 1989).

1.5 CYCLE OF REPLICATION

The first step in HIV infection is the binding of the external glycoprotein gp120 to CD4 expressed on the surface of CD4$^+$ lymphocytes (McDougal *et al.*, 1986). HIV infection can occur by direct fusion of the HIV envelope with the host cell membrane (Hoxie *et al.*, 1988; Bedinger *et al.*, 1988; Maddon *et al.*, 1988). HIV particles may also enter the cell by CD4 receptor-mediated endocytosis. Other cellular factors may also be critical for the HIV infection, since murine cells that express human CD4 are resistant to HIV infection (Maddon *et al.*, 1986).

As soon as an HIV virion enters the cell, the enzymes within the nucleoprotein complex become activated and initiate the viral reproductive cycle (Varmus, 1988). Viral reverse transcriptase transcribes viral RNA into double stranded (ds) DNA. Integrase then catalyzes dsDNA integration into genomic host DNA. Although in most retroviral systems the unintegrated viral DNA is short lived (Keshet and Temin, 1979), in some other systems

including HIV it can persist for long periods of time contributing to cytopathic effects upon the host cell (Ho *et al.*, 1987).

If the host cell is in a non-activated state, the provirus remains in a latent transcriptionally inactive stage for months or years. Upon host cell activation host transcriptional signals transactivate proviral transcription (Ho *et al.*, 1987). Proviral DNA is transcribed into genomic RNA and mRNA. mRNA is in turn spliced and translated into viral proteins that undergo post-translational modifications, including cleavage and glycosylation. Assembly of infectious particles begins by packaging full length virion RNA with core proteins including *gag* and *pol* which are required for the next cycle of integration (Ho *et al.*, 1987; Varmus, 1988). This nucleoprotein complex is then enclosed with viral proteins at the host cell membrane and the infectious virus is then released by a process of budding. Budding of the virus is associated with host cell lysis (Leonard *et al.*, 1988).

1.6 REGULATION

The HIV virus is an extremely complex virus that has evolved regulatory mechanisms that can alternatively facilitate high viral productivity with concomitant cell death, restrict viral production sparing the host cell, or perpetuate latent infection (Rosenberg and Fauci 1989b). Individuals with HIV infection indeed show an extended asymptomatic phase where p24 antigenemia is restricted. Late in the course of infection, concomitant with clinical manifestations, p24 levels rise indicating renewed viral replication.

Cellular factors play an important role in the activation of viral replication. Mitogens, such as PHA and PMA, have been shown to induce *in vitro* viral replication by inducing T-cell activation factors, such as NFκB that in turn bind enhancer elements on the HIV-LTR (Nabel and Baltimore 1987).

Antigenic stimulation also enhances HIV expression. HIV replication is up to hundred times higher in peripheral blood monocytes or leukocytes that had been previously antigenically activated (Margolick *et al.*, 1987).

Viral proteins from other viruses such as CMV, SIV, HSV, and EBV that exhibit similar cell tropism to HIV have been shown to upregulate HIV expression (Vaisnhav and Wong-Staal, 1991). CMV and HIV coinfection has been observed *in vivo* in brain cells of AIDS patients (Nelson *et al.*, 1988). These viral proteins exert their effects not only directly by binding distinct regions of HIV LTR, but also by stimulating HIV expression indirectly via induction of stress responses (Valerie *et al.*, 1988). For example, UV light and mitomycin C cause stress responses that can independently co-stimulate CAT activity in HIV LTR-CAT-transfected ceils, possibly by triggering a cellular "s.o.s" response (Stanley *et al.*, 1989).

Cytokines also play an important role in HIV regulation. GM colony-stimulating factor (GM-CSF) stimulates HIV expression in infected peripheral blood monocytes (Poli *et al.*, 1990). TNF-α and TNF-β enhance HIV transcription possibly by inducing the production of cellular factors that bind to the NFκB enhancer region (Durum *et al.*, 1991; Rosenberg and Fauci, 1990). Since TNF-α is secreted by monocytes in response to opportunistic infections, it can possibly accelerate progression to AIDS in asymptomatic individuals. Indeed high TNF-α levels were secreted by the peripheral blood monocytes of AIDS patients (Wright *et al.*, 1988). Another lymphokine IL-6 has also been shown to activate postranscriptionally the HIV virus (Poli *et al.*, 1990; Durum *et al.*, 1991; Rosenberg and Fauci, 1990; Breen *et al.*, 1990).

Inhibitory cytokines have also been identified. IFN-α can inhibit postranslational budding, while transforming growth factor β has been shown to block the induction of reverse transcriptase by PMA (Durum *et al.*, 1991; Brinchman *et al.*, 1991).

2. ADAPTIVE IMMUNE RESPONSES AGAINST HIV

2.1 HUMORAL IMMUNE RESPONSES

With the advent of AIDS, antibody responses against HIV molecules were identified (Imagawa *et al.*, 1989). This constitutes, to date, the basis for HIV detection by

the HIV enzyme-linked immunosorbent assay (ELISA). Seroconversion, in the majority of cases, occurs three weeks to three months post-HIV infection. Most antibodies are directed against gp120 and gp160 env glycoproteins, and they can be isolated from infected individuals throughout the course of HIV infection. Almost half of infected individuals produce non-neutralizing antibodies to a highly conserved 15 amino acid region (504-518) of the carboxy terminus of of gp120 (Palker *et al.*, 1987), while, antibodies against a 12 amino acid immunodominant sequence (598-609) in gp41 have been detected in all HIV-infected patients examined (Gnann *et al.*, 1987). The titer of p24 antibodies is higher in the beginning of the HIV infection and diminishes as the disease progresses (Pan *et al.*, 1987). Antibodies against reverse transcriptase (Pan *et al.*, 1987) and against several regulatory proteins (Barone *et al.*, 1986) have also been isolated.

Several neutralizing antibodies against gp120 have been isolated both from HIV-infected humans and animals (Lake *et al.*, 1992; Palker *et al.*, 1988). While some report no significant correlation between titers of neutralizing antibodies and disease status (Prince *et al.*, 1987) others show a negative correlation between antibody titers and disease progression (Sheppard *et al.*, 1991; Wendler *et al.*, 1987).

2.2 CELLULAR IMMUNE RESPONSES

2.2.1 T-cell proliferative responses

T-cell proliferation responses directed against p24 and gp120 HIV antigens have been reported (Wahren *et al.*, 1987;Ahearne *et al.*, 1988). However, the percentages of infected individuals who exhibit these responses vary from 25% (Wahren *et al.*, 1987) to 50% and 70% (Reddy *et al.*, 1987) among different studies. In direct contrast, in one study no responses against gp120 where observed (Krohn *et al.*, 1987).

Only the amino terminus of gp120 has been shown to elicit T-cell proliferative responses, indicating that the antigens stimulating neutralizing antibodies are distinct from the ones eliciting T-cell proliferative responses (Ahearne *et al.*, 1988; Walker *et al.*, 1988).

In one report, one quarter of the HIV seropositive individuals tested exhibited T-cell proliferation in response to an immunodominant epitope of gp41 (Schrier *et al.*, 1988).

Efforts for the design of a vaccine that can elicit both humoral and T-cell proliferative responses, have identified two conserved regions of gp120 (Cease *et al.*, 1987) that when injected into mice induce gp120-directed lymph node proliferation. In addition 60% of healthy individuals injected with vaccinia-*env* recombinant virus showed T-cell proliferative responses to at least one of these gp120 peptides (Berzofsky *et al.*, 1988).

2.2.2 Cytotoxic T-lymphocytes

Cytotoxic T-lymphocyte (CTL) responses have been detected in HIV-infected individuals. Indeed, the frequency of CTLs among peripheral blood lymphocytes (PBL) is so high that no prior *in vitro* antigenic stimulation was required for their detection, as is the case with most other human retroviruses. HIV-specific CTL clones, have been isolated from a variety of sources including PBL, alveolar, lymph node and CNS lymphocytes (Vanham *et al.*, 1990; Walker and Plata, 1990).

Of interest, HIV-specific CTLs have also been isolated from seronegative individuals after *in vitro* antigenic stimulation. Stimulation with HIV-1 infected autologous lymphocytes yielded $CD3^+CD8^+$ CTLs specific for *env* and *nef* while purified env gp120 yielded $CD4^+$ CTLs (Plata *et al.*, 1987; Huffenbach *et al.*, 1989; Siliciano *et al.*, 1988).

HLA-class I restricted CTLs specific for the *env* have been isolated from alveolar lymphocytes PBL and cerebrospinal fluid. In addition, peripheral blood class I restricted CTLs, specific for *gag*, *pol*, *vif* and *nef* (Walker and Plata, 1990) have been reported. Cohort studies demonstrate strong HIV-specific T-cell cytotoxicity in early asymptomatic patients that declines with disease progression (Autran *et al.*, 1991). Loss of HIV-specific CTLs has also been reported as a result of the ability of these cells to expand (Pantaleo *et al.*, 1990). Recent reports have indicated that $CD8^+CD57^+$ cells can exhibit inhibitory

effects on CD8 cytolytic activities (Joly *et al.*, 1989), via a lectin binding 20-30 kDa soluble molecule (Sadat-Sowti *et al.*, 1991).

Class II-restricted CTLs specific for the *env* protein have also been isolated from PBLs (Whitton *et al.*, 1987), and cerebrospinal fluid (Sethi *et al.*, 1988) of seropositive people upon *in vitro* antigenic stimulation. The *in vivo* role of these class II CTLs remains unclear since up to date no class II-restricted CTLs have been isolated from fresh tissues of seropositive individuals (Walker and Plata, 1990).

Several studies have focused on the epitopes HIV-specific CTLs are directed at. Elucidation of the precise immunogenic epitopes can prove useful to the design of vaccines. Although results from the mouse model suggested that CTL responses were directed at a single immunogenic epitope (Takahashi *et al.*, 1988), unfortunately studies from humans indicated that this was not the case. CTL responses in HIV infected people are heterogenous and multiple epitopes of the same proteins are involved (Walker and Plata, 1990).

The protective role of CTLs in HIV-seropositive individuals remains a controversy. Evidence for a beneficial role comes from experiments where $CD8^+$ T lymphocytes were able to block the in vitro replication of SIV and HIV in $CD4^+$-infected lymphoblasts (Walker *et al.*, 1986; Kannagi *et al.*, 1988). Although initially it was believed that this inhibition was mediated by a soluble factor, it has been later demonstrated that cell to cell contact was necessary (Tsubota *et al* 1989).

Alternatively HIV-specific CTLs can play a detrimental role in the outcome of disease by lysing HIV-infected host cells (Walker and Plata, 1990). In fact it has been proposed that AIDS is an autoimmune disease and T-lymphocyte tolerance to HIV might be the only solution (Walker *et al.*, 1986). CTLs have been isolated from inflammation sites (Sethi *et al.*, 1988). The $CD8^+$ lymphocytic pool is a heterogeneous one; CD8 cells can act as suppressor or cytotoxic cells or even exhibit natural killer cell activity. The

relationship, therefore between CD8 counts and AIDS progression is a complicated one, where different subsets of $CD8^+$ cells can play different roles (Anderson *et al.*, 1991).

3.IMMUNOPATHOGENESIS OF AIDS

3.1 DESTRUCTION OF CD4 CELLS

The hallmark of HIV infection is the severe depletion of CD4-expressing lymphocytes. CD4 lymphocytes play a central role in immunoregulation and therefore depletion of this population results in severe immunosuppression with the concomitant enhanced susceptibility to opportunistic infections (Bowen *et al.*, 1985). $CD4^+$ cell depletion by HIV has been proposed and/or demonstrated to occur through both direct and indirect mechanisms.

Direct mechanisms involve a) hole formation in the host cell, created by intense viral budding (Leonard *et al.*, 1988), b) loss of cell viability by increases in intracellular calcium levels as a result of changes in cell membrane ion permeability, caused by the insertion of HIV *env* proteins (Lynn *et al.*, 1988), c) accumulation in the cell cytoplasm of unintegrated viral DNA (Shaw *et al.*, 1984), or heterodisperse RNAs with repetitive sequences that do not contain long open reading frames (Koga *et al.*, 1988) and d) direct binding of *env* proteins to intracellular CD4 molecules which results in cell death (Hoxie *et al.*, 1986).

Indirect mechanisms include a) infection of CD4 precursor cells or other cells that secrete factors necessary for the propagation of this lymphocytic pool (Fauci, 1988), b) syncytia formation. These giant multinucleated cells result in vitro from the binding of gp120 expressed on infected cells to the CD4 molecule of uninfected CD4 cells. These cells die soon after they are formed (Lifson *et al.*, 1986), c) programmed cell death or apoptosis caused by signal transduction mediated by the interaction of CD4 with gp120 or anti CD4 antibodies; these signals cause the reemergence of the deletion program that operates during thymic education (Terai *et al.*, 1991; Groux *et al.*, 1992), and d) autoreactive phenomena

caused by the expression of gp120 on infected cells, or the binding of gp120 to uninfected $CD4^+$ cells that renders them susceptible to the host's immune system (Klatzmann and Gluckman, 1986). ADCC activity (Lyerly *et al.*, 1987), CD4-dependent autocytolytic mechanisms (Lanzavecchia *et al.*, 1988), autoantibodies (Ziegler and Stites, 1986) CTL- and NK-cell-mediated cytotoxicity are among the autoreactive mechanisms proposed to be involved in CD4 clearance (Walker and Plata, 1990; Brenner *et al.*, 1991).

Functional abnormalities of CD4 cells have been reported prior to their severe depletion, indeed months to years prior to critical $CD4^+$ cell reductions (Shearer and Clerici, 1991). CD4 cells from seropositive patients have been shown to be defective in their relative ability to induce B cells to secrete immunoglobulins and respond to alloantigens (Lane *et al.*, 1983). In addition, selective loss of T-helper function to recall or autologous antigens but not to T-cell mitogens or HLA alloantigens has been observed (Shearer and Clerici, 1991). This defect is not due to reduction in CD4-cell numbers, but rather to functional CD4 cell defects (Fauci *et al.*, 1991). Soluble factors secreted by HIV-1 infected monocytes have been shown to suppress T-lymphoproliferative responses to recall antigens (Foley *et al.*, 1992). Defective CD4 cell cloning efficiency (Margolick *et al.*, 1985), decreased IL-2R expression (Winkelstein *et al.*, 1988) and defective antigen and mitogen-induced IL-2 production (Antonen and Krohn, 1986; Prince *et al.*, 1988) have also been demonstrated throughout the course of HIV infection. At later stages these defects become more prominent.

Functional impairments in CD4 cells may not only be the result of non-cytopathic HIV infection. HIV or its products may interfere with proper CD4 cell-monocyte interactions (Fauci, 1987). The binding of gp120 may result in post-receptor signal transduction defects (Gupta and Vayuvegula, 1987; Linette *et al.*, 1988). In addition, a region of homology between gp120 and IL-2 has been reported, and binding of gp120 may directly or indirectly affect IL-2 activity (Reiher *et al.*, 1986). HIV and its products may also continually activate CD4-cell proliferation (Nair *et al.*, 1988) or induce IL-2 production

(Kornfeld *et al.*, 1988).This may suppress immune functions by making the cells less responsive to other immune signals.

3.2 THE ROLE OF PHAGOCYTES

Monocytes and macrophages are susceptible to HIV infection. HIV has been harvested from, or detected in monocytes and macrophages from peripheral blood (Ho *et al.*, 1986), lung (Salahuddin *et al.*, 1986) and brain (Gartner *et al.*, 1986) of seropositive individuals. Monocytes and macrophages can become infected by HIV either via direct phagocytosis of HIV particles, or through HIV attachment to the CD4 molecule present on the surface of monocytes and macrophages (Stewart *et al.*, 1986). HIV infection of monocytes and macrophages does not result in cytopathic effects or syncytia formation (Crow *et al.*, 1987; Salahuddin *et al.*, 1986).

Infected macrophages remain in tissues for extended periods of time (months), carrying large numbers of viral particles engulfed in cytoplasmic vacuoles, (Gartner *et al.*, 1986) sheltered from immune response. Binding of HIV to the CD4 molecule of monocytes and macrophages results in TNFα secretion of these cells (Merill *et al.*, 1989). In this way further activation of HIV expression may be attained.

Although reductions in monocyte-dependent T-cell proliferation (Shannon *et al.*, 1985), monocyte chemotaxis (Smith *et al.*, 1984) and receptor-mediated clearance (Bender *et al.*, 1987) have been reported in AIDS patients, most of the functions of macrophages such as TNF production, response to γ-interferon, superoxide anion release, candidacidal, antimicrobicidal and tumoricidal activities are normal (Meltzer *et al.*, 1990b).

The most important role of monocytes and macrophages in HIV pathogenesis is to function as reservoirs for viral infection and to spread the disease to the lung and brain (Meltzer *et al.*, 1990a).

3.3 HOW DOES HIV ESCAPE IMMUNOSURVEILLANCE?

Although HIV elicits a well established immune response both at the cellular and humoral level, all seropositive patients eventually progress into full blown AIDS and die.

Several theories have been proposed to explain how HIV evades immunosurveillance networks.

a) Generation of antigenic variants. The HIV virus has an extremely high mutational rate with the greatest variation in the most antigenic determinants of HIV, namely the *env* protein (Starcich *et al.*, 1986; Hahn *et al.*, 1986), b) Transactivation of HIV expression can result either from the induction of cellular signals as a response to antigenic stimuli, or directly, from cotransfection of other viruses that may express regulatory elements that transactivate HIV LTRs (see section 1.6), c) Decreased lymphokine production and deregulation of immunocompetent cells as a result of continuous antigenic stimulation (Rosenberg and Fauci, 1989a), and d) high and low virulence HIV variants. An individual gets infected with both high and low virulent HIV variants. High virulence variants exert their cytopathic effects immediately and elicit host immune responses, while low virulent variants persist for long periods of time in a latent stage, sparing the life of the host cell and escaping from immune responses. Gradually the low virulent population increases, generating more virulent variants. In this model, there is no true latent phase but rather a gradual increase of virulent virus accompanied by the concomitant decreases in $CD4^+$ cells. Full-blown AIDS results when the high virulent variants can no longer be dealt with because of severe $CD4^+$ cell depletion (Miedema *et al.*, 1990).

4.NATURAL KILLER CELLS

4.1 DEFINITION OF NK CELLS

During the 70's studies done on lymphocytes derived from cancer patients and healthy donors demonstrated the lysis of both allogeneic and syngeneic tumor cell lines via a non-MHC-restricted pathway independent of previous antigenic sensitization (Rosenberg *et al.*, 1972; Herberman *et al.*, 1975a,b). This non-MHC-restricted cell-mediated cytotoxicity was designated as natural cytotoxicity and the mediators of this activity NK cells (Herberman *et al.*, 1985). NK cells were initially defined by their large granular

lymphocyte morphology, their ability to functionally mediate spontaneous non-MHC-restricted cytotoxicity against a variety of tumor cell lines and virally infected targets, and their remarkably efficient *in vitro* and *in vivo* response to interferons and IL-2 (Herberman *et al.*, 1985).

A variety of target cell types has been used to measure NK activity. These targets include solid or hematopoietic tumor-derived cell lines and virus infected cells. The most sensitive and widely used target is K562 (West *et al.*, 1977), a cell line derived from a patient with chronic myeloid leukemia in blastic crisis (Lozzio *et al.*, 1976). This is the prototype NK target since it lacks both class-I and Class-II MHC antigen expression (Andersson *et al.*, 1979).

NK cell cytolysis is quantitated by ^{51}Cr-release assays in which a fixed number of ^{51}Cr (sodium chromate)-labelled target cells are incubated with NK-containing cell preparations at a single or multiple effector to target (E:T) ratios for 4 or 18 hours (Pross *et al.*, 1986). Cell mediated lysis is monitored by measuring the amount of ^{51}Cr released into the supernatant fluid.

The morphological identification of NK cells as LGLs was based on a) single-cell binding assays that indicated positive correlation between LGLs bound to K562 target cells and cytolytic ability observed among different normal individuals and b) the co-recovery of NK cell cytolytic activity and LGLs following discontinuous Percoll density gradient centrifugation.

It was indicated that at least 70% of peripheral blood LGLs exhibited NK-like activity (Timonen et al., 1981). NK cells have round or intended nuclei, condensed chromatin and unusually large nucleoli that result in high cytoplasmic to nucleus ratio. The cytoplasm contains a well-developed Golgi apparatus, prominent centrioles with the associated microtubules, numerous mitochondria and many lysosomal organelles including the frequently studied membrane granules (Trinchieri, 1989). Various organelles have been described, including granules with parallel tubular arrays (Caulfield *et al.*, 1987), myelin

membranes or crystalline lattices (Kang *et al.*, 1987). Electrolucent pinocytic vesicles, and large vacuoles are also observed in the NK cytoplasm. NK cell granules contain typical lysozymal proteins including acid phosphatase, trimetaphosphatase, aryl sulphatase, and β-glucoronidase (Trinchieri, 1989). Esterase activity was detected in NK cells, however staining pattern is different to T-cells, where esterase activity is confined to the Gall body or to clustered dense bodies (Prasthofer *et al.*, 1988).

NK cells represent a distinct third lineage of lymphoid cells (Lanier *et al.*, 1986b; Trinchieri and Perussia, 1984). Unlike T cells, NK cells lack the CD3 antigen associated with the T cell receptor. NK cells do not express any of the TCR-associated heterodimers α, β, γ, δ, nor do they rearrange TCR genes (Ritz *et al.*, 1985). NK cells lack antigen specificity and can effectively bind and kill target cells that lack class I or II antigens in a non-MHC restricted fashion (Ritz *et al.*, 1988; Trinchieri, 1989). It has been hypothesized that the sophisticated specific T-cell immunity has evolved from this more "primitive" natural immune system. Cytolytic T cells may have arisen from the acquisition by NK cell cells of a clonally distributed T-cell receptor (Janeway, 1989).

4.2 SURFACE PHENOTYPE OF NK CELLS

The use of monoclonal antibodies and molecular probes in conjunction with the improvement of various methods of identification and purification of NK cells have greatly aided NK phenotypic analysis. Although no surface markers unique to NK cells have been found, the characteristic array of NK cell surface antigens has firmly established their distinct cell lineage (Figure 1). These cell markers are not necessarily expressed on all NK cells, suggesting heterogeneity within the NK cell population. By and large NK cells do not express B-cell markers nor do they exhibit significant MHC class II expression (Trinchieri, 1989).

The most common surface antigens found on NK cells include:

a) **CD56 (Leu19,NKH-1).** A series of antibodies referred to as NKH-1 or Leu19 antigen react with most NK cells and precipitate a 200-220 kDa molecule. NKH-1 reacts

with an immature myeloid cell line KG1a and with most cells from patients with acute myeloid leukemia (Griffin *et al.*, 1983). In addition, CD56 expression is also observed in neurons, neuroblastoma cell lines and human teratocarcinoma cells (McGarry *et al.*, 1988). Leu19 recognizes the neural adhesion protein N-CAM that mediates homophilic binding (Lanier *et al.*, 1989). CD56 is the most representative NK marker isolated to date (Ortaldo and Longo, 1988). Although CD56 is expressed at low levels on all circulating NK cells, its expression and surface density increases dramatically upon *in vitro* NK stimulation and proliferation (Perussia *et al.*, 1987). N-CAM is expressed on 15% of circulating PBLs and 90% of LGLs (Lanier *et al.*, 1986a). In addition, it is expressed on the minor 5% of T cell population that mediates non-MHC-restricted cytotoxicity (Perussia *et al.*, 1987; Lanier *et al.*, 1986a). Experiments with Leu19 loss mutants and transfectants have indicated that N-CAM interactions do not play a major role in non-MHC-restricted lysis (Lanier *et al.*, 1991). In contrast, recent studies have suggested that CD56 constitutes an allorecognition system with restricted specificity (Suzuki *et al.*, 1991).

b) the **FcR (CD16)** antigen. Three types of FcR have been identified on hematopoietic cells. A high affinity FcRI that binds monomeric IgG is expressed on monocytes macrophages and upon IFN induction on PMNS; a low affinity receptor is expressed on monocytes, macrophages, and B-cells; and a third type FcγR (CD16) is present on the majority of NK cells and on macrophages. CD16 FcγR is a low affinity receptor that binds IgG only in immune complexes with membrane bound or soluble antigen. CD16 is expressed in peripheral blood on neutrophils, mature eosinophils but not basophils. Circulating monocytes do not express CD16 and only cultured monocytes can acquire CD16 expression (Perussia *et al.*, 1984).

CD16 is the receptor mediating ADCC and is expressed on 95% of PBL that exhibit cytotoxic activity against the NK-sensitive target cell line K562 and the NK-resistant Daudi target (Perussia *et al.*, 1983b). The degrees of CD16 glycosylation are different between FcγR on PMNs and FcγR on NK cells. On PMNs CD16 is linked to the membrane via the

FIGURE 1

Surface phenotype of human natural killer cells

glycosyl phosphatidylinositol phospholipid (GP-P-L)-linked carboxy terminus of membrane proteins, while evidence suggests that in NKs CD16 is a transmembrane protein (Simmons and Seed, 1988; Lanier *et al.*, 1988; Ravetch and Perussia, 1989). These two homologous CD16 molecules are encoded by two separate but closely linked genes on human chromosome 1 (Ravetch and Perussia, 1989). These structural differencies between the two FcγR on PMNs and NK cells seem to be associated with functional differencies (Unkeless 1989). In NK cells CD16 FcR can mediate ADCC as a signal-transducing element (Lanier *et al.*, 1988) while in PMNs only FcR II and nc CD16 can induce ADCC activity (Graziano *et al.*, 1989). In general only $CD16^+$ NK clones have ADCC activity (Perussia *et al.*, 1983b).

c) **HNK-1 (Leu7)**. HNK-1 was the original marker ascribed to NK cells (Abo and Balch, 1981). Further evidence now indicates, that this is not the case. Leu7 is an IgM antibody that reacts with 30-70% of PBL NK cells and precipitates a 110kDa antigen (Lanier *et al.*, 1983). However unlike NKH-1 and CD16, Leu7 does not correlate with NK cell cytotoxicity (Perussia *et al.*, 1983b). HNK-1 is present also on a small percentage of $CD3^+CD8^+$ and $HLADR^+$ T cells (Abo *et al.*,1982). Whereas in seronegative individuals, few cells coexpress the CD3 and CD57, in AIDS patients $CD3^+CD8^+CD57^+$ cells represent up to 50% of the circulating CD8 population (Landay *et al.*, 1990). In addition CD57 is expressed on a rare CD4 cell population that has LGL morphology and is associated with pathological conditions (Velardi *et al.*, 1985). $CD4^+Leu7^+$ cells are present under physiological conditions in germinal centers of lymphoid tissue and exhibit a decreased IL-2 and BSF secretion when compared to $Leu7^-$ $CD4^+$ helper T-cells (Velardi *et al.*, 1986). PBLs that react with CD16 and HNK-1 are subdivided to four categories where $CD3^-CD16^+HNK1^-$ NK cells mediate the highest cytotoxicity, $CD3^-CD16^+HNK1^+$ exhibit intermediate cytotoxicity, $CD3^+CD16^-HNK1^+$ have low or no cytotoxicity and $CD3^+CD16^-HNK1^-$ cells that do not mediate any NK activity (Lanier *et al.*, 1983; Abo *et*

al., 1982). In general Leu7 shows variable crossreactivity with T cells and should not be used as a marker for NK cells.

d) **CD11/CD18.** CD11/CD18 is a family of three molecules, CD11a or LFA-1, CD11b or CR3, and CD11c or p150, that share a common β subunit and different α subunits (Springer *et al.*, 1987), and are all expressed on NK cells (Timonen *et al.*, 1988). LFA-1 is expressed on all lymphocytes while C3 and p150 are preferentially expressed on NK cells (Timonen *et al.*, 1988). CD11b is dimly expressed on most NK cells, on some T cells and rapidly disappears from cultured NK cells (Perussia *et al.*, 1987). Recently it was reported that β-glucan, a CR3 ligand enhances NK activity (Di Renzo *et al.*, 1991).

e) T-cell associated antigens. NK cells can be classified by their lack of expression of the CD3 antigen. NK cells do not express CD4 (Perussia *et al.*, 1983a) nor CD5 (Perussia *et al.*, 1982,1983b) antigens. However 30-50% of NK cells have been reported to dimly express CD8. $CD8^+$ and $CD8^-$ NK cells exhibit similar cytotoxic and functional abilities (Perussia *et al.*, 1983a). 90% of NK cells react with CD2 antibodies that detect SRBC, however no correlation is found between CD2 and NK cytotoxicity (Perussia *et al.*, 1987). Most resting NK cells but not resting T cells dimly express CD38 (Ortaldo *et al.*, 1981). The same antigen (CD38), is strongly expressed on both *in vitro* activated NK and T cells (London *et al.*, 1985). Other markers expressed by both NK and T cells activated in vitro include HLA-DR, transferrin receptor, 4F2 antigen and IL-2 receptor TAC (CD25) (London *et al.*, 1985; Anegon *et al.*, 1988). Cytotoxic NK cells do not express the CD3 molecule (Perussia *et al.*, 1983b; Zarling and Kung, 1980). NK cells show no rearrangements of TCR α, β, γ or δ genes (Ritz *et al.*, 1985; Leiden *et al.*, 1988; Biondi *et al.*, 1989) genes.

4.3 ORIGIN DIFFERENTIATION AND TISSUE DISTRIBUTION OF NK CELLS

Considerable evidence derived from in vitro BM transplantations of laboratory animals (Migliorati *et al.*, 1989) and patients undergoing allogeneic BM transplantation

(Hercend *et al.*, 1986) has indicated that NK cells originate and at least in part differentiate in the BM. Thymic processing is not required (Pollack and Rosse, 1987; Migliorati *et al.*, 1989). Differentiated NK cells circulate in peripheral blood or migrate to the spleen, while very few are detected in the thymus or lymph nodes of healthy individuals (Reynolds and Ward, 1986). The life span of NK cells has not been established with reports ranging from a few days to several months (Pollack and Rosse, 1987). Murine models suggest that although mature NK cells are radioresistant with a life span of about two weeks, NK progenitors are radiosensitive (Hochman *et al.*, 1978). After bone marrow transplantation NK cells are the first to reconstitute the patients (Lum, 1987).

4.4 EFFECTOR MECHANISMS

NK cells are capable of dual cytolytic activity. They can mediate antibody-directed cellular cytotoxicity (ADCC) as well as spontaneous non-MHC restricted and antibody-independant NK cytotoxicity (Robertson and Ritz, 1990).

4.4.1. ADCC

In ADCC antibody-bound target cells direct Fc receptor bearing cells to lyse sensitized targets. Monocytes granulocytes and NK cells are capable of mediating ADCC function. ADCC in NK cells is mediated through the Fcγ CD16 receptor. ADCC function is inhibited by monoclonal antibodies directed against CD16, or by phorbol esters that downregulate CD16 expression (Perussia *et al.*, 1984; Trinchieri *et al.*, 1984). CD16 binds aggregated IgG1 and IgG3 but not IgG2 or IgG4 (Unkeless, 1989). As previously mentioned in section 4.2 the CD16 receptor present on NK cells is biochemically and serologically distinct from the one expressed in granulocytes, while CD16 can mediate ADCC only on NK cells and not on PMNs.

Although NK cells do not express the TCR, most NK cells express a TCR ζ-chain associated with CD16 (Anderson *et al.*, 1989). TCR ζ chain is tyrosine phosphorylated upon CD16 activation (Vivier *et al.*, 1991b; Stahls *et al.*, 1992).

Crosslinking of surface CD16 results in a rapid rise of cytosolic free calcium ions and the production of inositol 1,4,5 triphosphate through the activation of the (GP-P-L) pathway (Cassatella *et al.*, 1989; Stahls *et al.*, 1992). Both products are important in lymphocyte activation. Ultimately activation antigens are expressed and cytokines are secreted (Harris *et al.*, 1989)

4.4.2 NK activity

In contrast to the well characterized ADCC receptor, the receptors mediating NK cytotoxicity remain unknown. NK cells lyse homologous cells more efficiently than heterologous cells, although this phenomenon remains unexplained (Laskay and Kiessling, 1986; Timonen *et al.*, 1982).

Binding, although necessary, is not sufficient to induce target cell lysis (Timonen *et al.*, 1982). Conjugate formation is largely mediated by the binding of surface LFA-1 and CD2 to their respective target cell ligands ICAM-1 and LFA-3. Binding however of LFA-1 does not play a role in signal transduction and triggering of cytolysis (Robertson *et al.*, 1990). Glycoproteins from cell membranes of target cells inhibit conjugate formation but not cytolysis in a species restricted manner, indicating that short range lytic factors may play a role in cytolysis (Henkart *et al.*, 1986).

Susceptibility to lysis has been shown to be independent of the cell stage of the target cell (Landay *et al.*, 1987). Expression of class I molecules has been inversely correlated to NK cell lysis (Piontek *et al.*, 1985; Stern *et al.*, 1980). Contrasting results have been reported by other investigators where MHC class I expression plays no role in NK susceptibility (Gopas *et al.*, 1988; Dennert *et al.*, 1988). It is possible that class I MHC expression plays a role in some cases, while in others, different structures present on target cells may be recognized by NK cells.

Laminins and their receptors have also been implicated both in conjugate formation and activation of cytolysis (Schwartz and Hiserodt, 1988). Many molecules have been implicated in the search for the NK receptor, however neither the NK receptor nor the

structure(s) recognized on the target cells have been identified. Most "putative" NK receptors have proved to be adhesion enhancing elements (Trinchieri, 1989). So far only crosslinking of the CD2 has been implicated in triggering cytolytic mechanisms by inducing Ca^{2+} influx, release of cytolytic granules and enhanced cytolysis (Schmidt *et al.*,1988; Vivier *et al.*, 1991a).

A new important structure, involved both in recognition and triggering of NK cytolytic activities has recently been reported (Frey *et al.*, 1991). Antiidiotypic (anti-ID) antiserum raised against a monoclonal antibody that could block $CD3^-$ LGL-mediated K562 lysis, could inhibit LGL-mediated binding and subsequent lysis of tumor targets. In addition this anti-ID antiserum could trigger the release of serine esterases and IFN-γ from LGL. A synthetic peptide (p104) was constructed and p104 antiserum exhibited similar patterns to anti-ID antiserum. Although these results are promising, further experimentation employing transfection of this gene into cells lacking NK activity are necessary.

It is possible that a single NK receptor may not exist. In contrast multiple recognitive mechanisms may be operating. It has been hypothesized that NK cells belong to a more primitive immune system where less evolved and specialized cells are required to lyse a variety of foreign invaders and therefore be multispecific (Janeway, 1989).

4.5 CYTOLYTIC MECHANISMS

Although the receptors signaling ADCC, NK and LAK activity as well as CTL responses are distinct, target cell lysis is accomplished via cytolytic pathways that invoke the same terminal cytolytic responses (Young *et al.*, 1988; Carpen and Saksela, 1988; Podack *et al.*, 1991). Phosphorylation of the TCR ζ chain following CD16 or CD2 crosslinking has been shown to be important in triggering cytolysis (Moingeon *et al.*, 1992).

Binding of NK cells to targets can occur both at 4^o and 37^o requiring Mg^{2+} but not Ca^{2+}. After binding activation is optimal at 37^o and Ca^{2+} dependent (Quan *et al.*, 1982).

Following the induction of the phosphoinoside pathway, activation of protein kinase C appears to play a role in induction of cytolysis (Chow *et al.*, 1988). At this stage broad cell to cell interactions between NK and target cells occur. The following steps are Ca^{2+} and Mg^{2+} independent, relatively sensitive to reduced temperature, prostaglandin E2, heterologous anti-LGL antibodies, and proteolytic enzymes (Hiserodt *et al.*, 1982). Lysis is mediated by a vesicular secretory mechanism, where granules polarize towards the part of the NK membrane that binds the target cell, and then get secreted (Carpen and Saksela, 1988). Granule exocytosis involves perforins, serine esterases, and chondroitin sulfate proteoglycans. Perforin or pore-forming protein (PFP) is inserted into the target cell as a monomer where in the presence of Ca^{2+} polymerizes and forms cylindrical transmembrane pores on the target cell membrane, where rapid osmotic lysis is conferred. A regulatory mechanism where by PFP gets inactivated by another yet unknown soluble protein has been suggested. PFP exhibits high homology with the C9 protein of the complement system. NK cells are the only resting lymphocytes expressing constitutively detectable amounts of PFP (Young *et al.*, 1988; Podack *et al.*, 1991).

NK cells also secrete cytotoxins called NK cytotoxic factors (NKCF). TNFα appears to be one of the principle mediators of NKCF but other proteins seem to be involved as well (Wright and Bonnavida, 1987).

Apoptosis or programmed cell death provides an alternative cytolytic pathway. TNF can irreversibly activate target cell Ca^{++}-dependent endonuclease that cleaves genomic DNA into 180-200 base pair fragments (Duke *et al.*, 1986; McConkey *et al.*, 1990). Apoptosis occurs in the absence of bystander cell destruction and requires cell activation, and protein synthesis (McConkey *et al.*, 1990). In vitro experiments have demonstrated that DNA fragmentation requires at least 24 hours to occur (Groux *et al.*, 1992)

4.6 REGULATION OF NK FUNCTIONS

Many cytokines appear to be implicated in NK regulation. Of them, IFN and IL-2 appear to be the most important ones. All of the interferons including α, β, and γ have been

shown to enhance NK cytotoxicity. Virus or mycoplasma infected cells greatly enhance NK cytotoxicity by secreting interferons. IFN enhancement of NK cells is rapid, transient and requires protein synthesis but not NK cell proliferation (Ortaldo and Herberman, 1986; Ellis *et al.*, 1989). IFN-treated NK cells can efficiently lyse target cells that are not very sensitive to NK lysis (Ellis *et al.*, 1989), but not NK-resistant targets (Brunda *et al.*, 1986). IFNs induce NK cytotoxicity by increasing both the number of NK cells able to bind to targets, and the proportion of cytotoxic cells among the NK cell pool, by accelerating the kinetics of lysis, and by increasing the recycling ability of NK cells (Brunda *et al.*, 1986; Ellis *et al.*, 1989; Ortaldo and Herberman, 1986). NK exposure to IFN results in disappearance of cytoplasmic granules that contain PTA or electron dense matrices, and their replacement by vesicular structures that possess residual electron-dense matrices surrounded by round vesicles or membrane myelin figures (Zarcone *et al.*, 1987). IFN treatment in patients results in transient enhancement of NK cell activity (Lotzova *et al.*, 1982).

IL-2 greatly enhances NK activity both *in vivo* and *in vitro* (Smith, 1988). The IL-2 receptor consists of at least two subunits α and β (Siegel *et al.*, 1987; Smith, 1988): the low affinity receptor or CD25 (Tac antigen IL-2 Rp55α) that binds IL-2 with a K_D of 10^{-8} and has a dissociation time of seconds, and an intermediate affinity receptor (IL-2 Rp75β) that has a K_D of 10^{-9} and a dissociation time of 45 minutes. CD25 can not transmit any function signals while IL-2 Rp75 can. When both receptors α and β are expressed on the cell membrane they can form a high affinity heterodimer that binds IL-2 with a K_D of 10^{-11} and a dissociation time of 50 min (Siegel *et al.*, 1987; Smith, 1988). Only the intermediate affinity receptor is constitutively expressed on circulating $CD16^{+}CD56^{+}$ NK cells and therefore concentrations of IL-2 ~ 1-5nmol/L or approximately 100-500U/ml are required for NK stimulation (Kerlh *et al.*, 1988). However, the high affinity receptor is constitutively expressed on the $CD16^{-}CD56^{+bright}$ subset, and renders these cells

responsive to 100 fold lower IL-2 concentrations (Caliguiri *et al.*,1990; Nagler *et al.*, 1990).

Enhancement of NK cytotoxicity is mediated through the intermediate affinity receptor Rp75 and requires 4-6 hours of IL-2 exposure. Optimal stimulation requires at least 18 to 24 hours of incubation. Induction of NK proliferation requires continuous IL-2 exposure for 3-4 days and expression of the high affinity IL-2 receptor. Since the high affinity IL-2 receptor is not constitutively expressed on NK cells, it has been hypothesized that IL-2 induces IL-2Rp 55 synthesis and expression. IL-2Rp55 in turn associates with the constitutively expressed IL-2 Rp75 in order to form the high affinity IL-2 receptor (Siegler *et al.*, 1987; Kerlh *et al.*, 1988).

With regards to NK morphology IL-2 induces the expansion of the Golgi apparatus, and increases the number of electron-dense granules and vesicles, however unlike IFN-induced activation, no deaggregation of the electron-dense matrix is observed (Zarcone *et al.*, 1987). The biochemical events induced by IL-2 as well as the effects that result in increased cytolytic activity are not well understood. IL-2 induced synthesis of *c-myb* mRNA appears to play a role in cellular proliferation (Kerlh *et al.*, 1988; Kornbluth and Hoover, 1988). IL-2 induces increased expression of adhesion molecules which may augment NK binding to NK resistant targets (Robertson *et al.*, 1990). In addition IL-2 induces expression of proteases (Zarcone *et al.*, 1987). However these IL-2 effects require time and can not account for the early activation mechanisms.

IL-2 culture of PBL for few days results in the induction of non-MHC-restricted cytotoxic cells that can lyse a panel of NK-sensitive and NK-resistant targets. These cells are called lymphokine activated killer (LAK) cells (see section 5).

The recently discovered NKSF (Natural Killer Cell Stimulatory Factor) or IL-12 is a heterodimer produced by B cells that has been shown to exert multiple effects on NK cells (Chan *et al.*, 1992; Kobayashi *et al.*, 1989). These include augmentation of NK cell cytotoxicity, induction of NK and T cell proliferation, and induction of IFN-γ production

from both NK and T cells. In addition NKSF synergizes with IL-2 in stimulating IFN-γ induction by NK cells as well as generating LAK cells (Chan *et al.*, 1992).

Phorbol esters enhance NK cytotoxicity (Ramos *et al.*, 1983). Platelet derived growth factor (PDGF) inhibits cytolysis in resting NK cells (Gersuk *et al.*, 1988). Granulocytes and peripheral blood monocytes (Santoli *et al.*, 1978) suppress NK activity. Tumor-associated lymphocytes and macrophages from cancer patients also inhibit NK cytotoxicity (Uchida and Micksche, 1981).

Many products are preformed in NK granules and are released during cytolytic responses. These factors as previously mentioned in section 4.5 include NKCF, PFP, esterases proteoglycans and various enzymes. NK cells also, secrete TNF and IFN γ in response to IL-2 or CD16 crosslinking. In addition activated NK cells produce IL-3, GM-CSF, M-CSF, TNF α and TNF β (Cuturi *et al.*, 1989; Cassatella, *et al.*, 1989; Paya *et al.*, 1988).

4.7 NK CELLS IN TUMOR-SURVEILLANCE

Considerable evidence from experiments with mice indicates a direct relationship with NK cell activity and resistance to transplanted syngeneic tumors (Haller *et al.*, 1977). Reconstitution experiments in NK cell deficient or immunosuppressed mice showed that tumor metastasis was prevented by the adoptive transfer of purified NK cells (Hanna and Barton, 1981; Barlozzari *et al.*, 1985) or NK cell lines but not of polyclonal T cells or CTL clones (Barlozzari *et al.*, 1985; Warner and Dennert, 1982).

The high variability of NK cell number and activity among healthy individuals, the difficulty of quantifying the results, and the need for large number of patients have been major obstacles for the establishment of the prognostic relevance of NK cells. Cumulative findings have indicated depressed NK cell cytolytic activity in cancer patients, with highest NK suppression seen with advanced disease. It still remains unclear however whether depressed NK activity can predispose individuals to develop cancers or it is secondary to the malignant state (Trinchieri, 1989). Presence of glycoproteins and glycolipids as well as

prostaglandins released by monocytes and macrophages in cancer patients have been reported to inhibit NK cell activity (Pross and Baines, 1986). Patients suffering from Chediak-Higashi or X-linked lymphoproliferative syndromes have been shown to exhibit decreased NK activity but relatively normal B and T-cell function. In these patients the probability of developing a lymphoproliferative cancer has been shown to be increased (Pross, 1986).

Depressed NK activity has also been demonstrated in high-risk individuals with a genetic predisposition to melanoma and other cancers (Pross, 1986). Patients with high NK activity exhibited lower recurrence rate of melanoma (Pross, 1986) and head and neck (Schantz *et al.*, 1987) metastases.

4.8 ANTIMICROBIAL ACTIVITY OF NK CELLS

NK cells represent the first line of defense of the immune system against viral infections. NK cell responses are immediate and peak 3 days post viral infection. On the other hand, onset of CTL responses requires at least 4 days and peaks 7-9 days after viral invasion (Janeway, 1989). Many studies have indicated that NK cells can lyse a panel of virally-infected cells while at the same time spare uninfected cells (Welsh, 1986). Individuals with NK cell deficiencies have been shown to exhibit repeated and life threatening viral infections including varicella zoster, primary cytomegalovirus pneumonia, severe primary cutaneous herpes simplex infection and EBV infections (Fleischer *et al.*, 1982; Biron *et al.*, 1988,1989). In one well-characterized patient with severe viremia, complete absence of NK cells was demonstrated without any other lymphocyte deficiency. No NK cell-responses could be elicited in this individual, even after IL-2 and IFNα stimulation (Biron *et al.*, 1988, 1989).

Most evidence for the antiviral activity of NK cells comes from experiments on CMV and HSV-1 infections in murine model systems. Mice strains that are deficient in NK activity are more susceptible to CMV and HSV-1 infections. In addition while bone marrow transplants can confer resistance to these infections, inhibition of the NK cell

activity abrogates the conferred resistance (Welsh, 1986). Experiments with mice have also indicated a protective role of NK cells against murine hepatitis virus infection (Bukowski *et al.*, 1983) and influenza pneumonia (Fitzerald and Lopez, 1986). NK cells can lyse in vitro mammalian cells infected with herpes, vaccinia, measles, mumps and influenza viruses (Fitzerald and Lopez, 1986).

IFNα, secreted either by $HLADR^+$ cells or by NK cells, is believed to play a participatory role in NK-mediated viral immunosurveillance. IFNα treatment has been shown to protect uninfected cells while at the same time leave virally-infected cells susceptible to NK cell lysis (Trinchieri *et al.*, 1981). RNA and protein synthesis is required for this protection, since infected cells with LCMV (a virus that does not inhibit RNA and protein synthesis) can be rendered resistant to NK cell lysis (Welsh, 1986).

An IFN-independent mechanism has also been proposed for the NK lysis of paramyxovirus infected cells. This mechanism is defined as virus-dependent cellular cytotoxicity (VDCC). Viral glycoproteins evoke cytotoxicity 3-4 hours post PBL exposure. This lysis can be blocked by antibodies to viral glycoproteins, only prior to glycoprotein activation of effector cells (Alsheikhly *et al.*, 1985). Interestingly NK lysis of measle-infected targets occurs in two phases. An early phase occurs within 4 hours and can be blocked by antibodies to hemaglutinin but not to IFN-α. A second phase occurs 8-16 hours post infection and can be inhibited only by antibodies to IFN α (Casali and Oldstone, 1982).

NK cells are also involved in surveillance of bacterial and parasitic infections as indicated by the decreased NK activity of germ-free animals (Bartizal *et al.*, 1984). Increased NK activity is observed in mice infected with various bacterial strains (Williams *et al.*, 1987). NK-secreted soluble factors are implicated in bactericidal activity (Trinchieri, 1989). NK cells have been shown to lyse both extracellular as well as intracellular gram-positive and gram-negative bacteria, the fungi *Cryptococcus neoformans* and the protozoa *Toxoplasma gondii* and *Trypanosoma cruzi* (Fitzerald and Lopez, 1986; Trinchieri, 1989).

Depletion of NK cells has been shown to result in increased fungal clearance, but does not affect the long term survival of the animal. NK cells most likely play a role in the defense against microbial and fungal infections by secreting lymphokines and macrophage chemotactic factors and activating other effector cells of the immune system (Trinchieri, 1989).

4.9 NK ACTIVITY IN AIDS

There have been many reports of changes in NK responses in AIDS. However, these studies have not been comprehensive due to a limited number of available patients and limited blood volumes obtained from leukopenic AIDS patients.

Studies employing single or multiple E:T ratios, have indicated a reduced peripheral blood NK activity in PGL and AIDS patients when compared to asymptomatic HIV-seropositive individuals and healthy controls (Brenner *et al.*, 1989).Of interest, a number of reports, have indicated that asymptomatic seropositive homosexuals exhibited increased NK activity compared to healthy controls, while homosexuals with PGL and AIDS showed decreased NK activity. Others have demonstrated NK reductions throughout the course of HIV infection including the asymptomatic phase. Similar reductions of NK function are also observed among drug abusers with PGL and AIDS (Brenner *et al.*, 1989). In seronegative hemophiliacs, NK function has been shown to be normal or somewhat reduced. In HIV seropositive hemophiliacs NK function progressively decreases with advancing disease (Brenner *et al.*, 1989; Landay *et al.*, 1983).

Similarly, decreased NK activity in PGL and AIDS patients has been demonstrated against virally-infected targets including HSV-infected fibroblasts, HSV-infected RAJI cells, and HIV-infected and uninfected $CD4^+$ H9-cells (Brenner *et al.*, 1989).

Of interest $CD16^+$ NK cells derived from HIV-seropositive homosexuals exhibited an increased cytotoxicity against HIV-infected U-937 targets, when compared to U-937 uninfected counterparts (Rappocciolo *et al.*, 1989). In addition $CD16^+$ NK cells demonstrated an elevated capacity to lyse HIV-infected HUT cell lines comperatively to

uninfected HUT cells (Bandyopadhyay *et al.*, 1990). Lysis of cells infected with the HIV-IIIB or the WMJI strains of HIV-1 has been shown to be mediated by $CD16^+$ cells. Lysis of cells infected with the A1.25 HIV-1 strain however, required the presence of $HLA\text{-}DR^+$ and $CD16^+$ cells (Bandyopadhyay *et al.*, 1990). NK cytolytic activities against HIV-infected and uninfected U-937 targets have been reported to decline with disease progression (Rappocciolo *et al.*, 1989).

With regards to the NK cell population sizes, percentages of $CD56^+$ cells have been reported to be normal in asymptomatic seropositive individuals. Absolute numbers of CD56-expressing lymphocytes per ml of blood however, were reduced (Vuillier *et al.*, 1988). In another study, percentages of CD56-expressing peripheral blood mononuclear cells were found to be reduced in seropositive and ARC patients, but normal in AIDS patients . However, overall CD56 numbers (per ml of blood) were reduced throughout the course of HIV infection including AIDS (Landay *et al.*, 1990).

Levels of CD57-expressing cells have been shown to be normal (Landay *et al.*, 1983) or elevated (Lewis *et al.*, 1985; Gupta, 1986; Voth *et al.*, 1988) in homosexual drug abusers and hemophiliacs, and elevated in PGL patients (Gupta, 1986; Landay *et al.*, 1983). The absolute per ml of blood CD57 numbers are elevated in PGL and seropositive subjects, and significantly reduced in AIDS patients (Lewis *et al.*, 1985; Gupta, 1986; Landay *et al.*, 1983). This increase in CD57 (Leu-7)-expressing cells, is due to an increase in these cells that coexpress CD3 and CD8 but not CD16 (Gupta, 1986; Landay *et al.*, 1990). $CD57^+$ cells have been implicated in suppressor activity in AIDS patients (Joly *et al.*, 1989; Sadat-Sowti *et al.*, 1991).

Percentages of $CD16^+$ cells are reported to be normal in seropositive homosexuals and patients with PGL and AIDS (Creemers *et al.*, 1985; Lewis *et al.*, 1985; Vuillier *et al.*, 1988), however absolute numbers are decreased (Creemers *et al.*.; 1985; Lewis *et al.*, 1985; Voth *et al.*, 1988). This decrease is due to a depletion of the $CD16^+CD56^+$ subset, the subset that mediates the highest levels of NK cytolytic activity (Voth *et al.*, 1988).

Since these decreases in NK numbers are not incorporated into the cytolytic assays where fixed E:T ratios are used, single-cell-binding assays have been employed in order to assess number of effector-target conjugates and their relative lytic capacity (Pross *et al.*, 1986). NK activity was decreased in HIV-seropositive individuals and AIDS patients (Katzman and Lederman, 1986; Bonavida *et al.*, 1986). Nevertheless, the percentages of effector cells binding to U937 (Katz *et al.*, 1987) and K562 (Katzman and Lederman, 1986) target cells were comparable in healthy controls and seropositive populations. Cytolysis of these targets subsequent to cell binding was observed to be reduced (Katzman and Lederman, 1986; Katz *et al.*, 1987). Experiments with double targets have indicated that the same effector cell that can effectively lyse an ADCC target can not effectively lyse an NK target (Katz *et al.*, 1987). Recycling times of NK cells were comparable between seropositive individuals and control subjects (Katzman and Lederman, 1986). Cytolytic NK cells from AIDS patients exhibited a defect in the characteristic internal tubulin rearrangement and polarization of activated lymphocytes (Sirianni *et al.*, 1988).

Attempts to HIV-infect fresh NK cells have failed (Ruscetti *et al.*, 1986). IL-2 or PHA activated LGL (Ruscetti *et al.*, 1986; Robinson *et al.*, 1988) and NK cell lines (Chehimi *et al.*, 1991) have been infected *in vitro* with the HIV virus. Immunofluorescent assays have revealed the coexpression of CD16 and HIV antigens (Robinson *et al.*, 1988). Viral replication was also detected by p24 antigen intracellularly, and in culture supernatants, and by the presence of proviral HIV DNA within infected cells (Chehimi *et al.*, 1991). In addition supernatants of HIV-infected NK cell lines were able to infect PBLs, mononuclear cells and $CD4^+$ cell lines. A great number of NK cells died in HIV-infected cultures. However, both HIV-infected and uninfected NK cells exhibited similar cytotoxicities (Chehimi *et al.*, 1991). Todate, isolation of NK cells from AIDS patients harbouring the HIV virus has not been reported.

NK dysfunction may be the indirect effect of HIV infection. Virally encoded proteins and/or lymphocyte secreted proteins, may inhibit NK activity. A synthetic peptide

corresponding to 735-752 and 846-860 of the HIV gp41 was able to inhibit NK activity, by blocking lysis of the conjugated target at a post binding level (Cauda *et al.*, 1988).

The vasoactive intestinal peptide VIP shares a homology with the CD4 binding site of gp120. VIP receptors are found on NK cells and binding of VIP to its receptor has been shown to increase cAMP levels and decrease lytic NK activity. HIV binding to the VIP receptor may therefore elicit similar responses (Sirianni *et al.*, 1990).

In addition NK activity was inhibited by autologous serum derived from asymptomatic homosexuals and patients with PGL and AIDS but not from healthy controls (Creemers *et al.*, 1985).

4.10 ADCC RESPONSES IN AIDS

Sera from HIV-1 seropositive individuals have been used to arm PBL from healthy donors, with cytophilic antibody. This approach has been used to monitor the humoral component of HIV-directed ADCC. Depending on the target cell line and assay system, 40-100% of sera from seropositive donors could direct ADCC, primarily against the *env* proteins gp120 and gp41 (Brenner *et al.*, 1991). This method is very sensitive for the detection of circulating antibodies to gp120 and gp41. ADCC antibody titers were higher than neutralizing antibody titers (Bottinger *et al.*, 1988). In addition, following HIV infection, ADCC antibodies appeared earlier than neutralizing antibodies. No correlation between antibody titers and disease progression was observed, since ADCC antibody titers remain high throughout the course of HIV infection (Bottinger *et al.*, 1988; Lyerly *et al.*, 1988).

ADCC antibodies recognize epitopes common among many HIV strains that include potential antigenic sites of gp120. ADCC antibodies were directed against the carboxy end of gp120 and the transmembrane portion of gp41, including aminoacids 557-677 and 728-752 (Evans *et al.*, 1989). These epitopes appear to be quite different from corresponding epitopes, that evoke CTL responses that are directed against the aminoacid sequence 308-322, 381-392 and 410-429 of gp160 (Walker and Plata, 1990).

Studies comparing the relative efficacy of PBLs isolated from HIV seropositives and healthy controls, to lyse antibody-coated targets in the presence of sera, have given conflicting results. In many studies, decreased efficacies of PBLs derived from HIV seropositives and AIDS patients to elicit ADCC against a panel of different targets, have been observed compared to healthy individuals. In direct contrast, others have reported increased abilities of HIV infected individuals to evoke HIV-specific and non-specific ADCC responses (Brenner *et al.*, 1991).

NK cellular post-binding dysfunctions have been observed in AIDS patients (Sirianni *et al.*, 1988), that may account for the observed ADCC dysfunctions. However, single-cell binding assays with two different targets, one NK-resistant and one NK-sensitive, have indicated that although the NK target is not lysed the ADCC target bound to the same effector cell, is successfully killed (Katz *et al.*, 1987).

5. LYMPHOKINE ACTIVATED KILLER CELLS

5.1 DEFINITION

In 1976 Morgan and Ruscetti achieved selective growth of functional T-lymphocytes by culturing them with conditioned medium from phytohemagglutinin-stimulated lymphocytes (Morgan and Ruscetti, 1976). IL-2, contained within the T-cell growth factor (TCGF), has been identified as the lymphokine providing the obligatory second signal for clonal expansion of antigen-primed lymphocytes (Grimm *et al.*, 1982a). In 1981, Rosenberg's group, reported that normal splenocytes cultured *ex-vivo* in TCGF for 2-3 days could lyse NK-resistant cells isolated from fresh autologous tumors (Lotze *et al.*, 1981). No corresponding lysis of a panel of normal cells was observed. Cells mediating lysis of tumor cells were named LAK cells (Grimm *et al.*, 1982b) and their identity and function has been and still is the subject of intense study and debate.

IL-2 alone is sufficient and necessary for the generation of LAK cells. Antibodies against TAC (IL-2 receptor) block the generation of LAK cells (Grimm *et al.*, 1983).

Interferon γ can augment this IL-2 activation (Findley *et al.*, 1990). Optimal concentrations of IL-2 were reported to be 100U/ml or equivalent to 6.7 nM (Grimm and Wilson, 1985). However, it was later shown that incubation of lymphocytes with concentrations as low as 20U/ml (equivalent to 1-2nM) of IL-2 for at least 2-3, days could result in LAK precursor activation and the subsequent induction of LAK cells (Grimm, 1986). This IL-2 concentration is similar to the in vivo IL-2 circulating concentrations (15U/ml) (Lotze *et al.*, 1985) .

Inhibition of LAK cell induction by mitomycin pretreatment and irradiation of effector cells, has indicated that both differentiation and proliferation are required for LAK cell generation (Grimm and Wilson, 1985; Grimm, 1986; Ramsdell *et al.*, 1988).

LAK cells are highly cytotoxic against a variety of tumor and virally-infected cells, yet they are distinct from NK and CTLs with regards to kinetics of activation, recognition stimuli and target cell specificities (Ortaldo and Longo, 1988). NK-resistant targets including fresh solid tumors are susceptible to LAK cells (Grimm, 1986; Ortaldo and Longo, 1988; Grimm *et al.*, 1983). LAK-sensitive targets include autologous and allogeneic fresh melanoma, sarcoma, carcinoma glioma, benign schwanoma, as well as cultured tumor cells, placenta fetal tissue EBV-transformed B-cell lines and TNP-modified PBLs (Grimm, 1986; Grimm *et al.*, 1982b). Cells resistant to LAK-cell lysis include fresh normal bowel, colon, kidney, liver, and pancreas cells as well as conA-stimulated lymphoblasts (Grimm, 1986).

LAK cells can elicit cytolytic responses without antigenic specificity or MHC-restriction, thereby indicating their disparity with CTLs.

5.2 PRECURSOR AND EFFECTOR CELL PHENOTYPE OF LAK ACTIVITY

LAK cell activity has been induced from every lymphoid tissue available, including intestinal mucosa, spleen, thoracic duct fluid and PBLs (Skibber *et al.*, 1987; Grimm, 1986). Both CTL and NK cells have been shown to exhibit LAK cell activity upon IL-2

activation (Grimm, 1986;Skibber *et al.*, 1987; Geller *et al.*, 1991; Smyth *et al.*, 1991; Nitta *et al.*, 1991; Sawada *et al.*, 1986; Phillips *et al.*, 1986; Ortaldo *et al.*, 1986; London *et al.*, 1986). The relative contribution however of NK cells and CTLs depends on the source of lymphocytes and conditions of activation (Hercend and Schmidt, 1988). Lymphocytes from peripheral blood and spleen will produce LAK cells mainly from NK cell precursors (Skibber *et al.*, 1987; Phillips *et al.*, 1986; Ortaldo *et al.*, 1986; London *et al.*, 1986; Ibayashi *et al.*, 1990).

A third precursor cell population that expresses neither NK nor T cell markers has also been proposed. This precursor cell population might be identical to, or include the NK precursor cell (Morris and Pross 1989)

LAK cell generation requires expression of the high-affinity heterodimers (Siegel *et al.*, 1987). As previously mentioned only NK cells constitutively express the intermediate affinity IL-2 receptor β (kD 10-9,dissociation time of 45 minutes) and therefore low IL-2 concentrations of 100pM or 10-15U/ml are sufficient for LAK cell generation. In direct contrast, resting T-cells express only the low affinity IL-2 receptor α (kD 10-8, dissociation time of seconds) and require in addition to antigenic stimulation, high IL-2 concentrations (5000pM or approximately 500U/ml (Siegel *et al.*, 1987; Caliguiri *et al.*, 1990). Therefore, incubation with low IL-2 concentrations (15U/ml similar to the physiological levels) has been shown to preferentially activate NK cells (Siegel *et al.*, 1987; Caliguiri *et al.*, 1990; Robertson and Ritz, 1990). Addition of CD3 antibodies can augment non-MHC restricted LAK activity of CD3-expressing lymphocytes (Tovar *et al.*, 1988; Stohl *et al.*, 1990).

LAK cell activity was functionally defined as non-MHC-restricted lysis of NK-sensitive and NK-resistant target cells. It describes an activation phenomenon rather than a unique homogenous cell population; i.e. the ability of IL-2 to enhance NK cell cytolysis and endow other lymphocytes including some T-cells with non-MHC-restricted cytolytic capabilities (Trinchieri, 1989).

5.3 LAK CELLS IN TUMOR IMMUNOSURVEILLANCE

The ability of LAK cells to elicit cytotoxicity against fresh tumor targets has rendered them as a unique antitumor effector mechanism that could be employed for cancer immunotherapy. Initially numerous experiments were done in murine systems using infusions of IL-2 and LAK cells or IL-2 alone (Lafreniere and Rosenberg, 1985). With the advent of molecular biology, large amounts of recombinant IL-2 have become available (Rosenberg *et al.*, 1984). Results from experiments with mice have indicated that micrometastases in the liver and lung from immunogenic and non-immunogenic sarcomas, melanomas and adenocarcinomas could be inhibited with the administration of high doses of IL-2 or IL-2 combined with LAK cells (Rosenberg *et al.*, 1985b). A direct relationship between the increased number of LAK cells administered, and the therapeutic effect has been observed. In addition, host-proliferating lymphocytes, have been shown to be necessary for tumor regression (Lafreniere and Rosenberg, 1985). In murine systems, LAK cells were shown to be derived from an asialo-GM-1 (mouse NK marker)-expressing precursor population (Mule *et al.*, 1986).

In 1984, Rosenberg's group transfused human IL-2-activated lymphocytes along with IL-2 in mice and observed a reduction in the number of pulmonary metastic lesions (Mule *et al.*, 1984). Phase I trial studies established half life, immunological effects and toxicity of IL-2 plus LAK cell infusions (Rosenberg *et al.*, 1985a). Phase II and III trials in humans have demonstrated partial responses with significant tumor reductions that persisted for prolonged periods of time (Rosenberg *et al.*, 1987). Toxicity was severe mainly due to capillary permeability and subsequent tumor retention, however, with termination of therapy most cytotoxic effects ceased, and they all seemed to be a direct IL-2 effect (Rosenberg *et al.*, 1985a,1987).

Responsive tumors included renal cancers and melanomas. These are hard to treat cancers that do not respond to chemo-or radiotherapy (Rosenberg, 1989; Borden and Sondel 1990). LAK cells infused via tumor-feeding arteries have been shown to be

selectively localized in the tumor tissue, mainly in the kidney, spleen and liver but not in the lung (Morita *et al.*, 1987).

Several investigators have reported that administration of high dose IL-2 to cancer patients results in the induction of LAK cell activity mediated by Leu19 (CD56)-expressing PBLs as demonstrated by lysis of the NK-resistant but LAK-sensitive DAUDI target cell line (McMannis *et al.*, 1988; Weil-Hillman *et al.*, 1990).

5.4 IL-2 AND LAK CELLS IN AIDS

NK cells are highly susceptible to IL-2 immunomodulation. Decreases in IL-2 levels of HIV-seropositives and AIDS patients have been reported (Ernst *et al.*, 1986) . Limiting dilution analysis indicated a decrease in the number and proliferative capacity of IL-2 responding PBLs in seropositive individuals (Donnenberg *et al.*, 1989). In addition PHA-induced IL-2 production and PHA-induced IL-2 receptor (p55, Tac^+) generation is decreased in HIV seropositive individuals (Hofmann *et al.*, 1989). These IL-2 deficiencies have been shown to occur concurrently with a profound non-specific activation of the immune system (Schwartz and Merigan, 1990). Immune activation has been indicated by increases in $HLADR^+$ cells and elevated levels of β-microglobulin, neopterin and soluble IL-2 receptors (Schwartz and Merigan, 1990; Brenner *et al.*, 1991; Scott-Algara *et al.*, 1991). Increased levels of soluble IL-2 receptors have been associated with a number of immunopathologies, including hematologic malignancies, and viral and autoimmune diseases (Rubin and Nelson, 1990). The role and significance of IL-2 receptor increases in immunopathogenesis remains unclear.

It is suggested that the down-regulation of IL-2 responsiveness has resulted from continuous non-specific activation in HIV seropositive patients, that results in the subsequent inhibition of specific antigenic responses. High concentrations of IL-2 (>500U/ml) can partially restore *in vitro* NK cytotoxicity in patients with PGL and AIDS, induce release of NKCF and partially overcome NK suppression mediated by the 735-752 and 846-860 gp41 HIV synthetic peptides (Cauda *et al.*, 1988).

Trials using systemic IL-2 administration in ARC and AIDS patients have resulted in transient lymphocytosis of T cells, eosinophilia and IL-2 related toxicity without clinical improvement (Schwartz and Merigan, 1990). Other studies exhibited increased lectin PBL responsiveness partial restoration of NK cell levels, transient increases in $CD4^+$ cell numbers and decreases in CD57 subsets (Ernst *et al.*, 1986). In addition IL-2 administration has reversed the AZT induced NK activity reductions observed in patients receiving AZT therapy (Schwartz and Merigan, 1990). Of interest, in another study, *in vitro* addition of AZT resulted in inhibition of LAK cell activity derived from HIV infected individuals (Stine *et al.*, 1991).

Ex vivo incubation of PBL results in the induction of LAK-cell activity. LAK cell activity in HIV infection is not well characterized. In one study with a small subject group LAK cell activity of HIV seropositive and AIDS patients was comparable to healthy controls (Chin *et al.*, 1989). Contrasting results were obtained by another group where decreases in LAK cell activity were observed throughout the course of HIV infection (Cauda *et al.*, 1990). In addition, the same gp41 synthetic peptide that inhibited NK cell function was also found to inhibit LAK cell cytolysis (Cauda *et al.*, 1990).

6. HYPOTHESIS AND RATIONALE

When we began this study the vast majority of research was focusing on HIV detection, prevention and control (i.e. antiviral therapy). Very few studies, monitored changes on the immune system of HIV-seropositive individuals that could ultimately lead to the immunopathogenesis of AIDS. We therefore decided to study the effects of HIV infection upon natural immunity. We focused our studies specifically on LAK cell responses, since up to that date there were no reports on LAK cell activity of HIV-seropositive individuals and AIDS patients, and we believed that NK cells and their ex-vivo induced counterparts LAK cells can play a significant role in HIV-infection and the development of AIDS.

Infection with the HIV virus causes a progressive loss of $CD4^+$ lymphocytes that eventually leads to AIDS. AIDS is preceded by a long asymptomatic phase during which the immune system can successfully cope with foreign invaders despite the loss of up to 70% of $CD4^+$ cells. NK cells and their IL-2 inducible *ex-vivo* counterparts LAK cells have been shown to be mediators of antiviral and antitumor immunity. As such they may play a significant role in the host defense against the characteristic AIDS-associated opportunistic infections and neoplasias.

In addition NK cells are by and large the progenitors of LAK cells. Adoptive transfer of LAK cells has been shown to be an important immunotherapeutic avenue against solid tumors, and may therefore also be useful in the development of immunotherapeutic treatments against AIDS.

In direct contrast NK and LAK cells may contribute to the AIDS immunopathogenesis by lysing HIV-infected or gp120 coated uninfected $CD4^+$ cells.

NK cells do not express the CD4 antigen. Delineation therefore of the mechanisms that lead to NK dysfunctions may provide useful insight on the immunoregulatory circuits and the role of NK and LAK cells in viral immunosurveillance.

CHAPTER 2

In the beginning of our study we monitored the effects of HIV-1 infection upon LAK cell activity. HIV-seropositive patients were subclassified into two groups. The asymptomatic population included patients with no evidence of disease (CDC II or III), while patients with two or more AIDS-associated infections and/or cancers (CDC-IVC or IVD) constituted the overt AIDS population. Control groups consisted of age-matched healthy HIV-seronegative individuals.

PBL and LAK cell recoveries of HIV-seropositive and AIDS patients were contrasted to healthy controls. LAK cell cytolysis was monitored using Chromium release assays. Effector cells were derived from patient and control PBLs, incubated with IL-2 for 4-6 days, and assayed against a panel of chromium labelled targets. Cytolytic values were calculated using the following equation:

% cytotoxicity = (test sample release - spontaneous release)/(maximum release - spontaneous release)x100%, where spontaneous release is determined from control wells containing target cells only, and maximum release by the addition of a detergent to the appropriate wells.

Targets employed included the NK-prototype K562 cell line that lacks class I or II antigen expression and therefore can be lysed only in a non-MHC restricted fashion, the NK-resistant but LAK-sensitive RAJI cell line, and the U937 promonocytic cell line that can be chronically infected with the HIV virus.

Results indicated an impairment in inducible LAK responses in HIV-seropositive populations. Of interest, enhanced cytolysis of HIV-infected U-937 targets was observed in HIV-seropositive groups, but not in healthy controls.

DIMINUTION OF INDUCIBLE LYMPHOKINE-ACTIVATED KILLER CELL ACTIVITY IN INDIVIDUALS WITH AIDS-RELATED DISORDERS

ABSTRACT

We have compared the relative ability of lymphokine-activated killer (LAK) cells derived from peripheral blood of HIV-seropositive individuals, AIDS subjects, and healthy controls, to lyse a panel of natural killer (NK)-sensitive and NK-resistant tumor and virally-infected targets. We have found that LAK cells derived from HIV-seropositive populations show a significant albeit reduced capacity to lyse U937, K562, and RAJI target cell lines, in comparison to similarly derived cells from healthy controls. The diminishments of LAK activity in both HIV-seropositive asymptomatic and AIDS populations reflect a significant reduction in cytotoxic potential of individual LAK cells. The maximal LAK cytotoxic potentials of control, asymptomatic seropositive, and AIDS populations are comparable. LAK cells derived from HIV-seropositive populations show an enhanced capacity to lyse HIV-infected U937 targets relative to their uninfected counterparts. These enhancements in HIV-infected U937 vs U937 cytolysis arise from increases in the maximal cell-mediated cytolytic plateau. Depletion of NK ($CD56^+$) lymphocytes from peripheral blood prior to LAK cell generation markedly diminishes subsequent specific and total inducible LAK activity. In some subjects, peripheral blood T cell depletion prior to LAK cell generation results in LAK cells that are subsequently enriched for cytolytic activity; whereas in other subjects, similar T cell depletion impairs inducible LAK cell responses.

INTRODUCTION

Natural killer (NK) cells, as well as their *in vitro* induced counterparts, lymphokine-activated killer (LAK) cells constitute a distinct lymphocyte pool that provides natural resistance to both viral infection and neoplastic disease (1-4). These cells, closely associated with large granular lymphocytes (LGL), elicit a broad range of non-major histocompatibility complex (MHC)-restricted cytotoxic responses, including NK activity, antibody-dependent cellular cytotoxicity (ADCC), and LAK activity (1-3, 5-9). While NK cells express a characteristic array of surface markers ($CD56^+$, $CD16^+$, $HNK1^{\pm}$, $CD3^-$), they lack the HIV-1 viral receptor, CD4 (6-12). As such, they may be clinically relevant in AIDS, providing an alternative cytolytic pathway for immune depletion of both HIV-1-infected cells and HIV-1-induced secondary opportunistic infections.

Clear patterns of decreased peripheral blood NK function against the prototype NK target K562, U937, and H9 target cells have been observed in HIV-1 seropositive individuals with persistent generalized lymphadenopathy (PGL) and AIDS as compared to healthy controls and asymptomatic HIV-seropositive individuals (4,13-23). There appears to be a dysfunction in the lytic machinery of NK cells from PGL and AIDS populations with a failure to polarize tubulin and to release NKCF (natural killer cytotoxic factor) (16-20). In addition, progressive HIV-1-associated diseases appear to cause a diminution of both $CD16^+$ ($Leu\ 11^+$) and $CD\ 56^+$ ($Leu\ 7^+$) subsets that are active in NK cell-mediated lysis (4,24,25). At the same time, there is an increase in a non-cytotoxic NK pool that coexpresses HNK ($Leu\ 7^+$) and CD8 but lacks CD3 (4,14,16,25-27).

NK cells are particularly amenable to modulation by biological response modifiers (11). Their proliferation and differentiation of NK cells can be upregulated by interleukin-2 (IL-2) (1,4). In addition, NK cells can be activated *in vitro* by IL-2 to generate a novel cytotoxic lymphocyte population, i.e. LAK cells (2,28,29). LAK cells are highly cytolytic both *in vitro* and *in vivo* to a broad spectrum of tumor and virally-infected targets . Yet, they are distinct from NK cells with regard to their target cell specificity, recognizing and

killing both NK-resistant and NK-sensitive targets (2,6-9,28,29). There has been some debate as to the cell phenotype of the progenitors and effectors that mediate LAK cytolytic activity, particularly concerning the relative contribution of NK vs cytotoxic T cells (1,6,9). However, recent studies conclude that by and large the activated LAK cytotoxic phenomenon is derived from large granular lymphocyte (NK) precursors and is mediated by cells expressing the NK marker, CD56 (Leu 19^{+}) (1,6-9).

Whereas NK cell function in patients with AIDS has been extensively studied, LAK cell function remains poorly characterized. In a recent study, LAK activity in patients with AIDS has been reported to be equivalent to that of normal controls using K562 and RAJI targets (30). The results reported herein indicate that HIV-seropositive individuals have an ability albeit impaired to generate cytotoxic LAK cells. The phenotypes and target reactivities of LAK cells from HIV-seropositive, AIDS, and control populations have been detailed.

MATERIALS AND METHODS

Subjects

HIV-1 seropositive individuals were subclassified into two populations.The overt AIDS population, CDC group IVC and IVD, included subjects that have had two or more AIDS-associated secondary opportunistic infections and/or cancers, including *Pneumocystic carinii* pneumonia and/or Kaposi's sarcoma, with disseminated herpes, oral candidiasis, and/or oral hairy leukoplakia. The asymptomatic HIV-seropositive population included CDC group II and III subjects. Age-matched healthy HIV-seronegative individuals constituted the normal control population.

Effector LAK cells

Peripheral blood, obtained with informed consent, was collected in heparinized tubes and lymphocytes (PBLs) were isolated by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation (31). Cultured LAK cells were generated by *ex-vivo* incubation of PBLs (10^6 cells/ml) in RPMI-1640 medium containing 10% decomplemented FCS and 15 U/ml recombinant IL-2 (Boehringer-Mannheim Canada Ltee) for 4-6 days at 37°C in a humidified atmosphere of 5% CO_2 in air (31). LAK cell recovery was determined relative to the original number of PBL lymphocytes seeded. This recovery was similar using concentrations ranging from 10-100 U/ml IL-2 (unpublished results).

Depletion of lymphocyte subsets from peripheral blood lymphocytes

Prior to LAK cell generation, PBLs were selectively depleted of NK and T cell subsets using mouse monoclonal antibodies and magnetic goat anti-mouse immunoglobulin beads. Briefly, PBLs (12-18 x 10^6) were divided into three equal aliquots. To deplete NK and T cell subsets, PBLs (2 x 10^6 cells/ml) were incubated with 20 µl Leu 19 and 20 µl Leu 4, respectively (Becton Dickinson, Mountain View, CA). Following washing a 15-fold excess of magnetic goat anti-mouse IgG (Fc specific) (Advanced Magnetics Inc., Cambridge, Mass.) particles were added. Following a 30 min incubation on ice, cells binding to magnetic beads were removed by a magnetic separator (Advanced Magnetics

Inc., Cambridge, MA) and all three aliquots were incubated in IL-2 containing media to generate LAK cells as previously described.

Target cells

Human tumor cell lines, obtained from the American Type Culture Collection (Rockville, MD), included the NK-sensitive target K562 (derived from a patient with chronic myelogenous leukemia in blast crisis); U937 (a promonocytic cell line) (32); and the NK-resistant target RAJI (a Burkitts' lymphoma cell line containing the EBV genome (33). Cell lines were maintained in complete RPMI-1640 containing 10% FCS, 2 mM gluthamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 mM HEPES. Cells were passaged twice weekly.

HIV infection of U937 target cells

HIV-1 was harvested from cultures of H-9 cells infected with the HIV-111_B strain (kindly supplied by Dr. R.C. Gallo, NIH, Bethesda, MD). As previously described, U937 target cells were infected with HIV-111_B at a $TCID_{50}$ of $10^{5.5}$ (34). The HIV infection status of the U937 cells was determined by indirect immunofluorescence, as previously described (35). Murine monoclonal antibodies to HIV-1 viral proteins p17 and p24 (supplied by Dr. R.C. Gallo) and fluorescein-labelled goat anti-mouse immunoglobulin (Miles Laboratories, Ekhart, IN) were used to quantify the percentage of HIV-1-infected cells.

Cytotoxicity assays

LAK activity was monitored using 18 hr chromium release assays with LAK cells as effectors (E) and ^{51}Cr-labelled tumor cell lines as targets (T). Target cells were labelled with 150 µCi of sodium ^{51}Cr chromate (ICN Biomedicals Ltd, Montreal, Canada). LAK cells were adjusted to give appropriate E:T ratios in triplicate wells of U-bottom 96-well microdilution plates. Spontaneous isotope release was determined from control wells containing only target cells, and maximum release was determined by the addition of 2% Triton-X100 to appropriate wells. This spontaneous release represented approximately

20% total release for K562 and RAJI targets and 25% for U937 targets. The percent cytotoxicity was calculated according to the following formula: % cytotoxicity = (test sample release - spontaneous release)/maximum release - spontaneous release

Where indicated, exponential regression analysis of subject's cytolytic activities monitored at 4-6 E:T cell ratios was performed using computer software kindly provided by Dr. H.F. Pross (36,37). Lytic units are defined as the number of effectors required for 20% lysis of 1,000 target cells ($20\%/10^6$) calculated according to the equation of $y = A(1-e^{-kx})$ where y = fractional chromium release, x = E:T ratio, k = negative slope derived from plotting ln (A-y) (i.e. target survival) vs x, and A = asymptote of the curve. A and k are independent parameters, A representing the maximal amount of cell-mediated lysis and k representing the relative lytic potential of lymphocytes.

Statistical analysis

LAK activities, measured over a range of E:T cell ratios, were statistically compared in central and HIV-1 seropositive populations using 2-way ANOVAS. Where significant differences were observed, the sources of variations between pairs of averages were determined using the Newman-Keuls Test. Similarly, individual cytolytic parameters of LAK activity in subject populations, i.e. LU_{20}, A and k, were statistically contrasted using 1-way ANOVAS, and Newman-Keuls, or paired t-test where indicated. Statistical tests were calculated using software obtained from Lionheart Press (Alburg, Vermont, USA).

RESULTS

LAK cell generation

PBL numbers were significantly reduced in the HIV-seropositive and AIDS population when compared to healthy controls ($p<0.01$ 1-way ANOVA). Healthy controls had an average of $2.1 \pm 0.1 \times 10^6$ PBLs per ml of blood (mean ± sem, n=47), while for asymptomatic HIV-1 seropositive and AIDS subjects the average PBL count was $1.6 \pm 0.1 \times 10^6$ and $1.2 \pm 0.1 \times 10^6$ cells per ml of blood (mean ± sem, n=34 and 28, $p<0.05$ Newman Keuls test. The lymphocyte count of AIDS subjects was also significantly less than asymptomatic HIV-seropositive individuals ($p<0.025$, unpaired t-test).

The ability of PBLs to serve as progenitors for LAK cells was determined for the healthy control and HIV-seropositive populations by monitoring cell counts at day 6 relative to the original cell counts on day 0. LAK cell recovery for the healthy control population was $56.8 \pm 2.5\%$, significantly greater than the $45.8 \pm 2.7\%$ and $41.1 \pm 2.9\%$ recoveries observed for the HIV-1 seropositive asymptomatic and AIDS populations, respectively 1 way ANOVA, $p<0.05$, Newman-Keuls test. The relative LAK cells recoveries of HIV-seropositive asymptomatic versus AIDS populations were not significantly different.

LAK cell-mediated activity was monitored at E:T ratios ranging from 20:1 to 0.25:1 using four to six serially halved dilutions. Data was then subjected to exponential regression analysis (36,37). If experimentally observed data points agreed with extrapolated data points, lytic units and lytic parameters k and A were determined. LAK cells from healthy control and HIV-1 seropositive populations had the ability to demonstrate cytolytic function against all tested targets, including RAJI cells which are NK-resistant. These cytolytic activities represented by definition LAK cell-mediated cytolytic phenomena. In the absence of IL-2, lymphocytes as early as day 1 and always at days 4-6 lacked any cytolytic activity against all tested targets.

FIGURE 1

Lymphokine-activated killer (LAK) activity of HIV-seropositive and control populations against K562 targets. LAK cells derived from healthy controls (●,n=16), asymptomatic HIV-1 seropositive subjects (■n=8), and AIDS subjects (O, n=6) were assayed for their cytotoxicity against K562 targets. E: T , effector : target cell ratio.

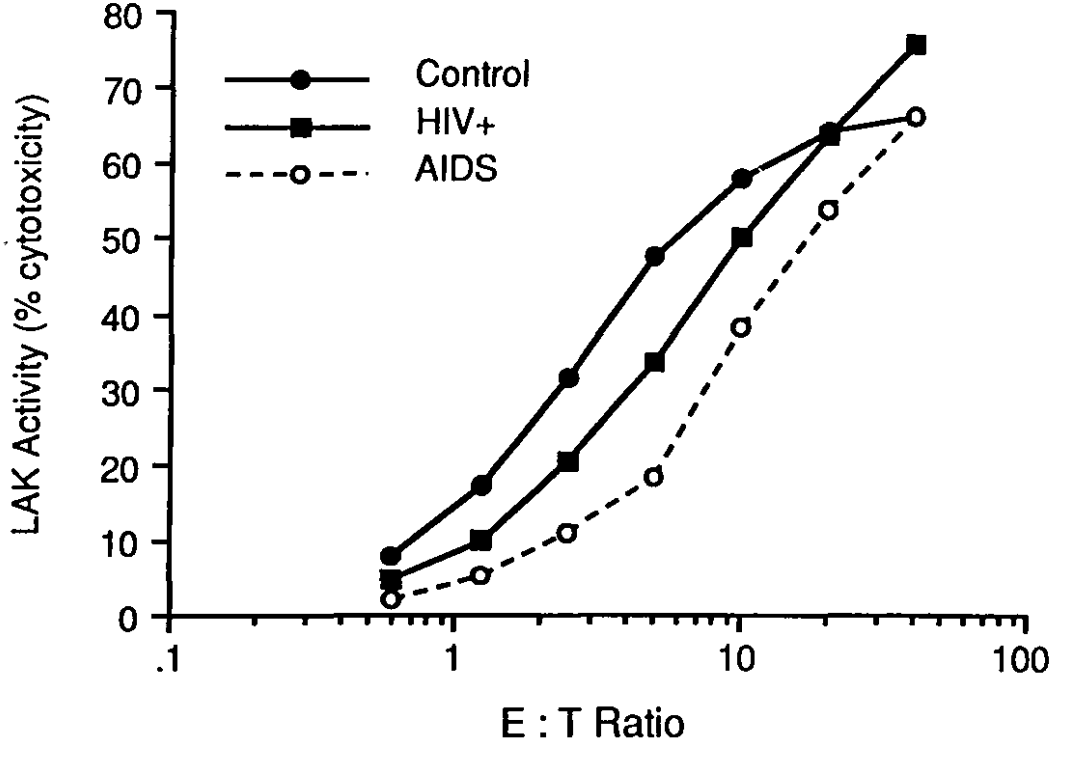
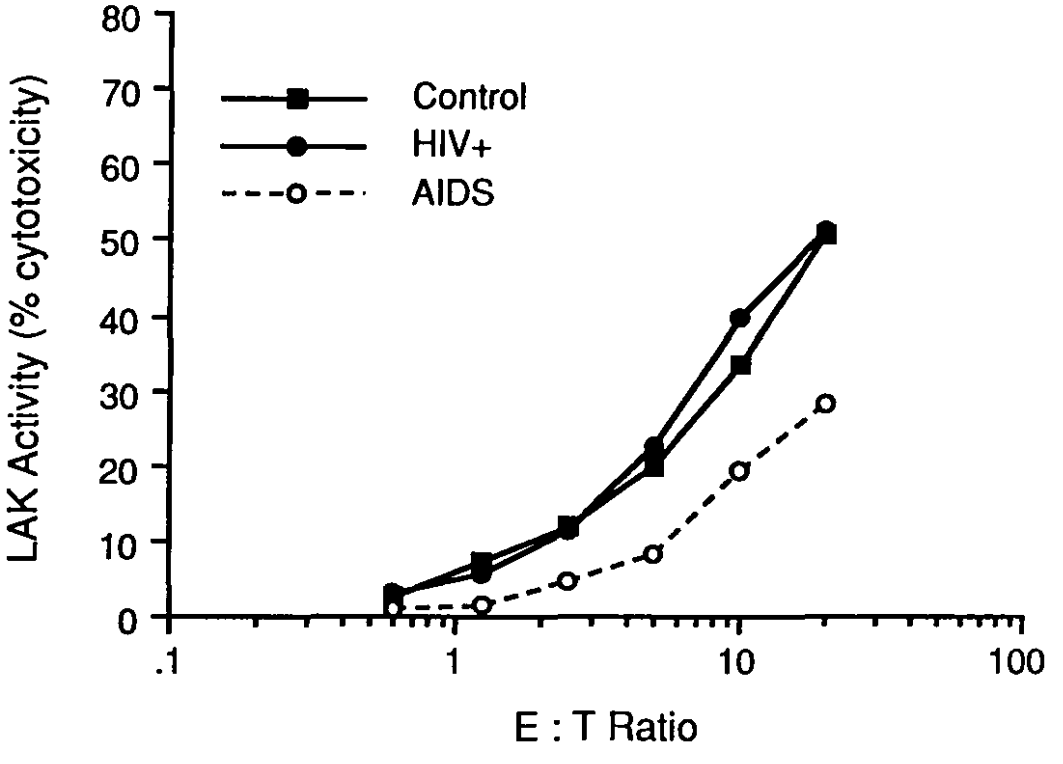

80
70
60
50
40
30
20
10
0
LAK Activity (% cytotoxicity)
Control
HIV+
AIDS
.1
1
10
100
E : T Ratio

FIGURE 2

Lymphokine-activated killer (LAK) activity of HIV-seropositive and control populations against RAJI targets. LAK cells derived from healthy controls (■,n=15), asymptomatic HIV-1 seropositive subjects (●,n=7), and AIDS subjects (O,n=6) were assayed for their cytotoxicity against RAJI targets.


80
70
60
50
40
30
20
10
0
LAK Activity (% cytotoxicity)
Control
HIV+
AIDS
.1
1
10
100
E : T Ratio

LAK activity against NK-sensitive and NK-resistant prototype targets

LAK cells derived from healthy control and HIV-seropositive populations showed significant differences in their ability to lyse the NK prototype target, K562 (Figure 1, $p<0.001$, 2 way anova). LAK cells derived from both asymptomatic and AIDS populations showed a reduced ability to lyse K562 targets when compared to similarly derived LAK cells from healthy individuals ($p<0.05$, Newman-Keuls Test). Moreover, the capacity of LAK cells from asymptomatic seropositive individuals to lyse K562 cells was significantly greater than corresponding LAK cells from individuals with overt AIDS, ($p<0.05$, Newman-Keuls Test).

Unlike resting PBLs, LAK cells, derived by *in vitro* incubation of PBLs in IL-2-containing media, acquire the ability to lyse NK-resistant RAJI cells. However, decreased levels of such killing were observed when LAK cells of HIV-seropositive populations were used (Figure 2, $p<0.01$ 2-way ANOVA). However, with RAJI cells, both healthy control and asymptomatic seropositive populations showed enhanced cytolytic activity when compared with individuals having overt AIDS ($p<0.05$, Newman-Keuls Test).

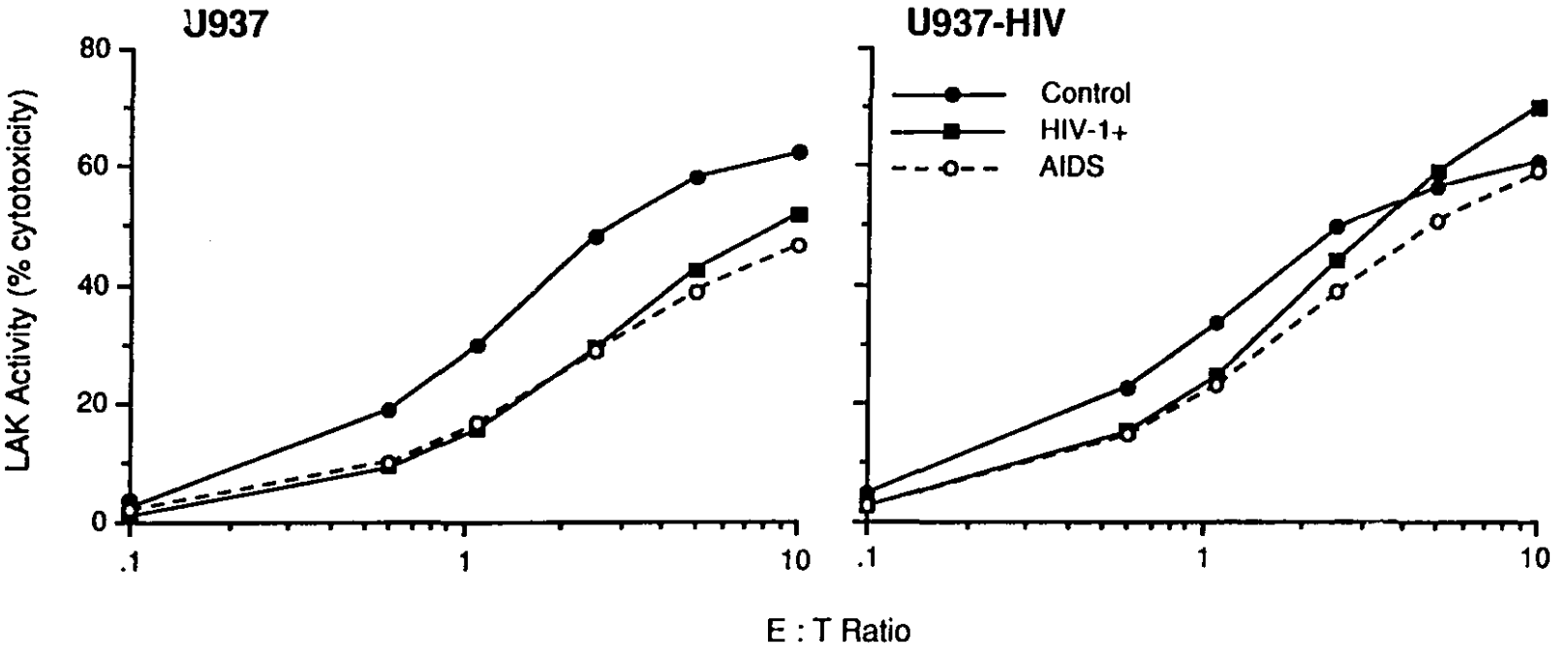
LAK activity using U937 targets and their HIV-infected counterparts

With regard to the U937 promonocytic target cell line, LAK cells derived from both HIV-seropositive populations showed a reduced capacity to lyse targets when compared to similarly derived LAK cells from healthy controls (Figure 3 $p<0.01$, 2-way ANOVA). The cytotoxic potential of LAK cells derived from both asymptomatic seropositive and AIDS subjects was significantly lower than that observed for the control population $P<0.05$, Newman Keuls test.

U937 cells were infected with HIV-1 as described in Materials and Methods. By indirect immunofluorescence using monoclonal antibodies against p17 and p24 viral proteins, 71-97% of these U937 cells were infected with HIV. In contrast to results obtained using non-infected U937 targets, the ability of LAK cells from asymptomatic seropositive, AIDS, and control groups to lyse HIV-infected U937 cells was statistically

FIGURE 3

Lymphokine-activated killer (LAK) activity of HIV-seropositive and control populations against HIV-infected and uninfected U937 tumor targets. LAK cells derived from healthy controls (●, n=13), asymptomatic HIV-1 seropositive subjects (■, n=7), and AIDS subjects (O, n=13) were assayed simultaneously for their cytotoxicity against uninfected and HIV-infected U937 target cells.


U937
U937-HIV
LAK Activity (% cytotoxicity)
80
60
40
20
0
.1
1
10
Control
HIV-1+
AIDS
E : T Ratio

comparable (Figure 3, 2way-ANOVA). This, cytolysis of U937 cells and their corresponding HIV-infected counterparts was significantly different for both HIV-seropositive population relative to healthy controls. LAK cells from both HIV-asymptomatic and AIDS groups showed a significant enhancement in their ability to kill HIV-infected U937 targets relative to U937 targets ($p<0.001$ and 0.001 respectively, 2-way ANOVA). In contrast, LAK cells from healthy controls showed comparable cytotoxic potentials against HIV-infected and non-infected U937 targets.

Characterization of LAK cytolytic parameters

To characterize the diminishment of LAK activity in HIV-1 seropositive subjects relative to healthy controls, the lytic parameters k and A were compared (Table 1). The k value is an index of the relative lytic capacity of individual LAK cells while the A value represents the maximal amounts of LAK cell-mediated cytotoxicity (see Materials and Methods). Regardless of the target cell line, the diminishment of LAK activity in seropositive individuals relative to healthy controls was due to a significant reduction in the lytic potential of LAK cells, i.e. the k values (Table 1). The maximal cytotoxic potential A was similar for all populations in using both U937 targets and their HIV-1 infected counterparts. The A value was actually increased in both seropositive populations relative to healthy controls using K526 targets.

The enhanced ability of HIV-seropositive population to lyse HIV-1 infected U937 targets vs their uninfected counterparts was due to significant enhancements in the maximal cytotoxic plateaus represented by the A values (Table 1). Data for RAJI was not detailed since, in the range of E: T ratios used, many HIV-1 seropositive individuals had cytolytic activities that did not converge.

Characterization of the lymphocyte population that mediates LAK activity

Peripheral blood lymphocytes from healthy controls and HIV-seropositive subjects were depleted of NK cells or T cells with Leu 19 (anti-CD56) and Leu 4 (anti-CD3) antibodies prior to LAK cell generation to determine the phenotype of the progenitors of

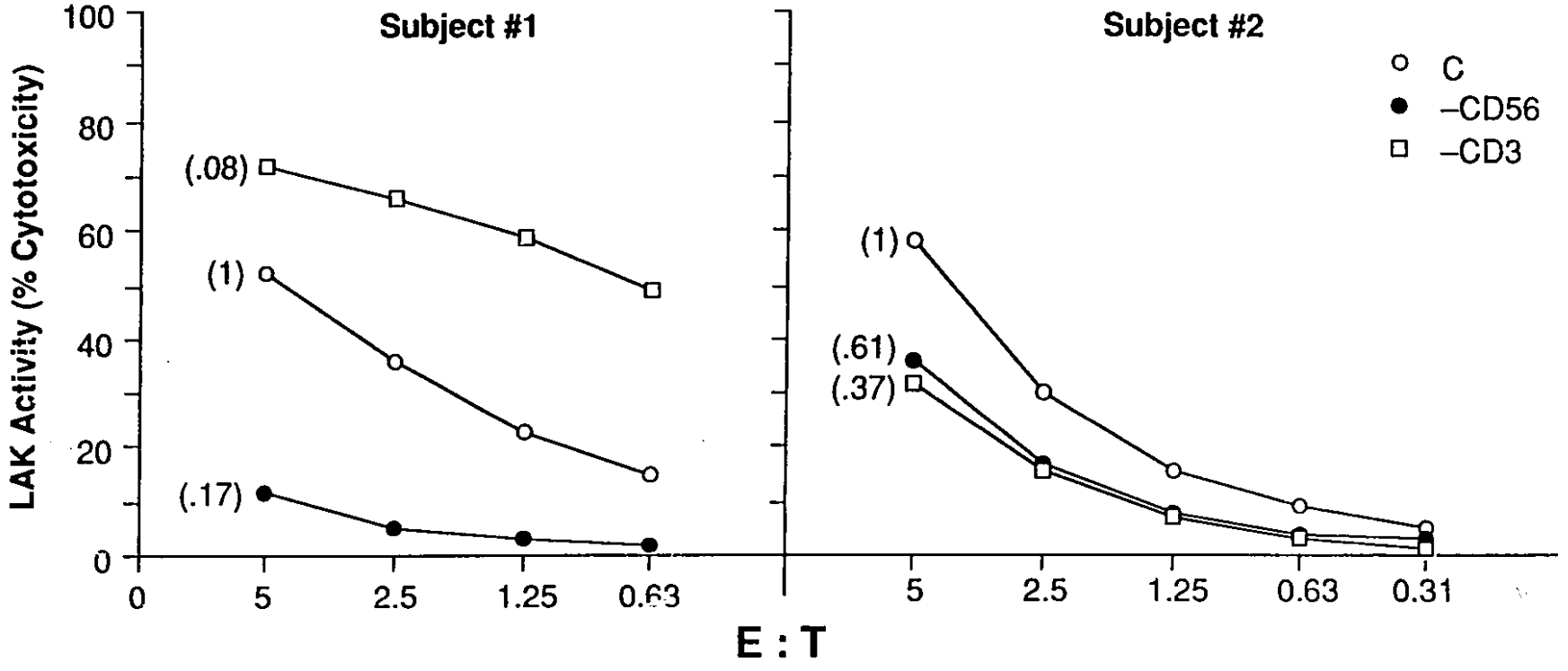
Table 1. Parameters of lymphokine-activated killer cell (LAK) cytolytic activity in HIV-seropositive and healthy control populations.

Cell source	Target	LU_{20}	k	A	n
Control	U937	160±19	0.59±0.07	65.±3.9	13
HIV+		66±14*	0.34±0.06	52.6±9.0	6
AIDS		70±18*	0.39±0.07*	54.3±6.9	13
Control	$U937_{HIV}$	159±30	0.72±0.14	62.7±5.0	13
HIV+		104±14	0.31±0.05	77.3±7.0‡	6
AIDS		114±29	0.39±0.09*	65.7±4.6‡	13
Control	K562	75±10	0.28±0.04	67.3±4.3	15
HIV+		52±21	0.13±0.05*	86.5±3.5†	8
AIDS		23±6*	0.06±0.02*	84.1±1.7†	6

Individual LAK data obtained for control, HIV-1-seropositive and AIDS subjects were subjected to exponential regression analysis (see Materials and methods). LAK cytolytic activity (LU_{20}) and lytic parameters k and A represent the mean ± s.e.m.; n= number of subjects. *Observed data for HIV-1-seropositive populations are significantly less than those for corresponding healthy controls, $p < 0.05$, Newman-Keuls test. †Observed data for HIV-1-seropositive populations are significantly greater than those for corresponding healthy controls, $p< 0.05$, Newman-Keuls test.‡Observed data for the HIV-infected U937 targets are significantly greater than corresponding values of uninfected U937 cells by two-way analyses of variance and paired t-tests.

FIGURE 4

Lymphokine-activated killer (LAK) activity of two subjects using peripheral blood lymphocytes (PBLs) and natural killer (NK)-cell (CD56) and T-cell (CD3) depleted PBLs to generate LAK cells. PBLs were depleted of $CD56^+$ and $CD3^+$ lymphocytes prior to LAK cell generation. The cytotoxicity of LAK cells derived from control (O), CD3 (❑), and CD56 (●) depleted PBLs was determined. Values in parentheses represent LAK cell recovery of depleted PBLs relative to control PBLs.


Subject #1
Subject #2
C
–CD56
–CD3
LAK Activity (% Cytotoxicity)
100
80
60
40
20
0
(.08)
(1)
(.17)
(1)
(.61)
(.37)
0
5
2.5
1.25
0.63
0.31
E : T

cytolytic LAK activity. Representative LAK activities against K562 targets by lymphocyte subpopulations from two subjects is depicted in Figure 4. The specific LAK activity of all subjects is significantly reduced when $CD56^+$ NK cells are depleted prior to LAK cell generation. Moreover, the overall recovery of LAK cells is reduced relative to the undepleted control (Figure 4). Thus, total LAK activity is markedly reduced upon $CD56^+$ cell depletion in both subjects.

In both control and HIV-seropositive populations, two patterns of changes in LAK activity were observed upon depletion of $CD3^+$ T cells as represented in Figure 4. In subject 1 (an asymptomatic seropositive individual), depletion of $CD3^+$ T cells on day 0 resulted in a marked activation of subsequent LAK activity (Figure 4). The total LAK activity, however, would have to take into account the marked reduction in recovery of LAK cells following T cell depletion relative to the undepleted control. In contrast, similar depletion of $CD3^+$ T cells prior to LAK cell generation in subject 2 (a healthy individual) resulted in a marked depletion of both specific and total LAK activity. Similar patterns to that seen in Figure 4 have also been observed in healthy and seropositive subjects when cytotoxicities were monitored against U937 targets and their HIV-infected counterparts (unpublished data).

DISCUSSION

The ability of natural killer (NK) cells and their *ex vivo* induced counterparts, lymphokine-activated killer (LAK) cells, to elicit potent anti-viral and anti-tumor immune responses has generated considerable interest as to their prognostic relevance and their potential usefulness in immunotherapy. A number of studies have demonstrated that patients with AIDS have diminished peripheral blood NK activity (4), however, comparable studies of IL-2 induced LAK activity have not been reported.

PBLs from both healthy controls and the HIV-seropositive individuals were contrasted with regard to their respective capacity to generate cytotoxic LAK cells after a 6 day incubation in media containing 15 U/ml IL-2. This IL-2 concentration corresponds to IL-2 concentration contained in T-cell growth factor, used to originally generate LAK cells (unpublished results, 28). Secondly, this concentration corresponds to the *in vivo* steady-state IL-2 concentrations contained in the serum of patients receiving IL-2 therapy (38). This *in vitro* IL-2 concentration generates LAK cells with cytolytic properties that are similar to those induced *in vivo* following IL-2 therapy (39). Moreover, preliminary studies indicate that low IL-2 concentrations at least *in vitro* selectively enhance NK cell responses in the AIDS and healthy control population with no concurrent enhancement of T cell responses (40,41).

In our study, not only were PBL levels reduced in HIV-seropositive individuals, but normalized numbers of lymphocytes generated fewer activated lymphocytes upon *in vitro* incubation with IL-2. These LAK cells from the AIDS population also show an impaired ability to lyse the prototype NK-sensitive target K562, the EBV-infected NK-resistant target, RAJI, and the promonocytic target U937. In comparison to AIDS subjects, LAK cells from asymptomatic HIV- seropositive individuals showed a similarly reduced capacity to lyse U937, and an enhanced capacity to lyse K562 targets and RAJI cells. Whether these differences in target cell susceptibility reflect distinct LAK effectors involved in their kill or differences in the size of asymptomatic cell pool awaits further investigation.

Studies in the literature have focused on quantitatively evaluating NK cytotoxicity by measuring lytic units (LU) defined as the number of effector cells required to lyse a given proportion (ideally 50%, but conventionally 20%) of target cells (1,6,36,37). We have found that evaluating the k and A values directly from the exponential fit equation provides the most useful information with regard to description of LAK cell-mediated cytotoxicity. The decline of LAK activity in both HIV-seropositive populations against all targets is due to reductions in the cytolytic parameter k, indices of the cytolytic potential of individual LAK cells. In control, HIV-seropositive, and AIDS populations, the maximal levels of cytotoxicity reflected by the asymptote A remain unchanged. These results are consistent with previous findings with NK cells using single cell binding assays where the impairment of NK activity in the AIDS population was not reflected by differences in lymphocyte numbers binding to targets but rather in impairments in the lytic machinery of NK cells post-conjugate formation (16-20,37).

The enhanced abilities of LAK cells derived from both HIV-seropositive groups to lyse HIV-infected U937 targets relative to their uninfected counterparts is due to significant increases in A, the asymptote or maximal achievable cytotoxicity. An increase in the V_{max} of NK cell lysis following viral infection of targets has been observed in other studies (1,42,43). Alternatively, we cannot exclude the possibility of recruitment of different effector cells in HIV-seropositive individuals mediating the killing of HIV-infected targets as compared to their uninfected counterparts. Our findings are consistent with Rappociolo *et al.* who demonstrated enhanced NK ($CD16^+$) cell-mediated cytolysis of HIV-infected U937 targets relative to their uninfected counterparts (26). However, while studies in our laboratory show reduced NK activity in HIV-seropositive subjects relative to controls, we do not show significant differences in the cytolysis of U937 targets and their HIV-infected counterparts (unpublished data).

Cell depletion studies were performed to ascertain the involvement of NK and T cell subsets in LAK cell-mediated cytolysis of targets. Depletion of NK cells resulted in marked

reduction in cytotoxic LAK cell responses. Depletion of T cells on day 0 resulted in two distinct patterns of response. In many seropositive and healthy subjects, depletion of T cell progenitors resulted in enhancement of LAK activity. This would be expected if T cells were not participating in the lytic process, whereupon T cell depletion would enrich for cytotoxic LAK cells derived from NK precursors. The second response pattern of diminished LAK activity upon T cell depletion indicates that T cells play a role in LAK cell generation. This may be due to a direct involvement of T cells in generating non-MHC restricted CTLs or in an immunoregulatory role of T cells on NK cell activation (44).

Cytotoxic T cells (CTLs) can be activated in culture with interleukin-2 (45-47) However, it is unlikely that the cytolysis monitored in this study is mediated by MHC-restricted CTLs. K562 lacks both class 1 and 2 MHC determinants required for CTL recognition (1). HIV-1 infection of U937 down-regulates class 2 HLA expression which would diminish rather than enhance CTL recognition and subsequent lysis of HIV-infected U937 targets relative to their uninfected counterparts (48). As further support to a physiologic role of natural immunity in HIV-seropositive individuals NK cells ($Leu7^+$ or $CD16^+$) rather than T cells ($CD3^+$, $CD4^+$, or $CD8^+$) have been recently observed to mediate cytolytic responses to autologous HIV-1, gp 120 envelope protein-coated $CD4^+$ cells (49).

To summarize, LAK cell-mediated cytolysis of both NK-sensitive (K562, U937, $U\text{-}937_{HIV}$) and NK-resistant (RAJI) targets is inducible in asymptomatic HIV-seropositive and AIDS subjects albeit less so than healthy individuals. These findings are consistent with comparable studies on NK cell function. The delineation of the regulatory role of both NK and LAK cell responses in prevention of HIV-induced disease progression awaits further study.

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Correspondence should be addressed to Dr. Bluma Brenner, Lady Davis Institute - Jewish General Hospital, 3755 Cote Ste-Catherine Road, Montreal, Canada H3T 1E2.

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CHAPTER 3

Since we established that HIV-seropositive and AIDS patients exhibit a decreased LAK activity compared to healthy subjects, exponential regression analysis was employed to compare the different cytolytic parameters *A* and *k* as well as LU_{20} values between the different populations. LU (lytic unit) is defined as the number of cells required to lyse a given proportion of cells (optimally 50% but usually 20%) in the assay period. LU are usually calculated by the exponential fit equation $y=A\ (1-e^{-kx})$, where y=fractional chromium release, x=E:T ratio, *k*=a constant which, for curves having the same asymptote, is directly proportional to NK lysis, and *A* = the asymptote of the curve (Fig.1). *A* and *k* are independent parameters representing the maximal amount of cell-mediated lysis and the relative lytic potential of the effector cells respectively. LU_{20} is a number derived by solving the equation for 20% cytolysis of 10,000 targets using *A* and *k* values, inverting, and then multiplying by 100. The use of regression analysis is useful for accurate comparisons of NK activity between individuals, since a series of dose-response data are reduced to single number that is directly proportional to NK cell lytic activity, namely the LU (Trinchieri 1989).

Concurrent studies in Dr. Brenner's laboratory detailed regression analysis values in cancer patients receiving and cancer patients not receiving chemotherapy. In this study viral-induced (HIV) and chemotherapy-induced immunocompromise was contrasted vis a vis NK and LAK cytotoxicity. PBL numbers and LAK cell recoveries were monitored in cancer and HIV-seropositive patients.

Since cytotoxic assays are always performed at constant E:T ratios, decreases observed in PBL numbers and LAK cell recoveries are therefore not incorporated in the LU_{20}. To correct for this, we incorporated PBL numbers and relative LAK cell recoveries in order to calculate absolute ALU_{20} values.

These studies demonstrated that in cancer patients receiving chemotherapy, NK and LAK progenitor cell pool sizes were decreased, while cytolytic ability of effector cells was not affected. In direct contrast, in HIV-seropositive individuals diminution of NK and LAK cell function was associated with reductions in both cell pool sizes and cytolytic functions.

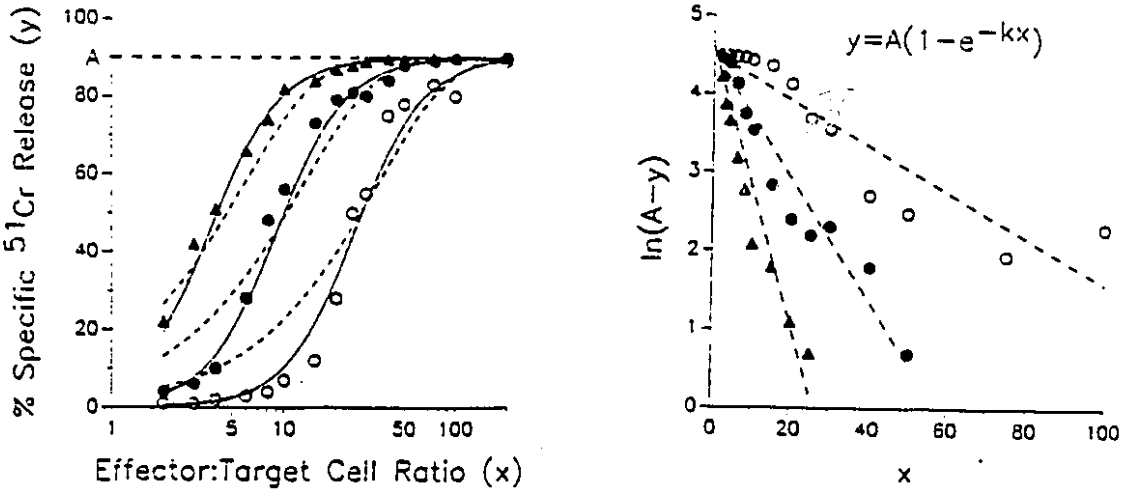

FIGURE 1. Analysis of the cytotoxic activity of PBLs from donors A (●), B (O), and C (▲) using the exponential fit equation. NK cell mediated cytotoxicity was quantitated by ^{51}Cr-release assays using a constant number (10^4) of ^{51}Cr-labelled target cells and a variable number of human PBLs as effector cells. The left panel depicts the best-fit sigmoidal curve for three donors using the exponential fit equation (broken lines). The right panel represents curves calculated according to the exponential fit equation and expressed as ln(A-y) versus x.

DIFFERENTIAL EFFECTS OF CHEMOTHERAPY-INDUCED AND HIV-1-INDUCED IMMUNOCOMPROMISE ON NK AND LAK ACTIVITIES USING BREAST CANCER AND HIV-1 SEROPOSITIVE PATIENT POPULATIONS.

ABSTRACT

This study contrasts the effect of chemotherapy-induced and viral-induced (HIV-1) immunocompromise on natural killer (NK) and lymphokine-activated killer (LAK) cell function. The ability of NK and LAK cells isolated from the peripheral blood of healthy controls, breast cancer patients receiving or not receiving adjuvant chemotherapy, and HIV-1 seropositive individuals to lyse K562 and U937 targets was determined. Exponential regression analysis of the cytolytic data was used to derive the cytolytic variables A (indicative of the maximal cytolytic kill of a target) and k (indicative of the lytic efficiency of individual effector cells). Overall LU_{20} values were ascertained and adjusted to incorporate absolute lymphocyte numbers. Such analysis indicates that the cytolytic NK and progenitor LAK cell pools are diminished in breast cancer patients receiving chemotherapy. However, the ability of individual NK and LAK cells from treated patients to lyse targets remain unchanged. In contrast, the diminution of NK and LAK cell function in HIV-1 seropositive individuals is associated with reductions in both NK and LAK cell pool sizes as well as their cytolytic functions.

INTRODUCTION

Natural killer (NK) cells have been distinguished by their characteristic expression of phenotypic markers ($CD56^+$, $CD16^+$, $CD57^{\pm}$, and $CD3^-$), as well as their functional involvement in non-major histocompatibility complex (MHC)- restricted cytolytic activities against virally-infected and tumor targets *in vitro* and *in vivo* (1-6). NK cell-mediated cytotoxic responses include NK activity, antibody-dependent cellular cytotoxicity (ADCC), and inducible NK and lymphokine-activated killer (LAK) cell activities (1-6).

It has been difficult to establish direct correlates between NK and LAK activities and resistance to tumor progression or opportunistic diseases. One major obstacle has been the high fluctuations and variabilities in NK cell numbers and activities within and between healthy donor and patient populations (1,7). NK cells are contained within the large granular lymphocyte (LGL) pool and as such represent less than 10% of circulating peripheral blood lymphocytes (PBLs). In addition, NK cells and their functions are exquisitely sensitive to endogenous and exogenous cytokine modulations (1-3). NK and LAK cells represent mixed cell populations that are both phenotypically and functionally heterogeneous (1-3,8). Furthermore, LAK cells are functionally dissimilar to NK cells, vis-a-vis target cell specificities, with LAK cells acquiring the capacities to lyse NK-resistant and NK-sensitive targets (1,2,8). The relative contributions of small proportions of T cells that coexpress NK determinants and lyse without MHC restriction remain unclear (1,3,8).

NK and LAK cell dysfunctions may occur secondary to cytotoxic drug therapies, viral infections, and overall anergy in patient populations (6,9-12). In contrast, other studies indicate that NK and LAK activities can be indirectly primed in patient populations by chemotherapy-induced or viral-induced inhibition of suppressor cell function (9,10,13). Overall, the effects of cytotoxic drug therapy on natural immune responses have not been detailed.

Studies in our laboratory have been independently evaluating the effect of chemotherapy-induced and HIV-1-induced immunocompromise on NK and LAK activities using breast cancer and HIV-1 seropositive patient populations, respectively (15-18). We have monitored lymphocyte numbers and recoveries as well as NK and LAK cytolytic activities. Exponential regression analysis of NK and LAK cytolytic data analyzes for changes in values of the independent parameters A and k, as well as LU_{20} values (18). Careful evaluation of these experimentally-derived values, incorporating lymphocyte numbers in the calculations, provides novel and useful information to compare and contrast healthy control and patient populations. Our studies indicate that cytotoxic drug therapy results in selective depletions of the basal and inducible NK pools. HIV-seropositive populations also show reductions in NK and LAK cell pools; however, overall cytolytic activities of individual NK and LAK cells are also impaired.

MATERIALS AND METHODS

Patient and Control Subjects.

With regard to studies involving breast cancer patient populations, healthy controls included research and hospital personnel and healthy female volunteers who have had no history of breast cancer. The chemotherapy-negative breast cancer patient population included both Stage I patients who have never received chemotherapy or received placebo in protocols evaluating tamoxifen therapy and Stage II patients who were two years post-adjuvant chemotherapy and disease-free. The breast cancer population who were receiving chemotherapy included individuals on a number of adjuvant chemotherapy protocols including CMF, CMFVP/VATH, CA, PF, PFAT, low dose adriamycin, or combined mitomycin-mitoxantrone (15). Blood samples from treated patients were obtained prior to administration of new cycles of chemotherapy.

With regard to studies involving HIV-seropositive subjects, healthy age-matched males constituted the normal control population. HIV-seropositive individuals were subclassified into two populations. The overt AIDS population, CDC group IV C and D, included subjects that have had two or more AIDS-associated secondary opportunistic infections and/or cancers, including *Pneumocystic carinii* pneumonia, and/or oral hairy leukoplakia. The asymptomatic HIV-seropositive population included CDC group II and III subjects free of opportunistic infections. The mean, CD4:CD8 ratios of the HIV-1^+ asymptomatic and AIDS populations were 1.1 and 0.2, respectively, significantly less than the corresponding value of 1.7 for healthy controls ($p<.05$, 1x ANOVA).

Cytotoxic effector and target cells.

Peripheral blood, samples obtained with informed consent, were collected in EDTA-containing (breast cancer patients) or heparinized (HIV-seropositive subjects) tubes and lymphocytes (PBLs) were isolated by Ficoll-Hypaque (Pharmacia, Piscataway, AJ) density gradient centrifugation (16,18). PBLs were the effector cell populations for assays of NK cell-mediated cytotoxicity.

Cultured LAK cells were generated by *ex vivo* incubation of PBLs (10^6 cells/ml) in RPMI-1640 medium containing 10% decomplemented FCS and 15 U/ml recombinant IL-2 (Boehringer-Mannheim, GmbH, West Germany) for 5-6 days at 37° in a humidified atmosphere of 5% CO_2 in air (16,18). LAK cell recoveries were determined relative to the original numbers of PBLs seeded. On the day of assay, LAK cells were washed twice in media containing RPMI-1640 and 10% FCS to remove IL-2 and constituted the cells used for assays of LAK cell-mediated cytolysis.

The target cell lines included the NK-sensitive K562 cell line (derived from a patient with chronic myelogenous leukemia in blast crisis) and the U937 cell line (a promonocytic cell line). Both cell lines, obtained from the American Type Culture Collection (Rockville, MD) were maintained in complete RPMI-1640 containing 10% FCS, 2mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 mM HEPES. Cells were passaged twice weekly.

Cytotoxicity assays.

NK and LAK activities were monitored using 18h chromium release assays with PBLs and LAK cells as respective effectors (E) and ^{51}[Cr]-labelled tumor cell lines as targets (T) (16-18). Target cells were labelled with 150 µCi of sodium [^{51}Cr] chromate (ICN Biomedicals Ltd, Montreal, Canada). LAK cells were adjusted to give appropriate E:T ratios in triplicate wells of U-bottom 96-well microdilution plates. Spontaneous isotope release was determined from control wells containing only target cells, and maximum release was determined by the addition of 2% Triton-X100 to appropriate wells. This spontaneous release represented approximately 20% and 25% total release for K562 and U937 targets respectively. The percent cytotoxicity was calculated according to the following formula: % cytotoxicity = (test sample release - spontaneous release) / maximum release - spontaneous release

Exponential regression analysis of subject's cytolytic activities monitored at 3-6 E:T cell ratios was performed using computer software kindly provided by Dr. H.F. Pross

(19). Cytolysis was calculated according to the equation of $y = A\ (1\text{-}e^{-kx})$ where y = fractional chromium release, x = E:T ratio, k = negative slope derived from plotting ln (A-y) (i.e. target survival) vs x, and A = asymptote of the curve. A and k are independent parameters, A representing the maximal amount of cell-mediated lysis and k representing the relative lytic potential of lymphocytes. Using A and k, the equations were solved for 20% cytolysis of 10,000 targets, inverted and multiplied by 100 to yield LU_{20} values. Where indicated lymphocyte counts were incorporated into LU_{20} values. Since healthy controls showed on average 1.75×10^6 cells/ml; individual LU_{20} were standardized as follows:

$$\text{Absolute } LU_{20} = \frac{\text{lymphocyte count (cells/ml) test sample}}{1.75 \times 10^6} \times \text{observed } LU_{20}$$

$$(ALU_{20})$$

Flow cytometric analysis.

Distribution of CD4, CD8, and CD56 antigens on PBLs and LAK cells was ascertained by cytofluorometric analysis using an EPICs analyzer (Coulter). Cells were stained with (FITC) fluorescein isothiocyanate conjugated and(PE) phycoerythrin-conjugated monoclonal antibodies. (Coulter Electronics, Burlington, Ontario, Canada).

Statistical analysis.

Levels of NK and LAK activities and lymphocyte counts in patient and control populations were statistically compared using unpaired 1-way (1x) ANOVAs. Where ANOVA significance was observed, sources of variations between pairs of averages were determined using Newman-Keuls tests. Similarly, the individual parameters of NK and LAK activities in subject populations, i.e. A, k, and LU_{20} were statistically contrasted. Statistical tests were calculated using software obtained from Lionheart Press (Alburg, VT).

RESULTS

NK cell-mediated cytolysis by breast cancer and healthy control PBLs.

PBL numbers were determined for healthy female controls and breast cancer patients. Patients who had mastectomies and were undergoing no subsequent treatment or patients who were greater than two years post-adjuvant chemotherapy comprised the no evidence of disease (ned)- no treatment population. Blood samples from patients receiving adjuvant chemotherapy were obtained prior to administration of new cycles of chemotherapy, thereby, eliminating acute or immediate toxic drug effects. Our results indicate that patients on chemotherapy show significant drops in their lymphocyte counts relative to both healthy controls and untreated patients (Table 1, $p<.05$, ANOVA and Newman-Keuls tests). There were no significant differences in lymphocyte numbers between the ned patient population and healthy controls (Table 1).

NK activities were monitored in all groups using 18h chromium release assays adjusting PBL numbers to give effector: target (E:T) ratio ranging from 40:1 to 2.5:1 using K562 cells as targets. Exponential regression analysis (see Materials and Methods) of cytolytic data yielded values for the independent cytolytic parameters A and k. The value A represents the maximal achievable plateau level of K562 lysis. The k value slope of target cell survival vs E:T ratio is indicative of the relative lytic effectiveness of PBLs. LU_{20} values, often quoted in the literature, were experimentally derived solving for 20% lysis of 10,000 target cells.

Results from data analysis indicates that patients with ned show significantly greater NK cytolysis of K562 targets than either healthy controls or patients receiving cytotoxic drug therapy (Table I, 1x-ANOVA, $p<.05$, Newman-Keuls tests). This elevated NK cell response in ned patients reflects a significant increase in the A value, i.e. the maximal attainable level of K562 kill (Table I, $p<.05$, Newman-Keuls tests).

The overall levels of NK cell-mediated cytolysis were adjusted to correct for levels of circulating lymphocytes. Individual LU_{20} values were normalized for PBL counts as

Table I. The effects of chemotherapeutic intervention on lymphocyte counts and parameters of NK cell-mediated cytolysis using healthy control and breast cancer patient populations.

Population	PBL Numbers (cells x10^{-6}/ml)	Parameters of NK activity LU_{20}	A	k	ALU_{20}
Female controls	1.80±0.10	11±2 (17)	37±3	0.10±0.01	9±1
Patients with ned	1.74±0.08	20±2 (59)[b]	43±3[b]	0.11±0.01	17±2[b]
Patients receiving chemotherapy	1.44±0.08[a]	11±1 (74)	35±2	0.14±0.01	5±1[a]

NK activities of PBLs against K652 targets were monitored in 18h chromium release assays at multiple E:T ratios. Exponential regression analysis of data as described in Materials and Methods yielded values for the independent cytolytic parameters A and k used to determine LU_{20} values. The absolute values for LU_{20}, ALU_{20}, incorporated lymphocyte counts into LU_{20} values. The values represent the mean ± SE for the designated number of subjects indicated in parenthesis. Following ANOVA analysis, Newman-Keuls tests indicated significant decreases relative to healthy controls[a] or significant enhancements in patients with ned relative to both controls and treated cancer patients[b].

described in Materials and Methods. Statistical analysis of such determinations indicate that patients receiving chemotherapy show significantly less absolute NK activity than healthy controls which in turn is significantly less than the ned cancer population (Table I, 1x-ANOVA, $p<.05$, Newman-Keuls tests). Thus, NK cell function is primed in breast cancer patients. Cytotoxic drug therapy markedly compromises such NK cell responses.

LAK cell-mediated cytolysis of breast cancer and healthy control populations.

The ability of PBLs to serve as progenitors of LAK cells was contrasted in control and breast cancer patients. The recoveries of LAK cells on Day 6 relative to the original number of PBLs incubated with IL-2 was significantly less in both treated and untreated patients (Table II, $p<.05$, 1 x ANOVA, and Newman-Keuls tests).

The cytolytic activities of the generated LAK cells were monitored against K562 targets using E:T ratios ranging from 10:1 to 0.3:1. Reduction of cytolytic data by exponential regression analysis showed no significant difference in the ability of healthy control and cancer patient populations to lyse K562 targets (Table II, 1x ANOVA). As expected, the cytolytic ability of LAK cells was significantly greater than corresponding NK cells, vis-a-vis, LU_{20}, A and k values (Table I and II).

NK cell-mediated cytolysis by HIV-1 seropositive and healthy male control PBLs.

The ability of PBLs from HIV-1 seropositive individuals with overt AIDS to lyse either K562 or U937 targets was monitored at E:T ratios ranging from 40:1 to 2.5:1. AIDS subjects showed an impaired ability to lyse both K562 and U937 targets (Table III). The reduced cytolytic potential of PBLs from HIV-seropositive individuals with AIDS is represented by significant declines in the experimentally-derived values A, k and LU_{20}. (Table III, $p<.05$, unpaired t-tests). Incorporation of lymphocyte levels into LU_{20} values shows a further marked reduction in absolute LU_{20} values for AIDS subjects when compared to healthy controls (Table III, $p<.05$ unpaired t-tests). Thus, the immune

Table II. The effects of chemotherapeutic intervention on LAK cell recoveries and cytolytic parameters of LAK activity using healthy control and breast cancer patient populations.

Population	LAK cell recoveries (% cells on Day 6 relative to Day 1)	Parameters of LAK activity LU_{20}	A	k
Female controls	49±6	155±43 (10)	82±3	0.43±0.11
Patients with ned	43±2[a]	134±17 (44)	77±2	0.36±0.06
Patients receiving chemotherapy	36±3[a]	100±18 (36)	73±4	0.36±0.06

LAK cells generated by IL-2 incubation were counted and assayed for cytolytic activities against K562 targets using multiple E:T ratios. Exponential regression analysis of data as described in Materials and Methods yielded values for the independent cytolytic parameters A and k used to determine LU_{20} values. The values represent the mean ± SE for the designated numbers of subjects indicated in parentheses. Following ANOVA analysis, observed values show significant decreases relative to healthy controls [a] as determined using Newman-Keuls tests.

Table III. NK activities of HIV-seropositive individuals with AIDS relative to healthy controls.

Population	Targets	Parameters of NK activity			
		LU_{20}	A	k	ALU_{20} (n)
Healthy males	K562	18±3	59±3	.08±.01	14±3 (6)
	U937	40±8	42±3	.28±.06	42±8 (12)
AIDS subjects	K562	9±4[a]	36±4[a]	.04±.01[a]	6±2[a] (6)
	U937	12±2[a]	28±1[a]	.15±.02[a]	9±1[a] (12)

NK activities of PBLs against K562 or U937 targets were monitored in 18h chromium release assays using multiple E:T ratios. Exponential regression analysis of data as described in Materials and Methods yielded values for the independent cytolytic parameters A and k used to determine LU_{20} values. The absolute values for LU_{20}, ALU_{20}, incorporated PBL counts into LU_{20} values. The values represent the mean ± SE with HIV seropositive subjects showing significant decreases[a] in values relative to healthy controls by t-tests.

compromise of NK cell responses in AIDS subjects represent both a selective depletion in the NK pool as well as impaired ability of NK cells to kill targets.

LAK cell-mediated cytolysis in HIV-seropositive and control populations.

The overall levels of circulating lymphocytes is significantly reduced in HIV-1 seropositive populations (Table IV, $p<.01$, 1 x ANOVA). The PBL numbers of AIDS subjects were significantly less than HIV-seropositive subjects who were as yet free of overt opportunistic infections who in turn were significantly less than healthy controls (Table IV, $p<.05$ Newman-Keuls tests). The ability of a normalized numbers of PBLs to serve as progenitors of LAK cells was significantly impaired in both HIV-seropositive populations relative to healthy controls ($p<.05$, 1-way ANOVA, Newman-Keuls tests).

The ability of the LAK cells to lyse K562 or U937 targets was monitored at E:T ratios ranging from 10:1 to 0.3:1. Exponential regression analysis of cytolytic data indicated that both HIV-seropositive populations show a significantly reduced abilities to lyse K562 or U937 targets (Table IV, $p<.05$, 1 way ANOVAs and Newman-Keuls tests). Furthermore, these impairments are reflected by overall reductions in the cytolytic parameters A and k (Table IV, $P<.05$, Newman-Keuls tests). Nonetheless, levels of LAK cell-mediated cytolysis against K562 and U937 targets is significantly greater than corresponding levels of NK cell-mediated cytolysis in all groups (Table III and IV). Thus, HIV-1 seropositive individuals can generate LAK cells albeit that their numbers and functions are reduced when compared to healthy controls.

Flow cytometric analysis of circulating PBLs and LAK cells.

We have determined the proportion of cells expressing NK ($CD56^+$), helper T ($CD4^+$) and suppressor T ($CD8^+$) cytotoxic cells in control and HIV-seropositive individuals with AIDS (Table V). Individuals with AIDS show a significant reduction in circulating $CD56^+$ and $CD4^+$ cells relative to healthy controls. In contrast, LAK cells from AIDS subjects show no significant differences from healthy controls with regard to $CD56^+$ cells. Thus, we have further evidence supporting a significant reduction of the NK cell pool in HIV-

Table IV. PBL numbers, LAK cell recoveries, and LAK activities in healthy control and HIV-seropositive populations.

Population	PBL Numbers (cells x10^6/ml)	LAK cell recoveries (% cells on Day 6 relative to Day 1)	Target	Parameters of LAK activity LU_{20}	A	k
Healthy controls	1.78±.09	58±3 (50)	K562	77±7 (29)	72±3	.28±.03
			U937	187±23 (27)	70±3	.59±.07
Asymptomatic HIV$^+$(OI$^-$CD2)	1.50±.12[a]	46±3[a] (33)	K562	46±9[a] (14)	80±4	.13±.03[a]
			U937	90±19[a] (16)	57±5	.34±.04[a]
AIDS subjects (HIV$^+$OI$^-$CD4)	1.22±.08[a]	43±2[a] (50)	K562	40±7[a] (20)	79±2	.13±.03[a]
			U937	60±8[a] (31)	61±4	.31±.04[a]

PBLs were used to generate LAK cells and assayed for cytolytic activities against K562 or U937 targets using multiple E:T ratios. Exponential regression analysis of data as described in Materials and Methods yielded values for the independent cytolytic parameters A and k used to determine LU_{20} values. The values represent the mean ± SE for the designated number of subjects indicated in parentheses. Following ANOVA analysis, observed values show significant decreases relative to healthy controls[a] as determined using Newman-Keuls tests.

Table V. Flow cytometric analysis of circulating PBLs and LAK cells from healthy controls and HIV-1 seropositive populations.

Population	Effector cells	Proportion (%) of cells expressing surface marker		
		CD56	CD4	CD8
Healthy Controls	PBLs	9.9±1.5(13)	47.5±2.6(13)	27.4±2.0(14)
	LAKs	7.9±1.3(9)	52.5±3.2(10)	32.2±2.1(11)
AIDS Subjects	PBLs	5.1±0.9(20)[a]	11.4±2.0[a](19)	58.8±2.5[b](20)
	LAKs	11.9±1.2(13)	9.0±2.6(15)[a]	60.5±6.0[b](16)

PBLs and LAK cells were evaluated for the expression of markers using an EPICs flow cytometer. Values represent the mean ± SE for the number of subjects indicated in parenthesis. Values for AIDS subjects are significantly less[a] or greater[b] than controls in unpaired t-tests, respectively.

seropositive populations. In addition, the diminution of LAK cell-mediated cytolysis by AIDS subjects occurs in the presence of a similar pool size, suggestive of a defective lytic machinery.

Discussion

NK cells and their *in vitro* induced counterparts LAK cells may be particularly important in the control of tumor progression and opportunistic infections. Any dysfunctions in natural immunity may contribute to disease pathogenesis. Immune compromise may arise via three distinct sources; cytotoxic effector pool size may be reduced, effector cell function may be impaired, or accessory immune cells may adversely regulate effector cell function. In this study, we have contrasted the effects of chemotherapy-induced and HIV-1-induced immunocompromise on NK and LAK cell functions.

Whereas adjuvant chemotherapy reduces tumor burden, such treatments destroy rapidly proliferating cells, including lymphocytes. The immunopathogenesis of AIDS has been primarily associated with a selective depletion of $CD4^+$ helper T cell population, which is essential to the proper orchestration of immunoregulatory responses (6,18,18). The causative agent of AIDS in the virus HIV-1, which infect lymphocytes by binding to CD4. While NK cells lack the surface receptor CD4, their functions can be indirectly impaired (6, 18,20-22). Thus adjuvant chemotherapy and HIV-infection represent drug-induced vs viral-induced sources of immunocompromise.

In this study, NK and LAK activities were monitored against K562 and U937 targets. Cytolytic data was subjected to exponential regression analysis, incorporating lymphocyte numbers into the calculations. Studies in the literature have focussed on evaluating NK cytotoxicity by measuring LU_{20} which essentially represents the inverse of the E:T ratio required to lyse 20% of a given proportion of targets using experimentally derived A and k values (1,7,19). We have found that evaluating the k and A values directly from the exponential fit equation provides the most useful information with regard to descriptions of natural immune processes. The maximal level of cytotoxicity is reflected by the asymptote A values. Enhancements in A represent increases in the cytolytic pool sizes.

The k value which takes into account the slope of the curve, provides an index of the relative cytolytic potentials of individuals NK or LAK cells.

Our data indicate that the effect of chemotherapy on NK and LAK-cell mediated cytolysis results in a diminished NK cytolytic pool size. In treated cancer patients, the lymphocyte numbers are diminished, A values representative of NK cytolytic plateaus are diminished, and the number of recovered LAK cells are diminished. However, k values, i.e. the lytic activities of individual NK and LAK cells, remain unchanged.

Of interest, NK cell responses in patients with no evidence of disease receiving no cytotoxic drug therapy are elevated relative to healthy controls. This primed response is associated with an increase in A. This increase in NK activity in breast cancer patients has been previously observed in other laboratories including our own (15,17,23,24). In addition, there is a significant increase in NK activity in male relative to female controls. The numbers of large granular lymphocytes, i.e. NK cells and their activities have been previously reported to be increased in males (25, 26).

Individuals who are HIV-seropositive show diminished NK and LAK cytolytic pool sizes as well as defective functions of individual NK and LAK cells. The lymphocyte numbers, LAK cell recoveries, the k values for LAK cells, and the k and the A values for NK cells are significantly reduced in HIV-seropositive populations.

Single cell binding studies in the literature confirm our observations of diminished NK activity in HIV-seropositive individuals, vis-a-vis, an impaired ability of individual NK cells to lyse NK targets (22,27,30). However, these single cell binding assays suggest that there is no defect in the binding of NK cells to their targets in HIV-1 seropositive individuals (22,27-29). In these studies, the number of PBLs forming conjugants with their targets in AIDS and healthy controls represent 15-20% PBLs (22,27-29). We have observed that $CD56^+$ NK cells represent 5% and 10% of circulating PBLs in AIDS subjects and healthy controls, respectively (Table V). Other investigators have also indicated that single cell binding studies are inaccurate grossly overestimating conjugate-

forming cells (1,7). Thus experimental regression analysis with regard to A values may be more relevant in determining overall cytolytic pool size than single cell binding assays.

LAK cell-mediated responses against targets have not been detailed in AIDS populations. In one report LAK cell-mediated responses in AIDS subjects were shown to be unimpaired (31). This expanded study confirms our previous findings of diminished LAK activity in HIV-seropositive individuals (18).

Thus, exponential regression analysis yields values for the independent cytolytic parameters A and k. These values, particularly when combined with lymphocyte counts, yield useful criteria to evaluate cytolytic pool size and lytic potential of NK and LAK cells. Whereas, chemotherapy diminishes NK and LAK cytolytic pools, HIV-1 induced immunocompromise reduces both the NK and LAK cytolytic pool sizes as well as NK and LAK cytolytic machineries.

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CHAPTER 4

One of the most interesting aspects that emerged from our first study (chapter 2) was that HIV-seropositive individuals exhibited enhanced LAK cytolysis against HIV-infected U-937 targets when compared to their U-937 uninfected counterparts. In direct contrast this pattern of reactivity was absent from healthy control groups where LAK cells exhibited similar cytolytic capacities against both the HIV-infected and uninfected targets. In order to further investigate this issue, we employed a number of U-937 subclones and tested the capacity of LAK cells derived from HIV-seropositive groups and healthy individuals to lyse these subclones and their infected counterparts.

Our HIV-patient groups were subdivided according to the presence or absence of AIDS-associated opportunistic infections. In addition we incorporated the $CD4^+$ PBL percentages into the classification as follows; CDC0: HIV-seronegative healthy controls, CDC2: patients asymptomatic with $CD4^+$ PBLs higher than 20%, CDC4CD: patients with Kaposi's Sarcoma but no other evidence of disease, and finally CD4CD: patients with two or more AIDS-associated opportunistic infections and or cancers with $CD4^+$ PBLs less than 10%.

This study indicated the emergence of an enhanced HIV-directed LAK cell response present only in HIV-seropositive individuals. In addition CD56-expressing PBLs, derived from healthy controls and all HIV-seropositive groups, were shown to be progenitors of LAK cells.

INCREASED LAK ACTIVITY AGAINST HIV-INFECTED CELL LINES IN HIV-1 SEROPOSITIVE INDIVIDUALS

SUMMARY

The role of natural killer (NK) cells and their inducible counterparts, lymphokine-activated killer (LAK) cells in AIDS with regard to HIV-1 viral immunosurveillance and the control of secondary opportunistic disease has yet to be established. In this study, we have demonstrated that LAK cells derived from all HIV-1 seropositive groups showed striking increases in their capacity to lyse HIV-1 infected U-937 cells relative to their uninfected U-937 counterparts. Surprisingly, similarly derived LAK cells from healthy seronegative controls showed no differences in their lysis of HIV-1 infected *vs* uninfected U-937 cells. The differential ability of LAK effectors from seropositive donors to lyse HIV-1 infected targets was demonstrable using a number of U-937 subclones and their HIV-1 infected counterparts. Again, no differences in LAK cell-mediated lysis of HIV-1 infected and uninfected U-937 subclones were observed in seronegative individuals. Our findings that HIV-1 seropositive individuals show selective expansion of non-major histocompatibility complex (MHC) restricted, HIV-1 directed cytotoxic LAK cells indicate that natural immunity may indeed play a role in HIV-1 viral immunosurveillance.

INTRODUCTION

The immunopathogenesis of AIDS has been primarily associated with a selective depletion of CD4-expressing T cells due to their susceptibility to human immunodeficiency virus type 1 (HIV-1) [1]. The clinical course of AIDS following HIV-1 infection has been shown to include a long asymptomatic period (typically 3 to 5 years). Cellular and humoral immune mechanisms may play a role in limiting viral replication and opportunistic diseases. Elucidation of HIV-specific immune responses in early phases of AIDS and their associated changes with progressive immunocompromise is crucial.

Natural killer (NK) cells and their inducible counterparts, lymphokine-activated killer (LAK) cells have been shown to elicit a number of broadly reactive and non-major histocompatibility complex (non-MHC) restricted cytolytic responses to virally-infected and tumor targets [2-4]. These responses include NK activity, antibody-dependent cellular cytotoxicity (ADCC), and LAK activity. Phenotypically, NK cells express an array of NK-associated surface markers ($CD56^+$, $CD16^+$, $CD57^-$) while lacking T-associated markers, including CD3, the T-cell receptor, and CD4, the HIV-1 viral receptor [2-4].

Patterns of decreased peripheral blood NK activity, ADCC responses and circulating $CD16^+$ and $CD56^+$ NK subsets have been observed in HIV-1 seropositive individuals with AIDS and persistent generalized lymphadenopathy (PGL) when compared to healthy seronegative and asymptomatic HIV-1 seropositive populations [5-12]. The effects of changes in natural immunity on disease course have yet to be established. Observed decreases may render seropositive individuals more susceptible to opportunistic diseases. Conversely, NK cell responses may be detrimental to seropositive populations. NK cells may potentially recognize and destroy virally-infected CD4 cells or virally-coated bystander uninfected cells [13-15].

IL-2 has been shown to play a central role in stimulating NK cell proliferation, differentiation and cytolytic activities. IL-2 in HIV-1 seropositive individuals has been observed to partially restore *in vitro* NK and ADCC functions [5,6,16]. A novel class of

effectors, LAK cells, have been generated via *ex vivo* incubation of peripheral blood lymphocytes (PBL) with IL-2 [17,18]. These LAK cells have been shown to mediate unique cytolytic activities killing both NK-sensitive and NK-resistant targets. NK cells have been demonstrated to be the primary progenitors and effectors of inducible LAK cell responses [19-21]. LAK cells, expressing NK antigenic phenotype, have also been induced *in vivo* following IL-2 therapy [22-25].

The relative abilities of inducible LAK cells to evoke anti-tumor and anti-viral cytolytic responses in seropositive populations have not been identified to date. Recent investigations in our laboratory have shown a diminished efficacy of PBL derived from HIV-1 seropositive individuals to generate cytotoxic LAK cells against K562, RAJI, and U-937 targets [13,26]. In this study, we have contrasted the ability of HIV-1 seropositive and seronegative individuals to generate LAK cell effectors cytotoxic to the U-937 cell line, isolated U-937 subclones, and their HIV-1 infected counterparts. LAK cells derived from all HIV-1 seropositive subgroups showed significantly increased cytolysis of HIV-1 infected U-937 targets relative to their uninfected counterparts. It should be emphasized that these enhanced non MHC-restricted, HIV-directed lytic responses were restricted to seropositive individuals.

PATIENTS AND METHODS

Study subjects

Blood samples were obtained upon informed written consent from HIV-1 seropositive individuals receiving treatment at our dental clinic. Our study group consisted primarily of seropositive homosexuals. Clinical status of these individuals was established according to standardized guidelines established by the Centers for Disease Control (CDC) [27]. The CDC2 subgroup included HIV-1 seropositive individuals with no signs or symptoms of AIDS. The CDC4D subgroup consisted of seropositive individuals with Kaposi's sarcoma who were otherwise asymptomatic. The CDC4CD subgroup included overt AIDS patients with two or more AIDS-associated secondary opportunistic infections (OI^+, CDCC1 and C2) and/or cancers (CDC4D) (e.g. Kaposi's sarcoma, *Pneumocystis carinii* pneumonia, oral candidiasis, oral hairy leukoplakia, and/or disseminated herpes). The CDCO subgroup was comprised of age-matched healthy, HIV-1 seronegative individuals recruited from hospital personnel.

Effector cells

Venous blood was collected in heparinized tubes and PBL were isolated by Ficoll Hypaque (Pharmacia, Piscataway, New Jersey, USA) density gradient centrifugation [28]. LAK cells were generated by *ex vivo* incubation of PBL (10^6 cells/ml) in RPMI-1640 (Gibco, Grand Island, NY) complete medium containing 10% decomplemented FCS, 15 U/ml IL-2 (Boehringer-Mannheim, GmbH, West Germany), 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 mM Hepes for 5-6 days at 37°C. On the day of assay, LAK cells were washed twice in complete media.

Target cells

The U-937 cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD). UC subclones were derived by a two time limiting serial dilution, seeding 0.15-.5 cells per well in 96-well microtiter plates [29]. The III_B strain of HIV-1 (kindly supplied by R.C. Gallo, NIH, Bethesda, MD) was used to infect the parental U-

937 and UC subclones as previously described using a multiplicity of infection of three ED_{50}/cell [29,30]. Murine monoclonal antibodies to HIV-1 viral proteins p17 and p24 (supplied by R.C. Gallo) and fluorescein-labelled goat anti-mouse immunoglobulin (Miles Laboratories, Ekhart, IN) were used to quantify the percentage of HIV-1-infected cells [30]. Virtually all chronically infected cells expressed HIV-1. All cell lines were passaged twice weekly.

Cytotoxicity assays

LAK activity was monitored using 18 h chromium release assays with LAK cells as effectors (E) and ^{51}Cr-labelled tumor cell lines as targets (T). Target cells were labelled with 150 μC sodium ^{51}Cr-chromate (ICN Biochemicals Ltd, Montreal, Canada). LAK cells were adjusted to give appropriate E:T ratios in triplicate wells of U-bottom 96-well microdilution plates. Spontaneous isotope release was determined from control wells containing only target cells, and maximum release was determined by the addition of 2% Triton-X-100 to appropriate cells. This spontaneous release represented approximately 20% total release. The percentage of cytotoxicity was calculated according to the formula: % cytotoxicity = (test sample release - spontaneous release)/(maximum release - spontaneous release) x 100%.

Exponential regression analysis of subjects' cytolytic activities monitored at 5 to 6 E:T cell ratios was performed using computer software kindly provided by Dr. H.F. Pross [31]. Cytolysis was calculated according to equation $y = A\ (1\text{-}e^{-kx})$ where y = fractional chromium release, x = E:T ratio, k = negative slope constant derived from plotting ln (A-y) (i.e. target survival) vs x, and A = asymptote of the curve. A and k are independent parameters with A representing the maximal amount of cell-mediated lysis and k representing the relative lytic potential of lymphocytes. Using A and k, the equations were solved for 20% cytolysis of 10,000 targets, inverted, and multiplied by 100 to yield LU_{20} values.

When indicated, lymphocyte counts and LAK cell recoveries were incorporated with LU_{20} values to yield absolute LU_{20} values, that represented the overall LAK cell lytic potential per ml of blood. Absolute LU_{20} = [lymphocyte count (cells/ml blood) x lymphocyte recovery (ratio of lymphocytes day 6/day 0) x observed LU_{20}]/100 [26].

Depletion of NK cells from PBL

Prior to LAK cell generation, PBL were depleted of $CD56^+$ NK cell subsets using Leu19 (Becton Dickinson, Mountain View, CA) and goat anti-mouse immunoglobulin. Briefly, PBL (8-10x10^6 cells/ml) were incubated in RPMI-1640 media containing 1% FCS and Leu19 (20 µl/10^6 cells) for 30 min at 4°C. After washing twice in HBSS (Gibco, Grand Island, NY), an excess (40 beads/cell) of magnetic goat anti-mouse-coated IgG beads (Fc specific, Advanced Magnetics Inc., Cambridge, MA) was added. Following a 30 min incubation on ice, those cells bound to magnetic beads were removed using a magnetic separator (Advanced Magnetics Inc.). This depletion was then serially repeated using 20 beads/cell. Depleted PBL were then used to generate LAK cells as previously described.

Flow cytometric analysis

Distributions of CD4, CD8, CD57 and CD56 antigens on PBL and LAK cells were ascertained by cytofluorometric analysis using an EPICs analyzer (Coulter Electronics, Burlington, Ontario). Cells were stained with relevant FITC or phycoerythrin-conjugated monoclonal antibodies (Coulter Electronics, Burlington, Ontario and Becton Dickinson, Mountain View, CA).

Statistical analysis

Cytolytic parameters, lymphocyte subset distributions, and lymphocyte counts were monitored in individual subjects with a single determination for any individual in any given population. Throughout the text and tables, data are represented as mean values ± SEM. Levels of activities and subset distributions were compared using one way ANOVA. Where a significant overall F value was obtained, post hoc comparisons were done using Tukey and Newman-Keuls tests. Comparisons of cytolytic parameters (LU_{20} values) of U-937

targets vs their HIV-infected counterparts were monitored using paired t-tests. LAK activities measured over a range of E:T ratios were compared in control and HIV-1 seropositive populations using two way ANOVA. Similarly, LAK activities monitored in parallel against HIV-1 infected and uninfected targets were contrasted using two way ANOVA. Statistical comparisons were calculated using software obtained from Systat Inc. (Evanston, IL) and Lionheart Press (Alburg, VT).

RESULTS

Cytotoxic lymphocyte distribution in AIDS

The relative numbers and subset distributions of PBL and LAK cells from HIV-1 seronegative (CDCO) and seropositive CDC2 (asymptomatic), CDC4D (KS), and CDC4CD (AIDS, OI^+) subgroups are shown in Table 1. PBL numbers declined with increasing duration of HIV-1 infection ($F=7.1$, $df=80$, $p<.001$). LAK cell recoveries on a per cell basis, however, were similar in all subgroups ($F=1.7$, $df=80$). Flow cytometric analysis indicated that the proportion of CD4-expressing cells progressively declined with advancing disease for PBL ($F=66.7$, $df=41$, $p<.001$) and LAK cells ($F=33.8$, $df=41$, $p<.001$). Conversely, significant increases in the proportions of $CD8^+$ PBL ($F=23.0$, $df=41$, $p<.001$), $CD8^+$ LAK cells ($F=3.6$, $df=41$, $p<.05$), $CD57^+$ PBL ($F=12.5$, $df=41$, $p<.001$), and $CD57^+$ LAK cells ($F=6.3$, $df=41$, $p<.001$) were observed in seropositive subgroups. CD57 was not representative of NK cells since only 11±1% of $CD57^+$ LAK cells co-expressed CD56 whereas 42±3% of $CD57^+$ LAK cells co-expressed CD8. It should be emphasized that corresponding proportions of CD56-expressing PBL ($F=.93$, $df=41$) and CD56-expressing LAK cells ($F=.63$, $df=41$) from seronegative and seropositive subgroups showed no significant differences.

LAK cell-mediated cytolysis of parental U-937 targets

As depicted in Figure 1, LAK cell-mediated cytotoxicity of uninfected U-937 targets progressively declined with disease progression ($F=4.7$, $df=84$, $p<.01$). Cytolysis of U-937 targets was significantly reduced in seropositive individuals with overt AIDS (CDC4CD) when compared to asymptomatic seropositive (CDC2) and seronegative (CDC0) controls ($p=.005$ and .037, respectively, Tukey tests). As shown in Figure 1, the ability of seropositive individuals to lyse HIV-1 infected U-937 cells was significantly enhanced relative to uninfected U-937 cells ($p = .001$, .038, and .001 for CDC2, CDC4D, and CDC4CD groups, respectively, paired t-tests). In direct contrast, there was no differential susceptibility of HIV-1 infected and uninfected U-937 targets to lysis by LAK

TABLE 1

Lymphocyte counts, lymphokine-activated killer (LAK) cell recoveries, times following HIV-1 seroconversion, and subset distributions in HIV-1$^+$ and HIV-1$^-$ populations

	CDC classification groups			
		HIV$^+$		
	HIV$^-$			
	CDC0	CDC2	CDC4D	CDC4CD
	(19)	(25)	(7)	(33)
PBL count	17 ± 1	15 ± 1	14 ± 3	10 ± 1[1,2]
LAK recovery	65 ± 4	52 ± 5	48 ± 8	51 ± 5
Time HIV$^+$(yr)	NA	2.6 ± 0.3[4]	3.0 ± 0.9	3.9 ± 0.4
Subsets (%)	(10)	(11)	(5)	(19)
CD4 PBL	45 ± 2	24 ± 2[1]	12 ± 4[1,2]	9 ± 2[1,2]
CD56 PBL	6 ± 1	5 ± 2	7 ± 3	8 ± 2
CD56 LAK	9 ± 1	7 ± 2	11 ± 4	10 ± 1
CD8 LAK	28 ± 2[3]	40 ± 3	51 ± 8	36 ± 4
CD57 LAK	4 ± 1[3,4]	15 ± 2[4]	24 ± 7	28 ± 4

PBL count (cells/ml blood x 10^{-5}), LAK cell recovery (% day 6/day 1), and selected lymphocyte subset distributions (%) in PBL or LAK cells are given. Number of subjects are indicated in parentheses.

[1,2,3,4] Values are significantly less than healthy controls[1] (CDC0), HIV-1$^+$ CDC2[2], CDC4D[3], or CDC4CD[4]

NA, not applicable; PBL, peripheral blood lymphocytes.

FIGURE 1

Lymphokine-activated killer (LAK) activity (LU_{20} values) against the uninfected (■) and HIV-1 infected (❑) U-937 cell lines using LAK cells from healthy control (CDC0) and HIV-1^+ individuals (CDC2, CDC4D and CDC4CD).

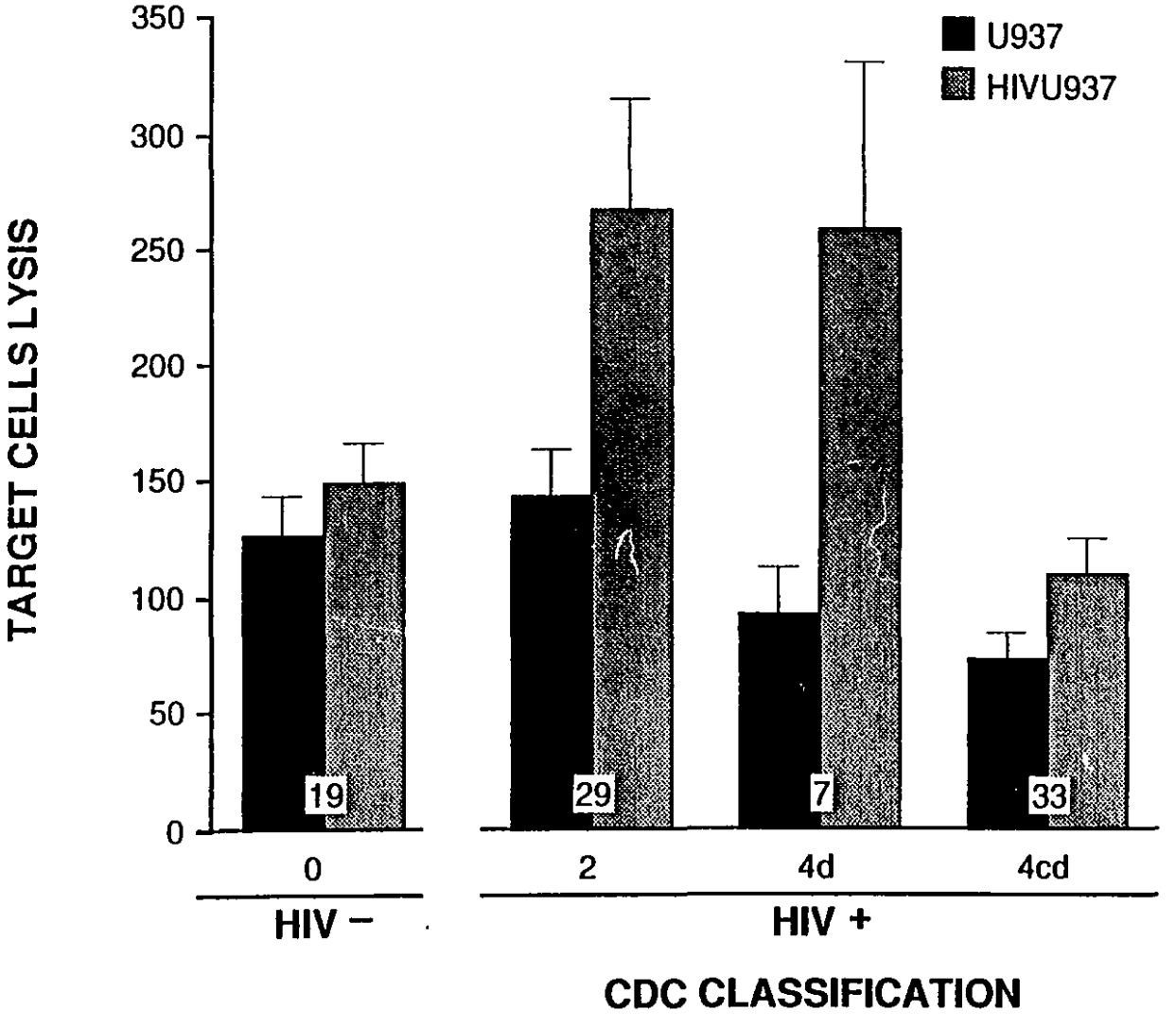
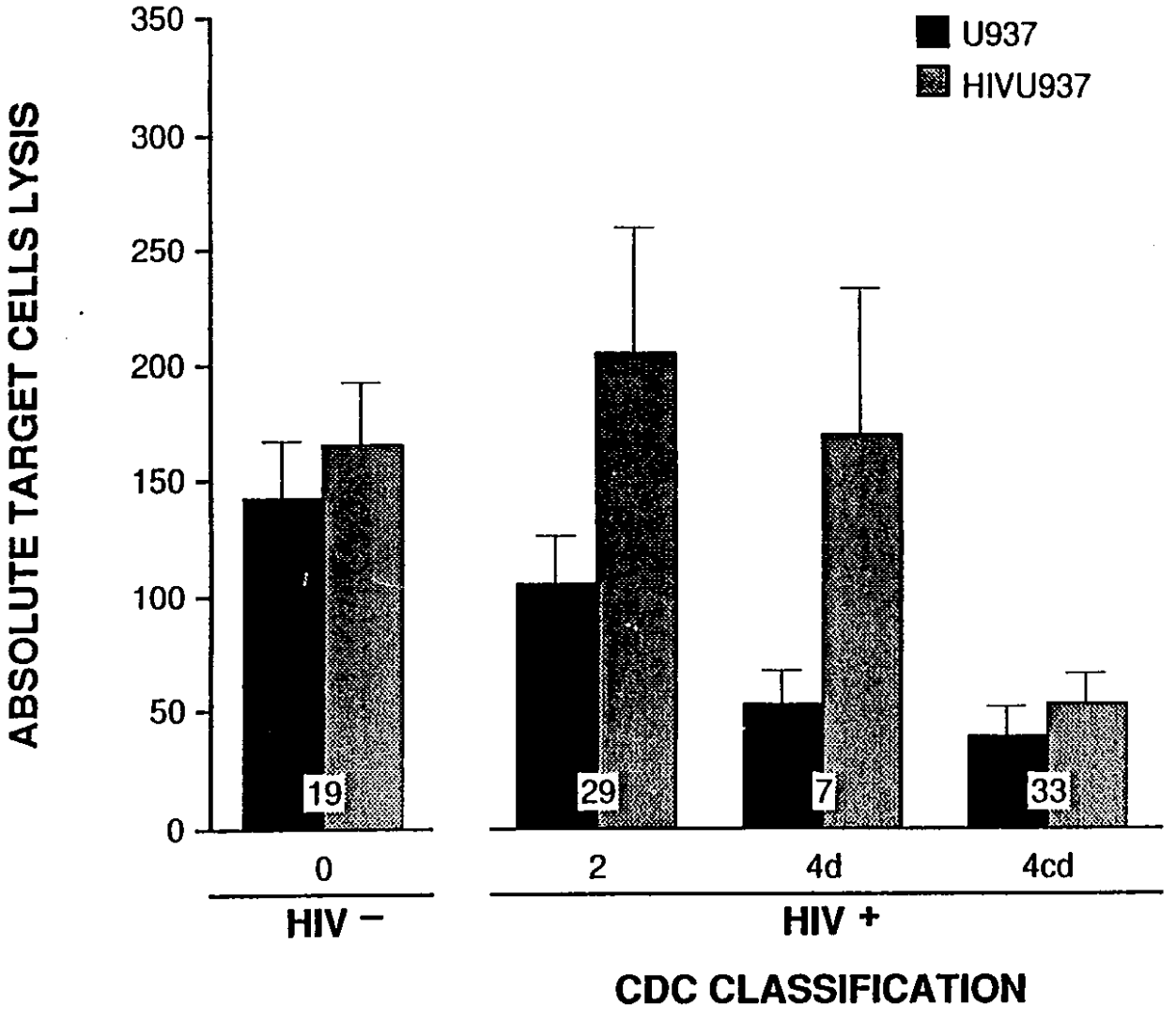

350
300
250
200
150
100
50
0
TARGET CELLS LYSIS
U937
HIVU937
19
29
7
33
0
2
4d
4cd
HIV −
HIV +
CDC CLASSIFICATION

FIGURE 2

Absolute target cell lysis of uninfected (■) and HIV-1 infected (❑) U-937 targets in HIV-1^- (CDC0) and HIV-1^+ individuals (CDC2, CDC4D, and CDC4CD). Absolute lysis incorporates peripheral blood lymphocyte (PBL) counts, lymphokine-activated killer (LAK) cell recoveries, and LU_{20} values as described in Patients and Methods.


ABSOLUTE TARGET CELLS LYSIS
350
300
250
200
150
100
50
0
U937
HIVU937
19
29
7
33
0
2
4d
4cd
HIV −
HIV +
CDC CLASSIFICATION

effectors isolated from healthy seronegative controls. There were significant differences in the overall ability to lyse HIV-1 infected targets among groups ($F=4.9$, $df=84$, $p<.01$). Post hoc Tukey tests ($p<.05$) yielded significant differences between CDC2 (asymptomatic) and CDC4CD (AIDS, OI^+) groups.

The absolute lytic potentials of LAK cells from seropositive and seronegative individuals were estimated by incorporating PBL lymphocyte counts and LAK cell recoveries into the calculated LU_{20} cytolytic values (Figure 2). Absolute lysis of uninfected U-937 cells declined progressively with increasing duration of infection ($F=6.7$, $df=84$, $p<.001$). The CDC4CD (AIDS) group showed significantly less absolute U-937 lysis than CDC2 (asymptomatic) and seronegative (CDC0) groups ($p=.043$ and $.001$, respectively, Tukey tests). The overall susceptibility of HIV-1 infected relative to uninfected U-937 targets was significantly increased in all seropositive individuals (paired t-tests). There were significant differences in absolute lysis of HIV-1 infected U-937 targets among groups ($F=3.6$, $df=84$, $p<.01$). Absolute lysis of HIV-1 infected targets in the CDC4CD (OI^+) group was significantly less than the CDC2 (asymptomatic) group ($p=.014$, Tukey test).

$CD56^+$ cells are the progenitors of cytotoxic LAK cells against U-937 and U-937 HIV targets

As depicted in Figure 3, CD56 cell depletion diminished LAK cell function directed against both U-937 uninfected and HIV-1 infected U-937 targets in all subgroups. The relative proportion of CD56-expressing cells in residual LAK cells from CD56-depleted PBL still represented ~50% of the corresponding proportions of CD56-expressing LAK cells from undepleted PBL (unpublished results). This may explain the failure to obtain complete abrogation of LAK cell function upon CD56 cell depletion.

LAK cell-mediated cytolysis of U-937 subclones

LAK cells from eight healthy seronegative and ten seropositive individuals were simultaneously assayed for their ability to lyse uninfected and HIV-1 infected UC11,

FIGURE 3

Lymphokine-activated killer (LAK) cell activity (LU_{20} values) against uninfected and HIV-1_{IIIB} infected U-937 targets by LAK cells derived from peripheral blood lymphocyte (PBL) or PBL depleted of CD56-expressing cells. Values represent the mean values for five CDC0, three CDC2, and five CDC4CD subjects.❑, LAK;■, CD56-depleted LAK.

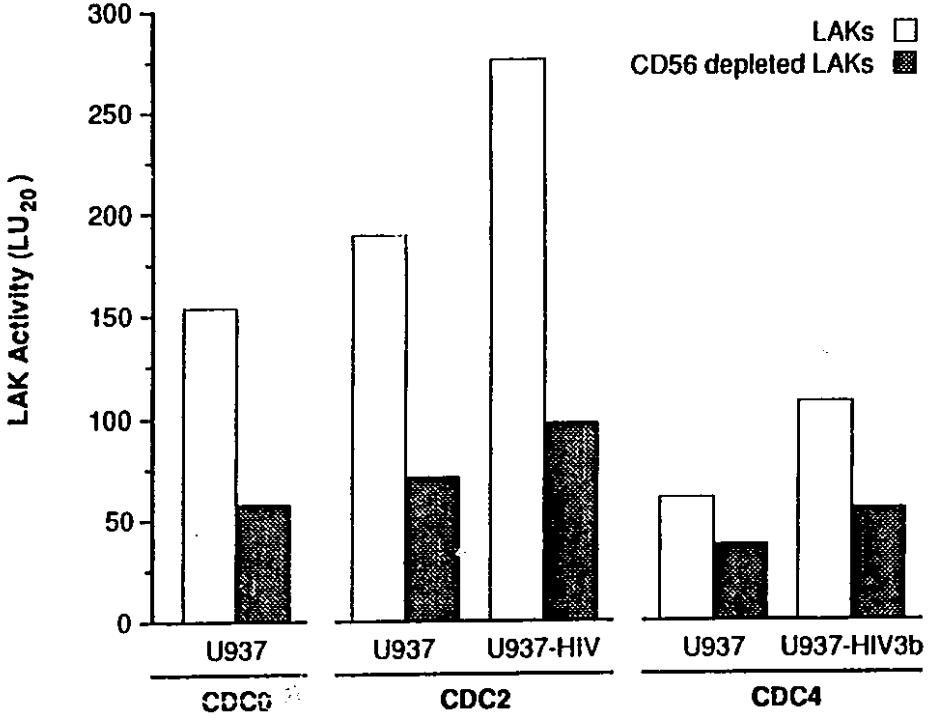
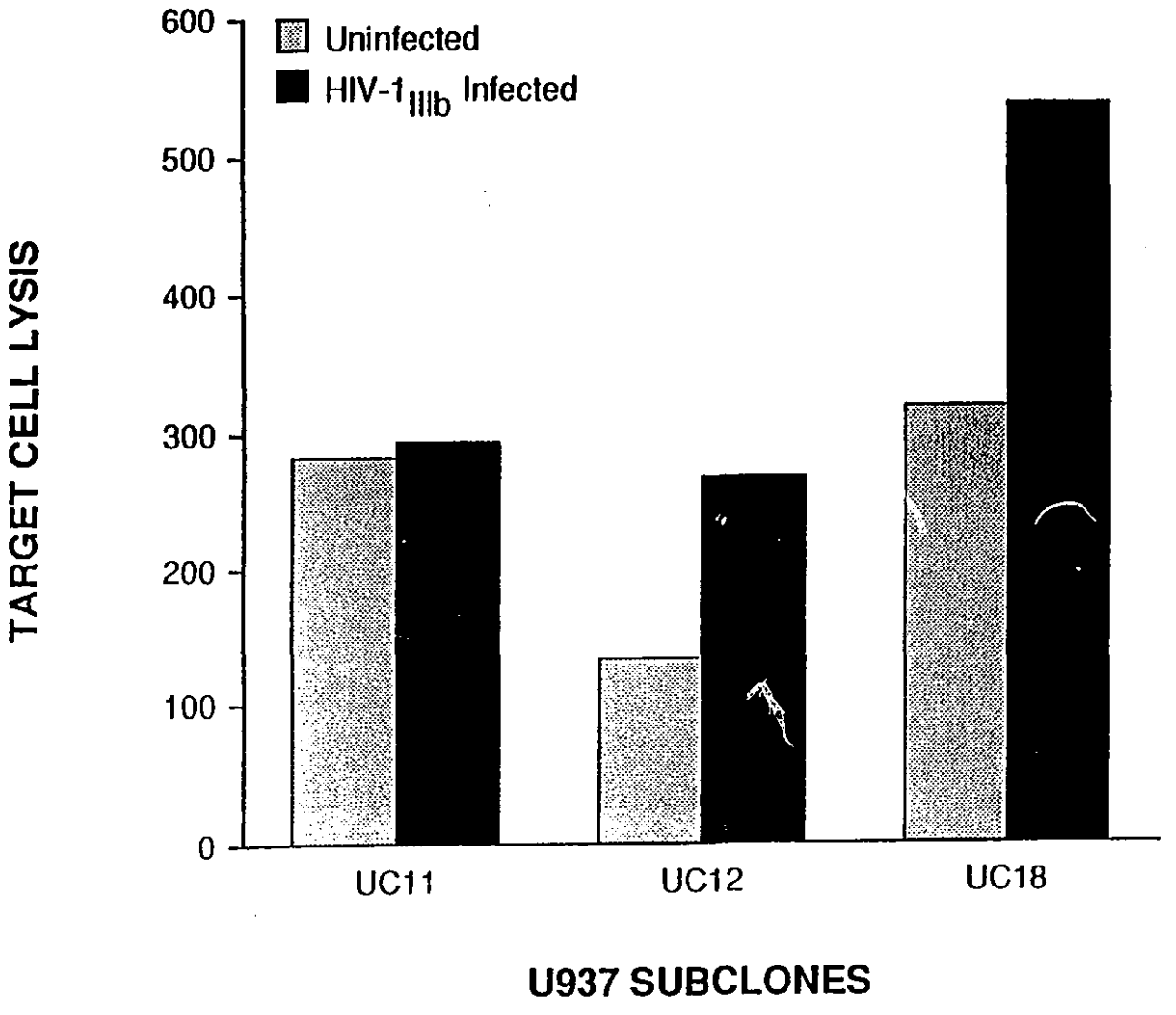

LAKs
CD56 depleted LAKs
LAK Activity (LU_{20})
300
250
200
150
100
50
0
U937
U937
U937-HIV
U937
U937-HIV3b
CDC0
CDC2
CDC4

FIGURE 4.

Average target cell lysis (LU_{20} values) of uninfected (❑) and HIV-1 infected ■ U-937 subclones, UC11, UC12, UC18, using lymphokine-activated killer (LAK) cells derived from 10 HIV-1^+ seropositive individuals.


600
500
400
300
200
100
0
Uninfected
HIV-1IIIb Infected
TARGET CELL LYSIS
UC11
UC12
UC18
U937 SUBCLONES

UC12, and UC18 subclones (Figure 4). Cytolysis of HIV-1 infected UC18 subclones was significantly greater than corresponding lysis of uninfected UC18 cells using LAK effectors from seropositive individuals ($F=6.18$, $df=1,36$, $p<.05$ for the CDC2 subgroup and $F=6.68$, $df=1,48$, $p<.05$ for the CDC4 subgroup). Cytolysis of HIV-1 infected UC12 subclones relative to their uninfected counterparts was higher in asymptomatic seropositives ($F=3.26$, $df=1,24$, $p=.08$). No significant differences, however, were observed in the corresponding lysis of uninfected and HIV-1 infected UC11 cells in these same seropositive groups ($F=.10$ and .43 for CDC2 and CDC4CD subgroups, respectively). There were no significant differences in the ability of seronegative controls (CDC0) to lyse HIV-1 infected UC11, UC12, and UC18 targets relative to their uninfected counterparts ($F=1.00$, 0.40, 0.65, HIV-1 infected vs uninfected UC11, UC12, and UC18 targets, respectively).

Levels of cytolysis of uninfected UC12 cells was significantly less than corresponding levels of UC11 and UC18 cytolysis ($p<.01$, paired t-tests). In this regard, UC12 are considerably larger and show a significantly slower doubling time than parental U-937, UC11, and UC18 cell lines [29].

DISCUSSION

The ability of *ex vivo*-induced LAK cells to elicit potent antiviral and antitumor immune responses has generated interest as to their prognostic relevance *in vivo* and their potential usefulness in immunotherapy. In this study, cytotoxic LAK cells from healthy control and HIV-1 seropositive populations were generated using 15 U/ml IL-2. This IL-2 concentration corresponds to *in vivo* steady state concentrations contained in the serum of patients receiving IL-2 therapy [22]. Low IL-2 concentrations, both *in vitro* and *in vivo*, have been shown to selectively augment NK cell responses in AIDS and healthy control populations with no concurrent enhancement of T cell responses [23,32-34].

Confirming our initial observations [13,26], we have found that inducible LAK cell function against both uninfected and HIV-infected U-937 targets was severely impaired in HIV-1 seropositive individuals with overt AIDS (CDC4CD) when compared to healthy seronegative controls. In contrast to our earlier studies [10,26], the cytolytic potential of LAK cells derived from the asymptomatic (CDC2) seropositive individuals generally fell within the range of healthy controls. Since LU_{20} values from both the healthy seronegative and overt AIDS group have not significantly changed from our previously obtained values, our larger asymptomatic population may now have included individuals with improved clinical status by way of earlier detection and improved treatments. This is the first comprehensive study of inducible LAK cell function in seropositive individuals. We are currently aware of only one other study of LAK cell function in AIDS where no significant differences in LAK activity against K562 and RAJI targets were observed in nine patients with AIDS as compared to seronegative controls [35].

We have observed a differential enhancement of LAK cell-mediated lysis of HIV-1 infected targets relative to their uninfected counterparts in seropositive populations. Corresponding increases in lysis of HIV-1 infected cells were absent in healthy seronegative individuals. Other studies have demonstrated that NK cells from seronegative heterosexuals, seronegative and seropositive homosexuals lyse HIV-1 infected targets more

effectively than corresponding uninfected targets [2,36-41]. The observation of elevated NK cell-mediated, HIV-1 directed lysis was dependent upon the target cell line and the HIV-1 strain [36,40,41]. Enhanced susceptibility of HIV-1 infected targets involved NK cells with or without accessory HLA-DR-expressing cells [36]. These observations combined with our present findings strongly suggest that multiple cytolytic effector subsets and pathways mediate NK and LAK cell-mediated cytolysis of HIV-1 infected targets. Furthermore, this study has shown that HIV-1-directed LAK effectors were preferentially activated in HIV-1 seropositive individuals.

It should be emphasized that the absence of HLA-matched targets strongly negates the involvement of classic viral specific cytotoxic T lymphocytes (CTL) in the HIV-1 directed lysis observed in this study. Increased lysis of HIV-1_{IIIB} infected relative to uninfected targets was demonstrable in 79 HLA-unmatched seropositive individuals. Lysis of uninfected and HIV-1 infected targets were diminished in CDC2 and CDC4CD subgroups upon depletion of CD56-expressing NK cells. Recent studies in our laboratory have identified the emergence of novel CD8-expressing LAK cytotoxic cells in asymptomatic (CDC2) seropositive individuals (manuscript submitted for publication). These CD8 subsets did not, however, elicit conventional MHC-restricted CTL responses. Lysis of K562, a target lacking both class 1 and 2 MHC determinants was reduced upon CD8 cell depletion (unpublished results). Thus both CD56 and CD8 non-MHC responses contributed to elevated LAK cell-mediated responses to HIV-1 infected targets in asymptomatic seropositives. Only CD56 responses mediated elevated HIV-1 directed responses in overt AIDS patients.

Our findings have shown that the enhanced susceptibility of HIV-1 infected U-937 targets was not universal. The same individuals that showed elevated lysis of HIV-1-infected UC12 and UC18 clones relative to their uninfected counterparts, showed no corresponding enhanced lysis of HIV-infected UC11 subclones. In this regard, UC11 is an interesting variant showing less accumulated viral DNA, long latency to HIV-1 infection,

and reduced levels of Sendai virus-inducible TNFα mRNA [29]. This target may be particularly useful in defining the target structures that mediate HIV-1 directed LAK cell-mediated cytolysis.

Reasons for the impairments of NK and inducible LAK activities in HIV-1 seropositive individuals remain unclear. It has been suggested that defective NK cytolytic machineries contribute to diminished NK cell function in AIDS subjects [11,12]. Other groups have reported that NK cells can be susceptible to *in vitro* HIV-1 infection despite their failure to express CD4 mRNA or surface CD4 [5,40,41]. Our findings confirm other studies that have indicated overall reductions in circulating CD56-expressing subsets in PBL of seropositive individuals [5,14,15]. We have, however, found no significant differences in the relative proportion of inducible CD56-expressing LAK cells in seropositive populations that could account for observed reductions in their LAK cell function.

A number of distinct cellular mechanisms have been implicated in HIV-1 immunosurveillance. These include cytotoxic function mediated by CTL [11,43,44], ADCC [6-8,45] and NK activity [5,22-24]. Our findings [6,13,21] indicate that inducible non-MHC restricted LAK activity derived from NK cell progenitors can also elicit anti-HIV responses. The emergence of novel HIV-1 directed immune responses in seropositive individuals indicates that they can contribute to the disease process. Whether these HIV-1 directed responses are beneficial or detrimental to AIDS progression awaits further study. Using our U-937 subclones and their HIV-1-infected counterparts, we have demonstrated that the differential cytotoxicity of HIV-1 infected targets relative to their uninfected counterparts is dictated both by the particular HIV-1-infected target and the source and inducibility of the effector cell population. These subclones will be particularly useful in the further identification, characterization, and expansion of HIV-1-directed cytolytic effectors.

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CHAPTER 5

Since LAK cell responses are diminished in HIV-seropositive individuals with advanced disease, studies were initiated to determine whether these effects were secondary to increases in the number of CD8-expressing lymphocytes.

Negative cell sorting employing immunomagnetic beads was performed either on PBLs prior to LAK cell generation (LAK cell progenitor depletions), or on LAK cells (cytotoxic effector-cell depletions).

Series of CD8 depletion studies both at the progenitor and effector cell level were performed. Furthermore we have depleted healthy control-derived PBLs of $CD4^+$ cells in order to study the effects of an artificially introduced $CD4^+$ cell depletion and the concomitant enrichment of $CD8^+$ cells in generating LAK cell activity.

Results have indicated the emergence of a non-MHC-restricted CD8-expressing population in HIV-seropositive asymptomatic patients. This CD8-expressing cytotoxic population was absent in healthy controls and AIDS patients. In contrast CD8 depletions in healthy controls and AIDS patients resulted in enhanced LAK cell cytolysis. In addition CD56-expressing cells were shown to mediate cytotoxic LAK cell responses in HIV-seropositive individuals.

NOVEL NON-MHC RESTRICTED CYTOTOXIC CELLS IN HIV-1 SEROPOSITIVE GROUPS

ABSTRACT

This study has contrasted the antigenic phenotype and cytotoxic properties of lymphokine-activated killer (LAK) cells isolated from PBL of HIV-1 seropositive and seronegative subgroups. LAK cells derived from HIV-1 seropositive individuals lacking secondary AIDS-associated opportunistic infections (Center for Disease Control subgroup 2, CDC2) showed levels of target cell lysis in the range of healthy seronegative controls. Corresponding LAK cells from individuals with overt AIDS (CDC4C or D) demonstrated significantly impaired target cell lysis. Both seropositive groups showed significantly enhanced capacities to lyse HIV-1 infected targets relative to their uninfected counterparts. The progenitors of these responses were established by depleting designated subsets from PBL. The effectors of LAK activity were determined by depleting select subsets just prior to assay. Cytolysis of K562, U-937, and HIV-1 infected U-937 targets was significantly reduced in all seronegative and HIV-1 seropositive subgroups upon depletion of CD56-expressing NK effectors. The primary effectors and progenitors of LAK cell function against K562 and U-937 targets in HIV-1 asymptomatic CDC2 seropositives were CD8-expressing subsets. CD8 progenitors and effectors did not, however, elicit cytotoxic responses in healthy seronegative controls and overt AIDS subjects. Cytolysis of targets in fact, increased upon CD8 progenitor depletion in the overt AIDS subgroup, concomitant with increased proportions of CD56-expressing subsets. Increasing the proportion of CD8-expressing subsets in healthy seronegative controls by depleting PBL of CD4-expressing subsets resulted in no significant changes in LAK cell responses. Taken together, these results indicate that dynamic changes in cytotoxic lymphocyte subset distributions occur upon HIV-1 associated immunocompromise. Novel cytotoxic subsets demonstrating enhanced non-MHC restricted cytolysis of HIV-1 infected targets emerge in early disease.

INTRODUCTION

Natural killer (NK) cells and their inducible counterparts, lymphokine-activated killer (LAK) cells comprise a distinct branch of the immune network that elicit broadly reactive and non-MHC restricted cytolytic responses to virally-infected and tumor cells (1-3). These large granular lymphocytes represent a heterogeneous population phenotypically defined by their expression of a characteristic array of NK-associated markers ($CD56^+$, $CD16^+$, $CD57^-$) (1-3). NK cells are distinct from T cells, lacking CD3, the T-cell receptor, and CD4, the HIV-1 viral receptor. NK can express CD8 on their surface, albeit at low density (1-3).

Resting and inducible NK responses may be relevant in AIDS, providing alternative cytotoxic mechanisms that can effectively limit HIV-1 viral spread and AIDS-associated opportunistic diseases (4-6). Cumulative information has indicated that NK cell responses are normal or elevated in HIV-1 seropositive individuals in early phases of AIDS (4-6). Progressive declines in NK cell responses and NK subsets are, however, observed in seropositive individuals with increasing duration of HIV-1 infection (4-6).

Interleukin-2 (IL-2) plays a vital role in stimulating NK cell proliferation, differentiation, and cytolytic functions (1,2,5,6,12). Moreover, incubation of PBL cultures *ex vivo* with IL-2 have revealed the inducibility of novel cytolytic effectors, LAK cells (13,14). These LAK cells elicit unique non-MHC restricted responses killing both NK-sensitive and NK-resistant targets (13,14). Delineation of the relative contribution of NK and T cells as progenitors and mediators of inducible LAK cell responses remain controversial (13-18). By and large, NK cells appear to be the primary source of inducible LAK activity (15-17). These LAK cells have been induced *in vivo* following IL-2 therapy with cells of NK antigenic phenotype eliciting LAK responses (19-22).

IL-2 has been shown to boost *in vitro* NK and ADCC responses in HIV-1 seropositive individuals (5,6,12). The corresponding potential of IL-2 to elicit inducible LAK cytolytic responses in seropositive individuals has not been established. Recent

investigations in our laboratory have demonstrated a diminished effectiveness of PBL from seropositive donors to develop cytotoxic LAK cells against K562, RAJI, and U-937 targets (9,23). In direct contrast, LAK cells derived from all HIV-1 seropositive subgroups showed significantly elevated cytotoxic responses to HIV-1 infected targets relative to uninfected counterparts (50). This preferential HIV-1 directed target cell response was absent in healthy seronegative controls (50).

This report has contrasted the functional and antigenic phenotype of lymphocytes mediating inducible LAK cell responses in HIV-1 seropositive and seronegative groups. Our results indicate the emergence of novel non-MHC restricted cytotoxic T cell populations inducible in seropositive individuals at early stages of AIDS. This cytotoxic population was absent in healthy controls and overt AIDS patients, where LAK responses were elicited by CD56-expressing lymphocytes.

SUBJECTS AND METHODS

Study subjects

Blood samples were obtained upon informed written consent from HIV-1 seropositive individuals receiving treatment at our dental clinic. Our study group consisted primarily of seropositive homosexuals. Clinical status of these individuals was established according to standardized guidelines established by the Centers for Disease Control (24). The CDC2 subgroup included HIV-1 seropositive individuals with no signs or symptoms of AIDS. The CDC4D population consisted of HIV-1 seropositive individuals with Kaposi's sarcoma who were otherwise asymptomatic. CDC4CD overt AIDS populations included individuals with two or more AIDS-associated secondary opportunistic infections (OI^+, CDC2C1 and C2) and cancers (CDC4D), including Kaposi's sarcoma, *Pneumocystis carinii* pneumonia, oral candidiasis, oral hairy leukoplakia, and/or disseminated herpes. Patients were classified according to the percentage of CD4-expressing cells in their PBL and LAK cells, where indicated. Age-matched healthy, HIV-1 seronegative individuals were recruited from hospital personnel and comprised the CDC0 population.

Effector cells

Venous blood was collected in heparinized tubes and PBL were isolated by Ficoll Hypaque (Pharmacia, Piscataway, New Jersey, USA) density gradient centrifugation (25). LAK cells were generated by *ex vivo* incubation of PBL (10^6 cells/ml) in RPMI-1640 (Gibco, Grand Island, NY) complete medium containing 10% decomplemented FCS, 15 U/ml IL-2 (Boehringer-Mannheim, GmbH, West Germany), 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 mM Hepes for 6-8 days at 37°C. On the day of assay, LAK cells were washed twice in complete media.

Target cells

The U-937 cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD). The CEM.NKR cell line was obtained through the AIDS

Research and Reagent Program, Division AIDS, NIAID, NIH: CEM.NKR from Dr. Peter Cresswell (26). The III_B strain of HIV-1 (kindly supplied by R.C. Gallo, NIH, Bethesda, MD) was used to chronically infect U-937 and acutely infect CEM.NKR as previously described using multiplicities of infection of three ED_{50}/cell (26,27). Murine monoclonal antibodies to HIV-1 viral proteins p17 and p24 (supplied by R.C. Gallo) and fluorescein-labelled goat anti-mouse immunoglobulin (Miles Laboratories, Ekhart, IN) were used to quantify the percentage (~97%) of HIV-1-infected U-937 cells (27). These cell lines were passaged twice weekly.

Cytotoxicity assays

LAK activity was monitored using 18 h chromium release assays with LAK cells as effectors (E) and ^{51}Cr-labelled tumor cell lines as targets (T). Target cells were labelled with 150 μC sodium ^{51}Cr-chromate (ICN Biochemicals Ltd, Montreal, Canada). LAK cells were adjusted to give appropriate E:T ratios in triplicate wells of U-bottom 96-well microdilution plates. Spontaneous isotope release was determined from control wells containing only target cells. Maximum release was determined by the addition of 2% Triton-X-100 to appropriate wells. This spontaneous release represented approximately 20% of total release. The percentages of cytotoxicity were calculated according to the formula: % cytotoxicity = (test sample release - spontaneous release)/(maximum release - spontaneous release).

Exponential regression analysis of subjects' cytolytic activities monitored at 5 to 6 E:T cell ratios was performed using computer software kindly provided by Dr. H.F. Pross (28). Cytolysis was calculated according to the equation $y = A\ (1\text{-}e^{-kx})$ where y = fractional chromium release, x = E:T ratio, k = negative slope constant derived from plotting ln (A-y) (i.e. target survival) vs x, and A = asymptote of the curve. A and k are independent parameters with A representing the maximal amount of cell-mediated lysis and k representing the relative lytic potential of lymphocytes. Using A and k, the equations

were solved for 20% cytolysis of 10,000 targets, inverted, and multiplied by 100 to yield LU_{20} values.

Serologic depletion of T and NK subsets

To determine the progenitors of inducible LAK cell responses, PBL were depleted of their designated subset and then incubated for 6-8 days as described above to obtain LAK cells. To determine the effectors of LAK activity, LAK cells isolated on the 6th-8th day were depleted of their designated subsets, prior to assay. Undepleted LAK cells were isolated under standard conditions and assayed in parallel. In all cases, lymphocyte counts were determined following subset depletion and cell dilutions were readjusted to obtain identical E:T ratios. Flow cytometric analysis of lymphocyte subsets was performed to evaluate subset distributional profiles.

Cells were negatively selected using mouse monoclonals to the designated subset followed by magnetic goat anti-mouse coated IgG beads (Fc specific, Advanced Magnetics Inc., Cambridge, MA or Dynal, Great Neck, N.Y.). CD56, CD8, and CD4 subsets were depleted using Leu19, OKT8, and Leu3a monoclonal antibodies, respectively. Leu19 and Leu3 were obtained commercially (Becton Dickinson, Mountain View, CA). The OKT8 cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD). OKT8 antibodies were purified by ammonium sulphate precipitation of culture supernatants performed as previously described (29).

Briefly, PBL or LAK cells were depleted of CD56, CD8, and CD4 using 20 µl, 100 µl and 200 µl, respectively, of antibody per 10^6 subset positive cells with incubations for 30 min at 4°C. After washing twice in HBSS (Gibco, Grand Island, N.Y.), an excess (50 beads/cell) of magnetic goat anti-mouse IgG beads was added. Following a 30 min incubation on ice, those cells bound to magnetic beads were removed using a magnetic separator (Advanced Magnetics, Inc., Cambridge, MA). This depletion was serially repeated using 20 magnetic beads/cell.

PBL and LAK cells were depleted of CD57 subsets using Leu7 purified from the HNK cell line obtained from the ATCC (Rockville, MD), as previously described (30). Briefly, cells were incubated with Leu7 for 45 min, washed twice, and resuspended in rabbit low-tox H complement (1:5 v/v, Cedarlane, Hornby, Ontario). Following incubation for 1 hr at 37°C, cells were washed twice.

Flow cytometric analysis

Distributions of CD4, CD8, CD3, CD57, and CD56 antigens on PBL and LAK cells were ascertained by cytofluorometric analysis using an EPICs analyzer (Coulter Electronics, Burlington, Ontario). Cells were stained with relevant FITC or phycoerythrin-conjugated monoclonal antibodies obtained from Becton Dickinson (Mountain View, CA) for CD56 and CD57 subsets and Coulter Electronics (Burlington, Ontario) for all other subsets. Since cytolysis of targets were determined at fixed cell numbers, subsets were represented throughout the text as their proportion of total PBL or LAK cells.

Statistical analysis

Throughout the text and tables, data was represented as mean values ± SEM. Levels of activities and subset distributions were compared between groups using one-way ANOVA. Where a significant overall F value was obtained, post-hoc comparisons were done using Tukey and Neuman Keuls tests. Cytolysis (LU_{20} values) of U-937 and CEM.NKR targets vs their HIV-infected counterparts were compared using paired t-tests. Statistical comparisons were calculated using software obtained from Systat Inc. (Evanston, IL).

RESULTS

LAK cell-mediated cytolysis in seropositive subgroups

LAK cell-mediated cytolysis of U-937 targets was monitored in HIV-1 seronegative and seropositive subgroups. A trend of elevated cytolysis of U-937 targets was found in the asymptomatic HIV-1 seropositive subgroup (CDC2, $CD4^+$PBL>20%). Box plots showed a higher median value with a wider range of cytolysis in asymptomatic seropositives relative to healthy controls (Figure 1) A progressive decline of LAK cell-mediated lysis occurred with advancing disease ($F=4.46$, $df=122$, $p=.005$ one way ANOVA). Cytolysis of U-937 targets by the overt AIDS group (CDC4CD, $CD4^+$PBL<10%) was significantly less than the corresponding lysis of the asymptomatic seropositive subgroup (CDC2, $CD4^+$PBL>20%) ($p=.002$ post-hoc Tukey tests).

LAK cell recoveries and PBL numbers were incorporated into calculations of U-937 lysis to estimate the absolute levels of LAK activity per unit blood volume. Absolute LAK activity varied in our groups ($F=7.82$, $df=122$, $p<.001$). Individuals with overt AIDS (40 ± 16) showed significantly less absolute target cell lysis than asymptomatic seropositives (133 ± 27) and seronegative controls (141 ± 25, $p<.001$, post-hoc Tukey tests). CDC2 seropositive individuals showed significantly increased cytolysis of CEM.NKR targets acutely infected with HIV-1 relative to uninfected CEM.NKR (Figure 2, $p=.008$, paired t-test).

$CD8^+$ subset depletion of LAK progenitors

PBL were depleted of CD8-expressing subsets prior to subsequent generation of LAK cells. LAK cells from CD8-depleted PBL were then compared to undepleted LAK cells, vis-à-vis LAK activity and T and NK antigenic distribution. Flow cytometric analysis revealed that $CD8^+$-subset depletion of PBL resulted in a complete loss of CD8-expressing cells in subsequently isolated LAK cells from both seropositive and seronegative groups (Figure 3). Cytometric analysis indicated that recoveries of $CD56^+$ LAK cells from CD8-depleted PBL differed significantly in HIV-1 seronegative and seropositive subgroups

FIGURE 1

Cytolysis of U-937 targets by LAK cells isolated from PBL of HIV-1 seropositive and seronegative subgroups. Target cell lysis (LU_{20} values) of LAK cells from healthy controls (n = 26), CDC2 (CD4>20%, n = 31), HIV-1^+ subgroup 20%<CD4>10% (n = 21), and overt AIDS CDC4 (CD4<10%, n = 51) seropositives are represented by box plots showing 25th, 50th (median), and 75th percentiles.

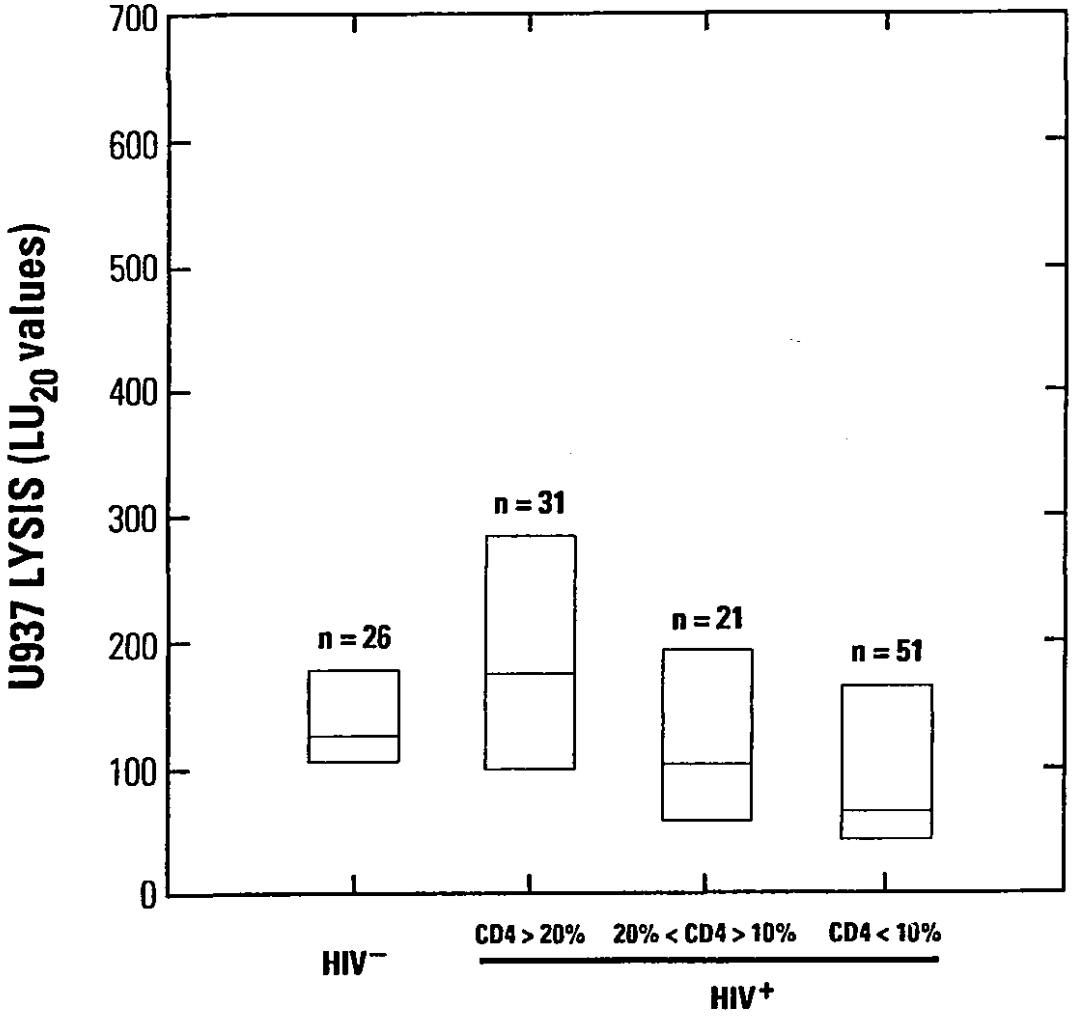

U937 LYSIS (LU_{20} values)
700
600
500
400
300
200
100
0
n = 26
n = 31
n = 21
n = 51
HIV⁻
CD4 > 20%
20% < CD4 > 10%
CD4 < 10%
HIV⁺

FIGURE 2

Cell-mediated cytolysis of HIV-1 infected U-937 and CEM.NKR targets and their uninfected counterparts by LAK cells isolated from PBL of 5 asymptomatic (CDC2) individuals.

LAK CYTOLYSIS OF HIV-1 INFECTED TARGETS vs THEIR UNINFECTED COUNTERPARTS

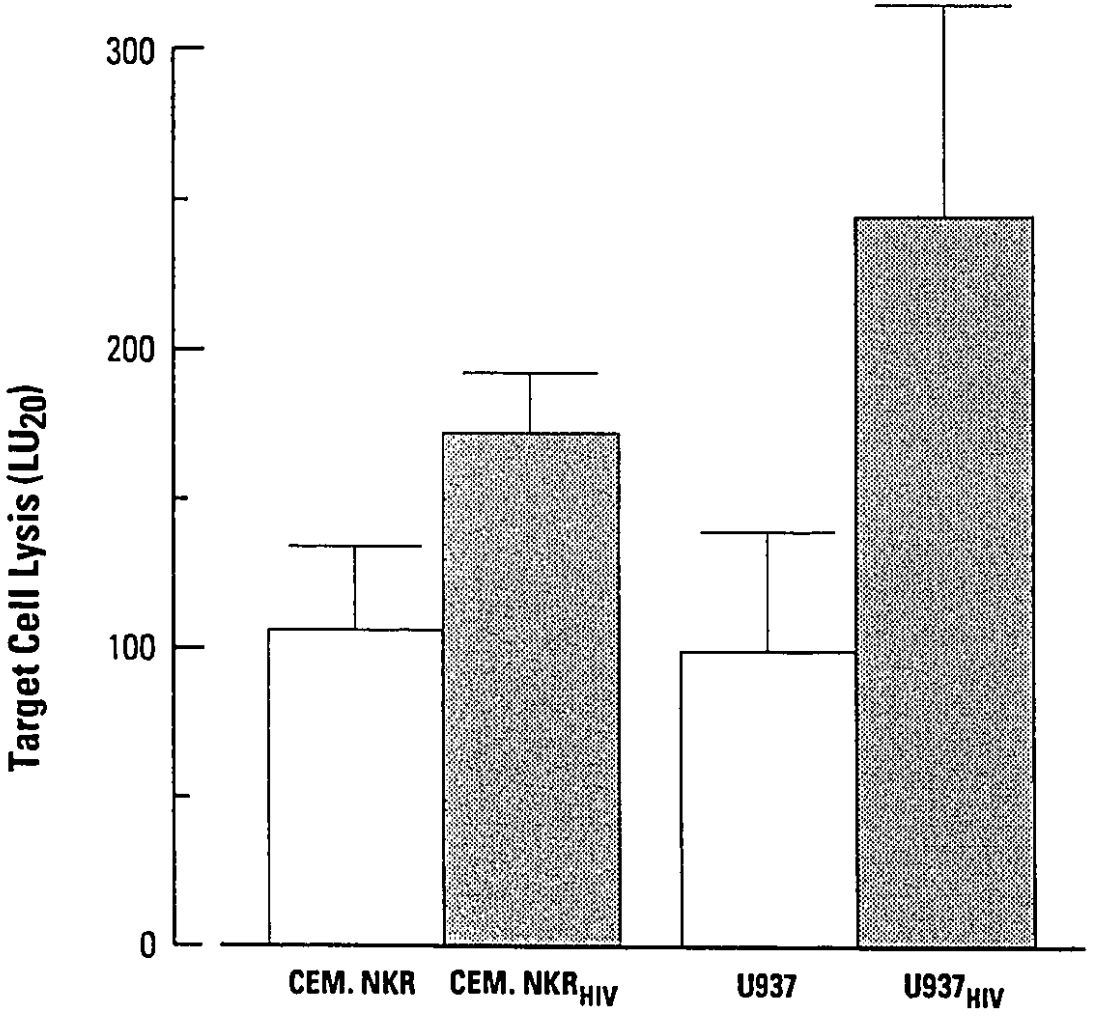
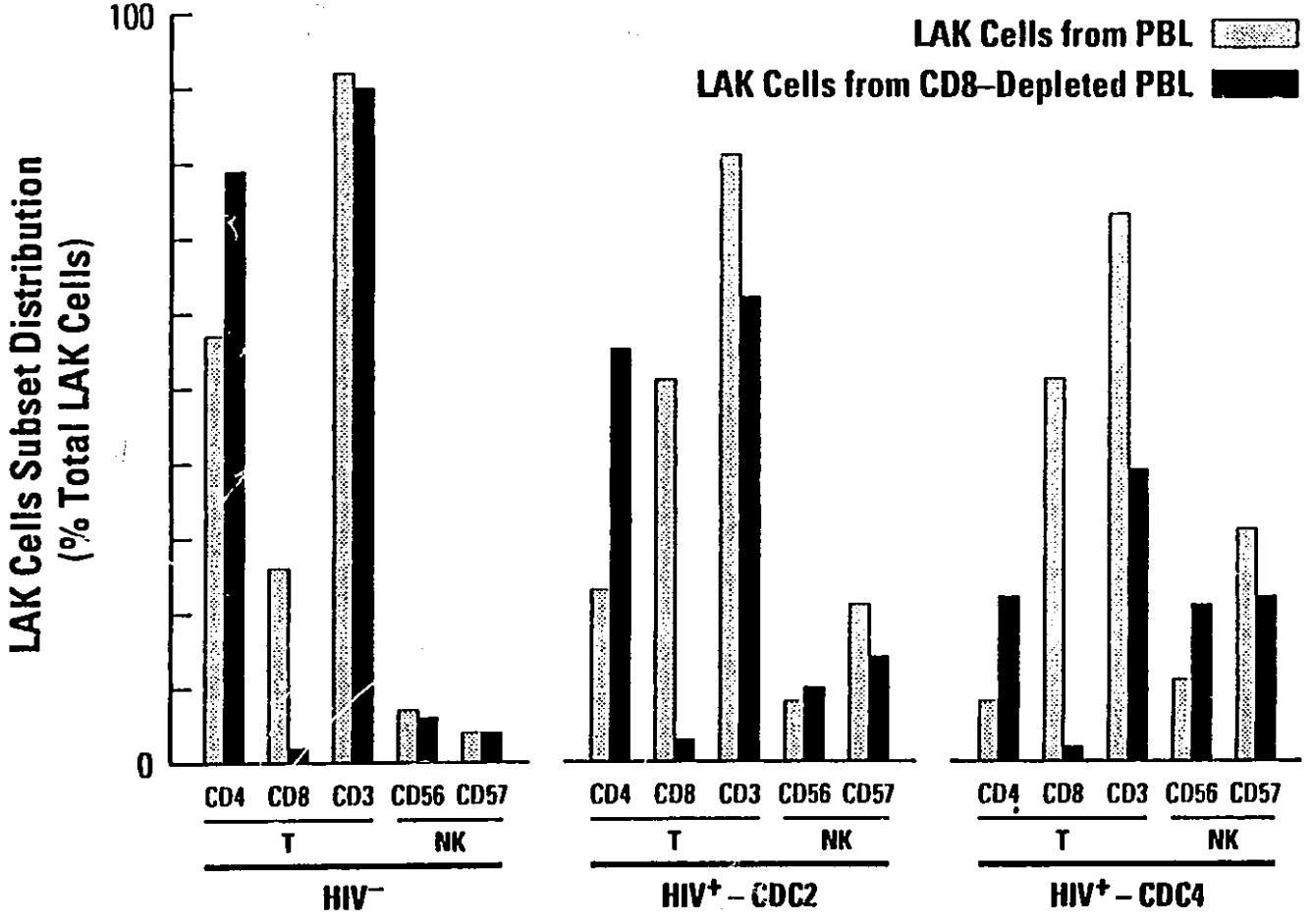


FIGURE 3

LAK cells derived from PBL of seronegative (n = 7), HIV-1 seropositive subgroup CDC2 (n = 5) and HIV-1 seropositive subgroup CDC4 (n = 5) were depleted of CD8 progenitors prior to LAK cell generation. Flow cytometric analysis of CD4, CD8, CD3, CD56, and CD57 lymphocyte subsets were monitored on LAK cells isolated from depleted and undepleted PBL.

FLOW CYTOMETRY
CD8+ PROGENITOR CELL DEPLETION



(F=8.44, df=13, p=.004). In both healthy controls and HIV-1 seropositive CDC2 asymptomatics, depletion of CD8 progenitor cells resulted in no significant enrichment of $CD56^+$ cells. In AIDS patients (subgroup CDC4CD), however, a significant enrichment of $CD56^+$ cells in LAK cells derived from CD8-depleted PBL was observed (p=.004 and .05 post-hoc Tukey test, CDC4CD vs CDC0 and CDC2, respectively).

Interestingly, CD57-expressing cells were not representative of $CD56^+$ NK cells. Two colour flow cytometric analysis indicated that 15±3%, 7±6%, and 6±1% of $CD57^+$ PBL co-expressed CD56 in CDC0, CDC2, and CDC4CD subgroups respectively (F=5.35, df=.007, CDC0>CDC2 and CDC4CD groups, p<.05, post-hoc Tukey tests). Corresponding proportions of $CD57^+$ PBL co-expressing CD8 were 37±8%, 62±5%, and 58±7%, in CDC0, CDC2, and CDC4CD groups, respectively. No significant changes were noted in the proportions of residual $CD57^+$ cells in LAK cells derived from undepleted vs CD8-depleted PBL in each group (Figure 3).

LAK cells isolated from these undepleted and CD8-depleted PBL were contrasted with regard to their efficacies in eliciting cytolysis of U-937 targets. Lysis of U-937 targets, when using undepleted LAK cells, was elevated in the CDC2 seropositive subgroup when compared to healthy controls and overt AIDS patients (Figure 4, F=5.14, df=14, p=.021, one-way ANOVA, p=.028 and .045 post-hoc Tukey comparisons of CDC2 with CDC0 and CDC4 groups, respectively). LAK cells derived from CD8-depleted progenitors showed marked declines in their relative effectiveness in eliciting U-937 target cell lysis in asymptomatic seropositives when compared to their undepleted counterparts (p=.029, paired t-test). Conversely, both seronegative controls and overt AIDS patients showed significant increases in the relative susceptibility of U-937 to lysis by LAK cells derived from CD8-depleted progenitors (Figure 4, p=.046 and p=.008, paired t-test undepleted vs CD8 progenitor-depleted LAK activities for CDC0 and CDC4CD subgroups, respectively). Susceptibility of U-937 targets to lysis by LAK cells from CD8-depleted PBL was significantly greater in overt AIDS patients than corresponding CD8-depleted

FIGURE 4

LAK activity of seronegative (n = 7), CDC2 (n = 5) and CDC4 (n = 5) individuals against U-937 and K562 targets using LAK cells or LAK cells derived from CD8-depleted PBL.

LAK CELL FUNCTION
CD8+ PROGENITOR CELL DEPLETION

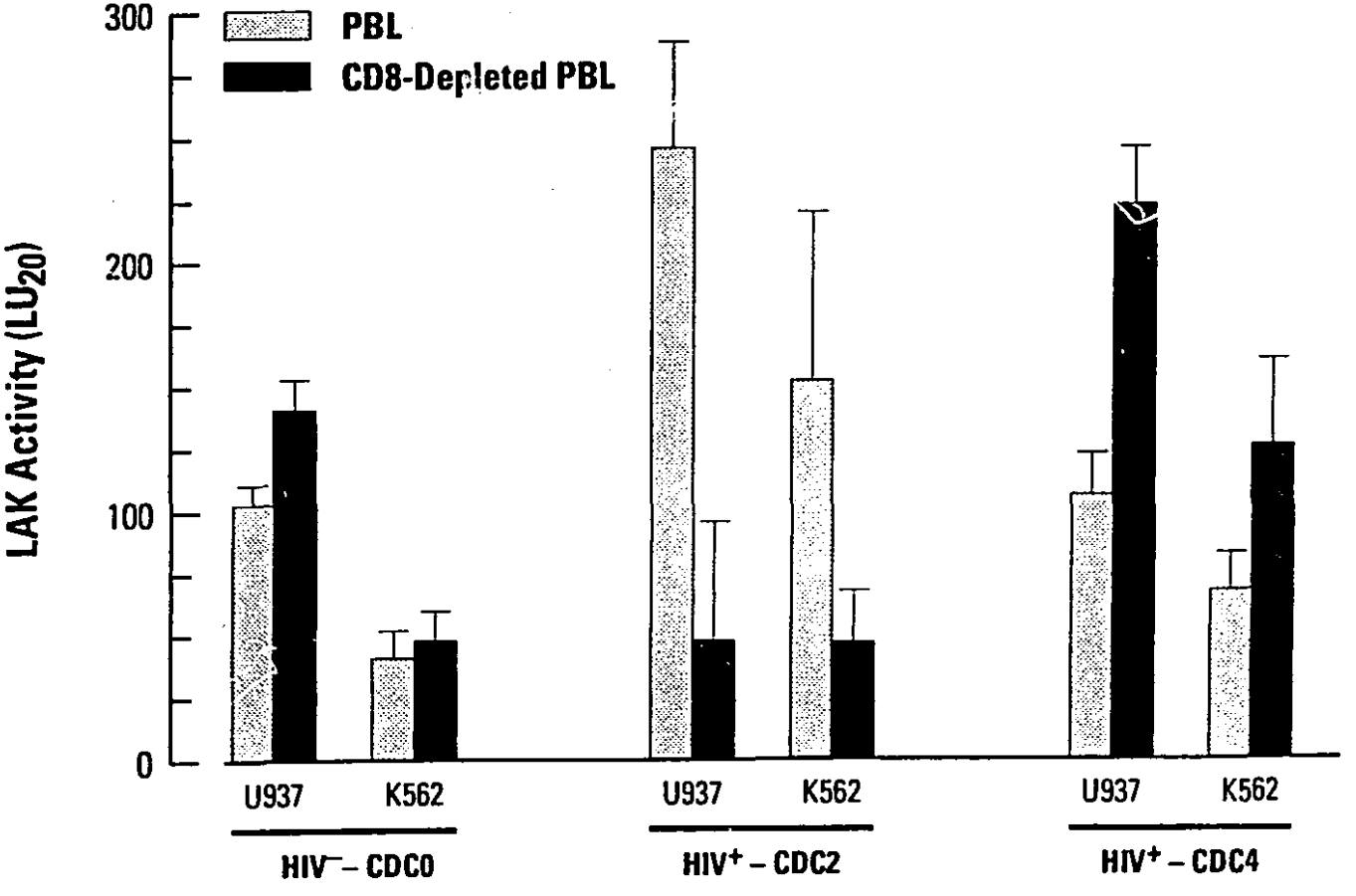
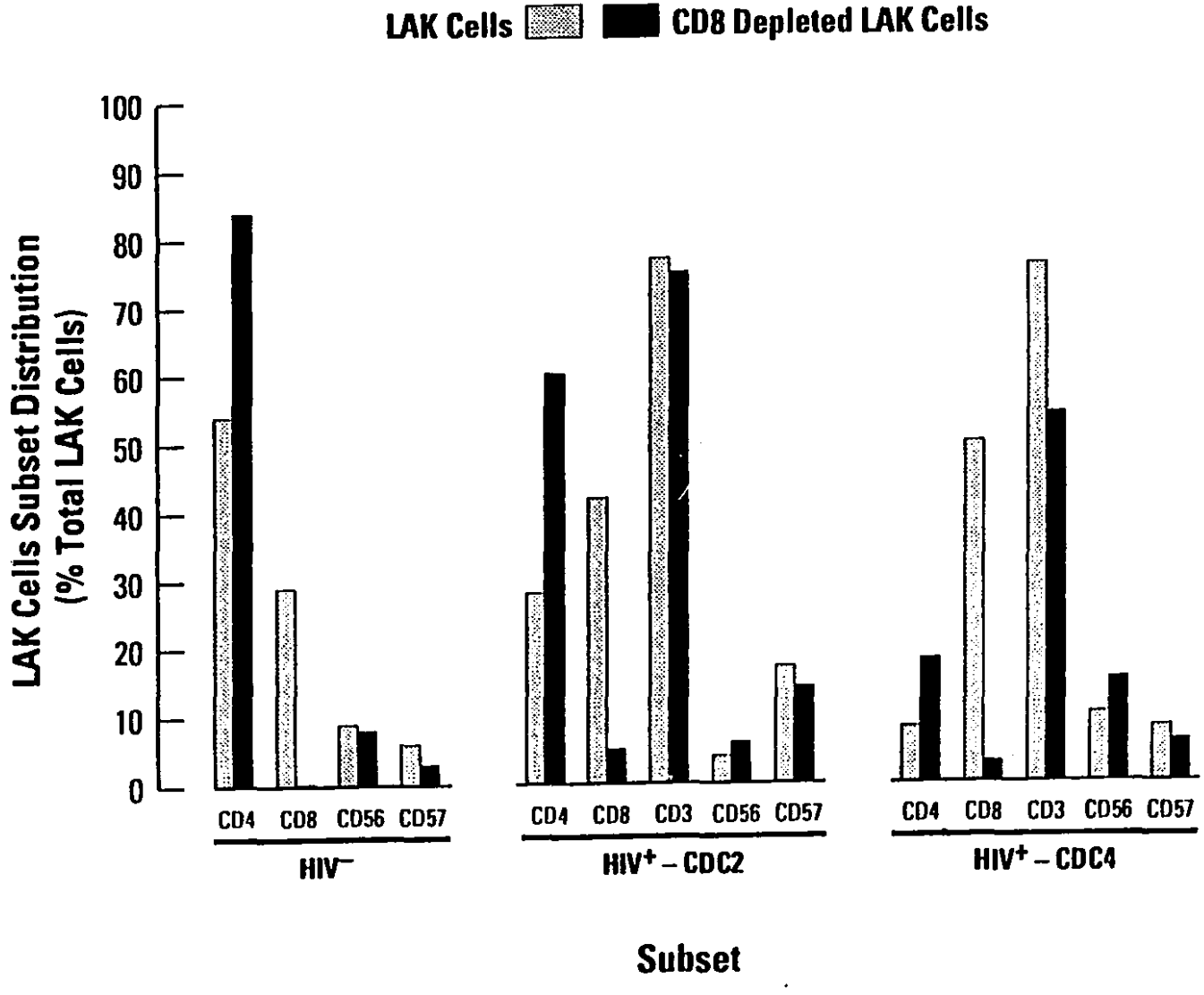


FIGURE 5

Flow cytometric profile of LAK cells from healthy control (n=3) HIV-1 CDC2 seropositive (n=7) and HIV-1 CDC4CD (n=6) seropositives before and after depletion of CD8-expressing effectors.

FLOW CYTOMETRY
CD8+ EFFECTOR CELL DEPLETION



LAK cells from HIV-1$^+$ CDC2 asymptomatics (Figure 4, F=4.81, p=.026, one-way ANOVA, p = .022, Tukey tests). This elevated cytolysis of U-937 targets in AIDS patients occurred concomitant with a three-fold enrichment of CD56-expressing lymphocytes.

It is important to note that similar patterns of cytolysis of K562 targets by undepleted vs CD8 progenitor-depleted LAK cells were observed. (Figure 4). Thus, CD8 progenitors were required to generate non-MHC restricted cytotoxic LAK cells in asymptomatic CDC2 seropositives. LAK cytotoxic function in seronegative controls was increased subsequent to CD8$^+$ progenitor cell depletion. In the absence of an inducible CD4 cell population, CD8 progenitor depletion of AIDS patient PBL selectively expanded CD56$^+$ cytotoxic LAK cells.

CD8$^+$ subset depletion of LAK effectors

LAK cells were depleted of CD8 subsets on the day of the cytolytic assay to monitor which subsets mediate LAK activity. Subset depletions resulted in variable non-specific losses of cells. Following depletion, cell counts were readjusted to identical E:T ratios and flow cytometric analysis was performed to evaluate subset distribution.

CD8 depletion of LAK cells totally depleted CD8 LAK effectors (Figure 5). In healthy controls and asymptomatic CDC2 seropositives, this resulted in a selective enrichment of the proportion of CD4 effectors (p=.008 and .001, paired t-test depleted vs undepleted CD4$^+$ LAK cells for CDC0 and CDC2 groups, respectively). In overt AIDS patients, the primary enriched population of effectors were apparently derived from CD3-expressing lymphocytes lacking both CD4 and CD8. No significant differences in the proportions of CD56-expressing effectors between groups were observed (Figure 5).

In CDC2 seropositive individuals, CD8 depletions significantly decreased LAK activity against both uninfected and HIV-1 infected U-937 targets (Figure 6, p=.02 and .009, paired t-tests using U-937 and HIV-1 infected U-937 targets, respectively). CD8 depletions resulted in no changes in LAK activity against both uninfected and HIV-1

FIGURE 6

Cell-mediated cytolysis of HIV-1 infected and uninfected U-937 targets by undepleted and $CD8^+$ effector-depleted LAK cells derived from seronegative controls (n=3), CDC2 (n=7) and CDC4CD seropositives (n=6).

LAK CELL FUNCTION
CD8+ EFFECTOR CELL DEPLETION

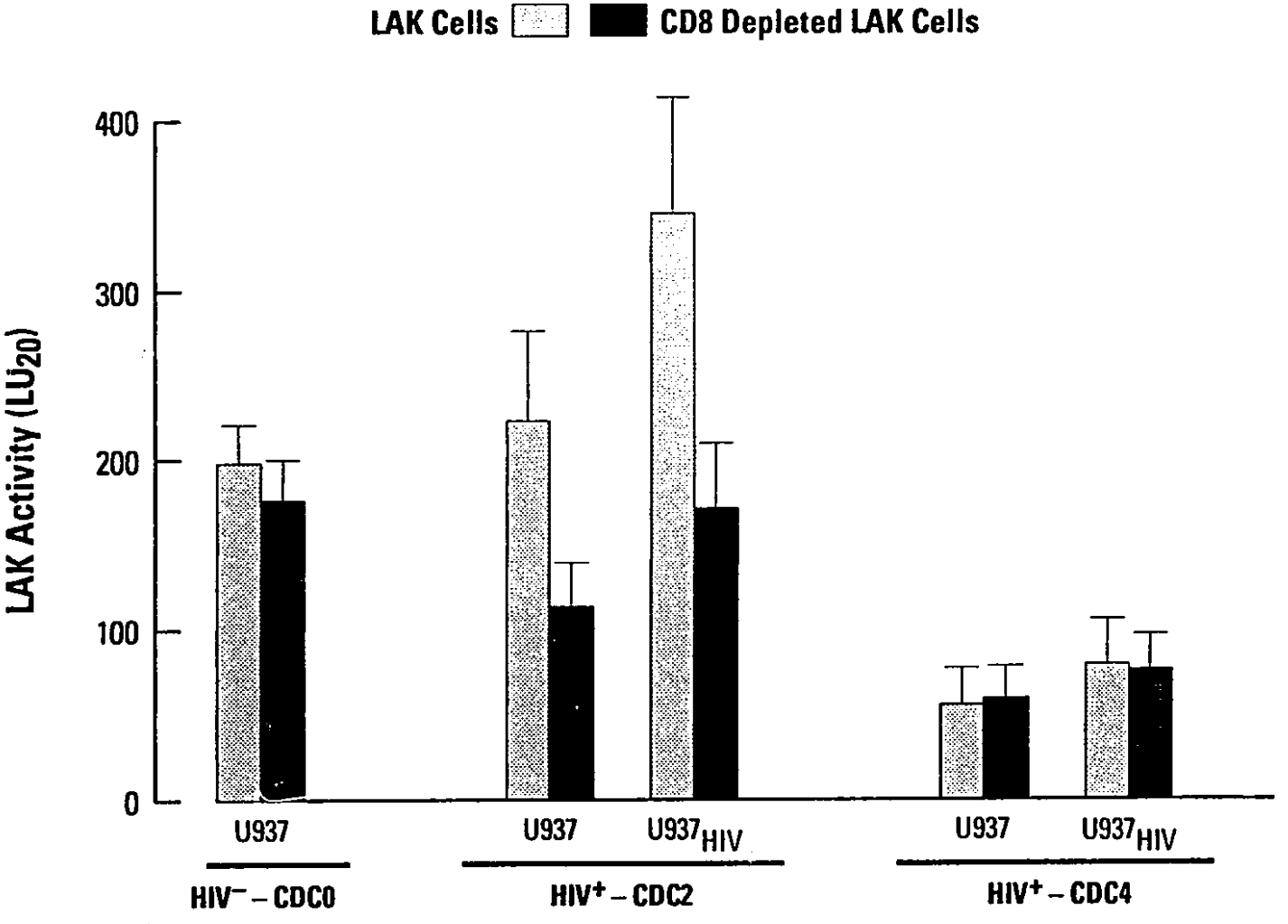
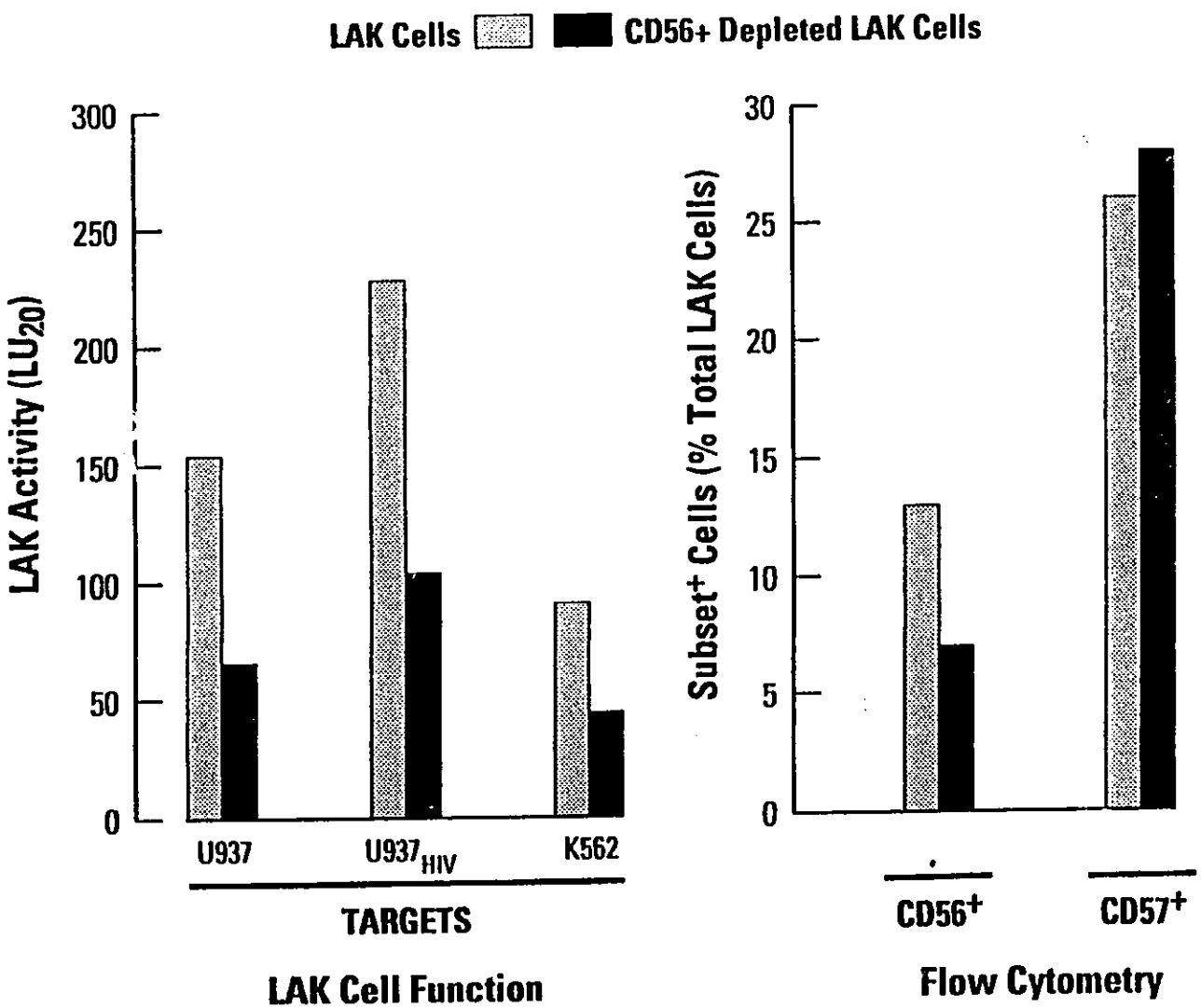


FIGURE 7

CD56 expression and LAK activity against HIV-1 infected and uninfected U-937 and K562 targets before and after CD56 depletion of LAK cells isolated from 10 seropositive individuals.

CD56+ EFFECTOR CELL DEPLETION



infected U-937 targets in seronegative controls and overt AIDS CDC4 patients (Figure 6). Despite total depletion of CD8 cells in all groups, significant lysis of all targets persisted.

$CD56^+$ and $CD57^+$ subset depletion of LAK cells

Previous studies in our laboratory indicated that CD56 depletion of PBL could effectively deplete out a significant proportion of cytotoxic LAK cells (50). To confirm that CD56 cells elicited cytotoxic LAK responses, LAK cells were depleted of CD56-expressing subsets prior to assay. As shown (Figure 7), flow cytometric analysis indicated that CD56 cells could be significantly decreased but not completely removed by our negative selection techniques ($p=.05$, paired t-tests). Using LAK cells from HIV-1 seropositive donors, the observed decreases in the proportion of NK subsets resulted in corresponding significant decreases in lysis of K562, uninfected and HIV-1 infected U-937 targets ($p<.05$ paired t-tests).

In two HIV-1 asymptomatic subjects, complement-mediated depletion of CD57 subsets at the progenitor and effector levels resulted in no change in cytolysis of uninfected and HIV-1 infected U-937 targets. Flow cytometric analysis showed complete depletion of CD57 subsets, no changes in CD56 and CD8 subsets, and increased proportions of CD4 subsets (unpublished results).

$CD4^+$ subset depletion of healthy control PBL

Since HIV-1 seropositive individuals show marked declines in CD4 subsets and corresponding increases in CD8 subsets, healthy control PBL were depleted of CD4 subsets to see whether such depletion could mimic the phenotypic and functional aberrations observed in individuals with AIDS. As observed in Table 1, CD4 depletions markedly enriched for CD8 subsets with modest but significant increases in CD56 subsets. No significant changes in LAK cell-mediated lysis of any of the tested targets were observed upon CD4 depletions.

TABLE I. Lymphocyte distribution and LAK cell function of healthy control LAK cells and corresponding LAK cells from CD4 depleted PBL

	LAK cells	LAK cells from CD4 depleted PBL
Subset (%)		
CD4	62 ± 3	2 ± 1*
CD8	26 ± 2	77 ± 4*
CD56	7 ± 1	9 ± 2*
CD57	6 ± 2	8 ± 2
Target cell lysis (LU_{20})		
U-937	133 ± 17	198 ± 48
$U\text{-}937_{HIV}$	145 ± 34	186 ± 11
K562	46 ± 10	74 ± 37

*$p<.05$, paired t-test

DISCUSSION

The ability of IL-2 to induce LAK cells that elicit potent anti-viral and anti-tumor responses generates interest as to their specific prognostic role *in vivo* and their potential usefulness in adoptive immunotherapy. In this study, induced LAK cells from seronegative and seropositive individuals were contrasted with regard to their effectiveness in eliciting cytotoxic responses to a panel of targets. Moreover, the progenitors and effectors of these responses were established. Our findings have indicated that novel LAK effectors mediate cytolysis in HIV-1 asymptomatic seropositive individuals. Asymptomatic CDC2 seropositives have LAK progenitors and effectors that are depleted subsequent to CD8 subset removal. Secondly, asymptomatics showed marked increases in LAK activity against HIV-1 infected targets relative to their uninfected counterparts (50). The failure to observe HIV-1 directed LAK responses or CD8-mediated cytotoxicity in healthy controls, suggests that primed immune responses in seropositive and seronegative individuals are quite distinct.

The inability to elicit CD8-mediated cytotoxic responses and limited enhanced responses to HIV-1 infected targets in seropositive individuals with overt AIDS is intriguing. Studies reported herein and elsewhere (9,23,50) indicate an overall anergy with regard to both NK and LAK cell-mediated immune responses in overt AIDS subjects. We have, however, observed an interesting pattern in overt AIDS patients following CD8 subset depletion at the progenitor and effector cell level. Depletion of CD8 progenitors could effectively increase the proportion of $CD56^+$ LAK cells and their function. Depletion of CD8 effectors, however, resulted in no corresponding enrichment in LAK activity. Removal of $CD8^+$ effectors resulted in an enrichment of $CD3^+CD4^-CD8^-$ cell subsets. Recent studies indicate that $CD3^+CD4^-CD8^-$ cells represent small proportions of circulating lymphocytes in healthy controls and bear either $\alpha\beta$ or $\gamma\delta$ T-cell receptors (31-36). It is important to note that such subsets have been expanded in disease states and have been characterized by their failure or reduced ability to elicit cytolytic responses (33,34,36).

Such a novel T lymphocyte population has been observed in a HIV-1 seropositive individual (32). Our findings, in agreement with other studies (1,5,37), indicate that expanded $CD57^+CD8^+$, $CD57^+CD8^-$, $CD57^+CD56^-$ subsets in seropositive individuals are not effective killers.

Other studies have indicated that defective NK cytolytic machineries post-target cell conjugation were observed in seropositive individuals (7,8). Others have reported that NK cells can be susceptible to HIV-1 infection despite their failure to express CD4 mRNA and surface CD4 proteins (38-40). Our findings confirm other studies that indicate overall reductions in absolute levels of circulating CD56-expressing subsets in PBL of HIV-1 seropositive individuals (5,10,11). This study indicates that NK cells mediate a significant proportion of LAK activity in asymptomatic seropositive individuals and the majority of LAK activity in overt AIDS patients. We have found no significant differences in the proportion of inducible CD56-expressing LAK cells from undepleted PBL in seronegative and seropositive populations that could account for all the reduction in LAK activity in overt AIDS patients (9,50).

CD8-mediated and HIV-1 enhanced cytolytic responses were non-MHC restricted. CD8 depletion of cytolytic responses was observed in all tested asymptomatic individuals with simultaneous depletion in the cytolysis of K562, a target cell line that lacks class 1 and class 2 MHC (1,2). Moreover, elevated lysis of HIV-1 infected CEM.NKR vs CEM.NKR was observed in asymptomatic seropositive individuals. CEM.NKR is a target that is NK-insensitive, alluding to an induced non-MHC restricted LAK cytolytic response.

Other laboratories have demonstrated that NK cells from healthy seronegative individuals, seronegative homosexuals, and seropositive individuals can lyse HIV-1 infected targets more effectively than corresponding uninfected targets (40-46). Whether elevated non-MHC restricted, HIV-1 directed lysis was observed depended on the target cell line, the HIV-1 cell strain, and the presence of NK with or without accessory $HLA\text{-}DR^+$ cells (38,41,50). Our study, however, confirming our previous findings would

indicate that only LAK cells from seropositive individuals show primed responses to HIV-1 directed targets (9,23,50). These combined observations strongly suggest that multiple cytolytic effector mechanisms and pathways mediate NK and LAK cell-mediated cytolysis of HIV-1 infected targets. In HIV-1 seropositive individuals, concomitant with CD4 subset depletion, dynamic changes in cytotoxic lymphocyte profiles and their functions occur as disease progresses.

A number of distinct cell-mediated cytotoxic mechanisms have been implicated in HIV-1 viral immunosurveillance. These include MHC restricted cytotoxic function mediated by cytotoxic $CD8^+$ T cells (10,47,48) as well as non-MHC restricted ADCC (6,45,46) and NK activity (5) mediated by NK cells. Findings reported herein and elsewhere indicate that inducible non-MHC restricted LAK activity derived from NK and T cell progenitors can elicit anti-HIV responses (9,23,49,50). The demonstration that novel LAK effectors can be induced in asymptomatic individuals but not controls or AIDS patients suggests that non-MHC restricted responses may be primed in seropositive individuals and may play an important role both in HIV-1 viral immunosurveillance and the control of opportunistic infection.

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CHAPTER 6

DISCUSSION

The ability of IL-2 to induce cytotoxic LAK cells that elicit potent anti-viral and anti-tumor responses, generates interest as to their participatory role *in vivo* and their usefulness in immunotherapy. Our study has focused on detailing LAK cell responses in HIV-seropositive individuals and healthy controls. LAK cells have been operationally defined as IL-2 induced lymphocytes mediating non-MHC-restricted and antigen-nonspecific cytolysis against a panel of virally-infected and neoplastic heterologous target cells (Trinchieri, 1989; Ortaldo and Longo, 1988). Due to the absence of MHC restriction, LAK cell responses represent an ideal system for in vitro analysis of inducible immune responses.

Seropositive subjects were stratified into two major groups: the CDC2 group, included individuals in the earlier phases of HIV-infection, with no clinically apparent opportunistic diseases; and the CDC4 group that consisted of the overt AIDS patients that suffered from at least two AIDS-associated opportunistic infections and/or cancers. Overall lymphocytic numbers, relative LAK cell recoveries and LAK cell cytotoxicities were monitored in both groups and contrasted to seronegative (CDC0) individuals. The contribution of regulatory and cytotoxic lymphocyte subpopulations in evoking LAK cell responses was subsequently detailed.

Our results confirmed the selective depletion of $CD4^+$ PBLs with advancing disease, and the concomitant diminution of the total circulating lymphocytic cell numbers. We observed that the relative ability of PBLs to generate LAK cells became impaired at early stages of HIV-infection, prior to the onset of clinical symptoms. To our surprise the relative proportion of $CD56^+$ cells remained unchanged in both CDC2 and CDC4 seropositive groups relative to seronegative controls. This confirms other studies (Vuillier *et al.*, 1988; Landay *et al.*, 1990) that indicate an overall reduction in NK cell numbers (absolute $CD56^+$ cell numbers per ml of blood) with disease onset. Of importance, our

studies have demonstrated that $CD57^+$ subsets in seropositive individuals represent a non-NK cell population where more than 50% of CD57 subsets coexpress CD8 cell and less than 10% express CD56. Studies in the literature (Lewis *et al.*, 1985; Gupta, 1986; Voth *et al.*, 1988,) that have indicated unchanged or augmented NK cell numbers upon HIV-infection, have monitored CD57 cell numbers. Nevertheless, the constant proportion of $CD56^+$ cell numbers in all groups allowed us to evaluate the relative functional changes in NK and LAK cell responses in AIDS.

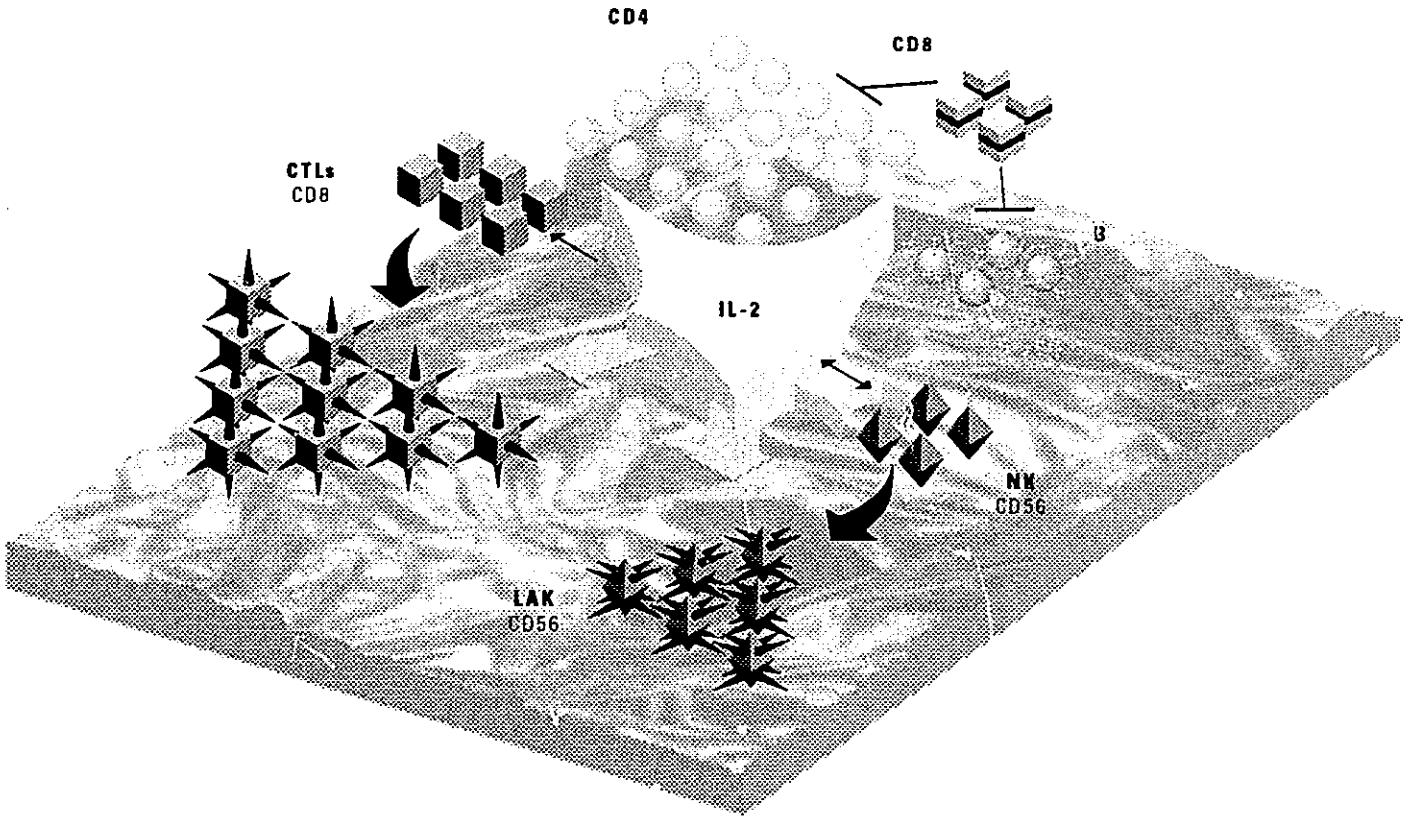
Since we started our study there were two other reports on LAK cell activity in HIV infected patients. In one study, LAK cell activity appeared to be at control levels for both ARC and AIDS patients (Chin *et al.*, 1990) however, the sample size was small for both groups (n=9). In the other study, NK and LAK activity was decreased in AIDS (n=9), ARC (n=10) and HIV-seropositive asymptomatic (n=12) patients in comparison to healthy individuals (Cauda *et al.*, 1990). Up to date, to our knowledge we are the only group that has extensively studied inducible LAK cell responses in HIV-1 seropositive individuals, employing large numbers of patients and simultaneously assaying them against series of different targets. Our studies indicated that inducible LAK cell function remained effective on early disease and became compromised only at late stages of HIV infection.

Much to our surprise, we have demonstrated an enhanced ability of HIV-1 seropositive individuals to lyse HIV-1 infected targets. This was shown to occur against targets such as U-937 that maintain chronic HIV-1 infection, as well as acutely HIV-1 infected CEM.NKR. The observation of enhanced cytolysis of the HIV-infected U-937 subclones strongly indicated that this was an HIV-1 directed response rather than a non-specific target cell activation.

Our studies then focused on establishing the cellular basis of the observed changes in LAK cytolytic responses in seropositive individuals. As illustrated in Figure.1, cytotoxic

FIGURE 1

The immune system and the central role of IL-2


CD4
CD8
CTLs
CD8
B
IL-2
NK
CD56
LAK
CD56

effectors include $CD8^+$ CTLs, activated $CD8^+$ CTLs, NK cells and activated $CD56^+$ LAK cells. In fact there is some evidence that $CD4^+$ cells may evoke cytotoxicity (Yasukawa *et al.*, 1991). $CD4^+$ cells are involved in antigen presentation and IL-2 inducibility (Bierer *et al.*, 1989; Parnes, 1989).

Target cell recognition in CTLs, NK and LAK cells is different. T-cells are both antigen specific and MHC-restricted. They recognize antigen only within the context of class I or class II MHC molecules (Schwartz, 1985; Rothbart and Gefter, 1991). In direct contrast, NK and LAK cells are not restricted by the MHC, nor are they antigen specific (Trinchieri, 1989; Ortaldo and Longo, 1990). In fact NK and LAK cells show broad target reactivity. In addition ADCC responses can be mediated by NK cells. ADCC responses by LAK cells have not been established to date, and in general while LAK cells express CD56, they often don't express CD16, the Fc receptor for antibody (Lanier *et al.*, 1986b). Thus, all aspects of the immune network may be fundamentally involved in our observed LAK cell responses.

In order to identify the phenotype of the effector cells mediating our observed LAK activity, we performed series of depletion studies that involved negative immunomagnetic selection systems. We have observed that negative selection systems are more efficient and reproducible than positive selection approaches. PBLs were depleted prior to LAK cell generation, to study the role of distinct subsets in generating LAK cells. Alternatively, LAK cells were depleted just prior to the assay, in order to determine the phenotype of cytotoxic LAK cells.

CD56-expressing lymphocytes participated as progenitors as well as effectors of LAK cell activity in healthy individuals and all HIV-seropositive groups. Depletion of $CD56^+$ PBLs as well as $CD56^+$ LAK cells resulted in marked reductions of LAK activity. Total abrogation of LAK activity was never achieved. CD56 is a dim marker, weakly expressed on the majority of NK cells (Lanier *et al.*, 1986b) and therefore total $CD56^+$ cell depletion was not possible. Of great importance, we established, that CD56-expressing

LAK cells mediated non-MHC-restricted cytolysis of HIV-infected targets in seropositive individuals. In addition, in as yet unpublished results, we have demonstrated marked enrichment in LAK cell responses by adherent LAK cells, that show a 5-10 fold enrichment of CD56-expressing cells.

The role of NK and inducible LAK cells in the immunopathogenesis of AIDS is not clear. Several investigators have suggested that NK cells may lyse HIV-infected CD4 cells or gp120 bound bystander CD4 cells and by this way contributing to the depletion of $CD4^+$ cells (Rosenberg and Fauci, 1989). In vitro studies indicated that NK cells ($CD5^+$ $CD57^+$) could lyse gp120 coated $CD4^+$ cells (Tyler et al., 1989; Weinhold *et al.*, 1989). This is a very interesting issue that merits further investigation.

$CD8^+$ cells mediate both cytotoxic and suppressor cell functions (Asherson *et al.*, 1986; Sercarch and Krzych 1991). $CD8^+$ lymphocytes are well established as cells capable of suppressor activity. In addition novel $CD8^+CD57^+$ T cells, expanded in AIDS patients, have been shown to suppress NK and LAK cell activity via a lectin-binding soluble factor (Sadat-Sowti *et al.*, 1991). To assess the role of $CD8^+$ lymphocytes in our observed LAK cell responses we performed series of depletions both at the progenitor and effector cell level. In healthy controls, depletion of CD8-expressing PBLs enhanced the generation of cytotoxic LAK cells. Depletion of $CD8^+$ PBLs resulted in an enriched LAK cytolysis of K562 and U-937 targets, with no concomitant $CD56^+$ LAK cell enrichment. In contrast, depletion of $CD8^+$ cells following LAK cell generation did not affect cytotoxic LAK cell responses. This suggests that CD8 lymphocytes suppress the proliferation of cytotoxic LAK cells but not their cytolytic capacity.

In overt CDC4 AIDS patients, similar to healthy controls, CD8 depletion of PBLs markedly increased cytotoxicity against K562 and U-937 targets. However since in AIDS patients overall CD8-expressing lymphocytes represent 60-70% of PBLs, depletion of CD8 cells enriched the relative proportion of $CD56^+$ cells by approximately 3-fold. We cannot exclude therefore, that the enhanced cytolysis observed upon CD8 effector cell depletion

was due to an increase in the proportion of cytolytic CD56-expressing LAK cells. This observation, raises intriguing possibilities, in that, depletion of CD8 cells may in fact allow for the selective proliferation of CD56-expressing cells. The consequences of this observation with adoptive immunotherapeutic strategies are important.

Depletion of CD8 cells following the generation of LAK cells in overt AIDS patients, generated another important finding. Unlike progenitor CD8 depletions, there was no selective enrichment of CD56-expressing cells nor enhancement of LAK cell responses. However, there was marked selective enrichment of $CD3^+$ $CD4^-$ $CD8^-$ cells rather than CD56-expressing LAK cells. Such cells have been described in healthy controls as a minor proportion of circulating lymphocytes that may express $\alpha\beta$ or $\gamma\delta$ receptors. Expansion of these cells has been associated with other immunodeficient disease states (Carbonari *et al.*, 1990). These cells have been reported to lack cytolytic activity (Haas *et al.*, 1990). Others have also observed these cells in HIV-seropositive populations (De Paoli *et al.*, 1991). We have shown that these cells do not contribute to our LAK cell responses. A selective enrichment of $CD3^+CD4^-CD8^-$ cells as a result of $CD8^+$ effector cell depletion did not correlate with an increase in LAK cytolysis. Taken together, CD8 depletions in AIDS patients, have indicated that $CD8^+$ cells may inhibit proliferation of LAK cells, however they do not appear to mediate cytotoxic LAK cell responses.

In contrast, to our great surprise, CD8 depletions in asymptomatic HIV-seropositive individuals resulted in significant decreases of LAK cell mediated cytotoxicity. These decreases were observed against both K562 and U937 targets, as a result of both progenitor and effector $CD8^+$ cell depletions. In addition since $CD56^+$ PBL and LAK cell percentages did not change, we could not directly attribute this decrease to a depletion of the NK cell pool. Cytotoxic T-cell responses to HIV-1 targets have been observed in HIV-seropositive patients and tend to diminish along with disease progression (Walker and Plata, 1990; Pantaleo *et al.*, 1990). These responses however were directed against distinct HIV viral epitopes and were recognized only within the context of self MHC (Walker and

Plata, 1990). The CD8 cells mediating LAK activity in our system do not represent classical CTLs. CD8-mediated cytolysis was not MHC-restricted since reduction of cytotoxicity upon CD8 depletion was observed against targets including K562 that lack class I or II antigen expression. In addition, it was not HIV-directed because, both K562 and U937 were not infected by HIV and therefore, these CD8-expressing IL-2 induced cells could not have recognised an HIV-associated viral antigen expressed on the surface of the target cells. Moreover, reductions in cytolysis were observed as a result of both progenitor and effector $CD8^+$ cell depletions, employing nine different patients that were not HLA matched.

Administration of IL-2 *in vivo*, has been shown to activate a highly cytotoxic expressing population called tumor infiltrating lymphocytes (TILs). These $CD8^+$ or $CD4^+$ TILs have been shown to be MHC-restricted and tumor specific (Melief *et al.*, 1992). In general, acquisition of non-MHC-restricted LAK activity by activated CTLs is associated with loss of antigen specificity and novel expression of the CD56 antigen on T cells. $CD8^{+dim}CD56^+$ mediating cytotoxic activity have been reported (Perussia *et al.*, 1983a; Robertson and Ritz 1990). We therefore do not know whether the cytotoxic CD8 cells induced in HIV-seropositive asymptomatic individuals, coexpressed the CD56 antigen. In our cytometric analysis, using an EPICs II analyzer we were unable to detect dim $CD8^+$ $CD56^+$ cells and therefore evaluate for their specific depletion. The failure to observe similar responses in healthy individuals and overt AIDS patients suggests that this $CD8^+$ cell population represents a novel expanded cytotoxic subset, present only in HIV-seropositive asymptomatic individuals.

Preferential lysis of HIV-infected U-937 and CEM.NKR targets in comparison to uninfected U-937 and CEM.NKR cells can be related with the HIV-specific CTL responses. One hypothesis might be that highly activated HIV-specific T-LAK cells, may recognize HIV antigen expression on the surface of infected targets within the context of foreign MHC, either by mistaken the foreign class I antigen as self or, just by binding to

the HIV-associated antigen with lower affinity and triggering cytolysis via a non-MHC restricted fashion. Similar mechanism have been proposed to explain alloreactive CTLs present in graft rejection (Lechler *et al.*,1990). Crossreactivity among different MHC molecules and recognition of peptide alone can be sufficient for triggering CTL-mediated cytolysis. In addition alloreactive T-cell clones have been shown to also mediate self-MHC-restricted and antigen specific cytolysis (Lechler *et al.*, 1990).

$CD56^+$ NK cells do not express the CD4 antigen associated with HIV-viral entry and infection. Although several investigators have *in vitro* infected cultured NK cell lines, in vivo infection of NK cells has never been observed. It was possible therefore, that the reductions observed in LAK activity of AIDS patients were due to severe reductions of $CD4^+$ T-helper cells or the concomitant proportional increases of CD8-expressing lymphocytes. Our experiments however, albeit in healthy individuals, gave no evidence to support this theory, since *in vitro* depletion of $CD4^+$ PBLs in healthy controls had no effect upon the subsequent generation of cytotoxic LAK cells.

We were then left with an intriguing problem. On an equal or somewhat elevated relative number of $CD56^+$ cells in overt AIDS patients, there was significant reduction in cytolysis relative to asymptomatic seropositives and healthy controls. Even in CDC2 asymptomatic group, if one considers the participation of $CD8^+$ cells in LAK cell responses, then we have a markedly diminished LAK activity per unit $CD56^+$ cell. Therefore $CD56^+$ cells in seropositive individuals are defective in their lytic capacity.

We have shown that this is not the indirect result of CD4 cell pool depletion; nor does it seem to be the result of direct *in-vivo* HIV infection of NK cells. Single cell-binding assays have indicated that although in lymphocytes derived from AIDS patients effector - target cell conjugation is normal, subsequent target cell cytolysis is impaired (Katzman and Lederman, 1986; Katz *et al.*, 1987). In a more mechanistic approach, defective tubulin polarization of NK cells at a post binding level, has also been observed in LGLs of AIDS patients (Sirianni *et al.*, 1988). Alternatively HIV-1 may inhibit LAK cell responses. A

synthetic gp41 peptide has been shown to *in vitro* inhibit NK and LAK cell activity (Cauda *et al.*, 1988,1990). Suppression of NK and LAK cell responses mediated by soluble factors secreted by $CD8^+CD57^+$ lymphocytes in AIDS patients has also been reported (Sadat-Sowti *et al.*, 1991). Decreased NK activity as a result of gp120 binding to the VIP receptor present on the surface of NK cells, has also been a postulated mechanism for decreased NK responses (Sirianni *et al.*, 1990).

Although NK cell suppression may not be the direct result of NK HIV-infection, infection of accessory cells may inhibit NK and LAK cell function. Indeed dendritic cells are susceptible to HIV-infection, and their function has been shown to be suppressed in HIV-seropositive individuals (Macatonia *et al.*, 1989). HLA-DR cells have been shown by several investigators to mediate necessary accessory functions for induction of NK activity (Bandyopadhyay *et al.*, 1990). Such cells may also be required for inducible LAK cell responses.

Alternatively, chronic HIV-infection and subsequent changes in immune cell homeostasis, may result in the propagation of immature NK cell populations in seropositive individuals with defective cytotoxic abilities. In our laboratory, we are studying HIV-associated deficiencies in the induction of adhesion molecules of NK cells during IL-2 activation (unpublished data). We are currently working with adherent LAK cells from HIV-seropositive patients and we have observed marked reductions in the number of adherent LAK cells, as well as their individual cytotoxic capacity. In addition the ability of LAK cells to secrete lymphokines and cytotoxic factors awaits further investigation. Such decreases have been observed in NK cells of HIV-1 seropositive individuals (Bonavida *et al.*, 1986).

The continuous non-specific stimulation of the immune system in AIDS patients may also result in overstimulation of NK or LAK cells and their subsequent inactivation or anergy. This has also been a proposed mechanism for B-cell and T-cell deficiencies

observed in AIDS patients (Rosenberg and Fauci 1989, Amadori and Chieco-Bianchi 1990).

The mechanism by which HIV-infection suppresses NK and LAK activities is not clear up to date. However, our results have established that HIV-infection results in decreased LAK cell activity, and have provided useful insight into possible scenarios that merit further investigation.

HIV infection results directly or indirectly in profound changes upon all cells of the immune system. It is intriguing that despite massive losses of 60-70% of circulating $CD4^+$ cells, individuals can remain symptom-free. Clearly establishing which cytotoxic responses maintain immunocompetence is crucial.

In our study we have provided a thorough analysis of the effects of HIV-infection upon the natural immune system and more specifically the IL-2 inducible LAK cell responses. We have shown evidence that this distinct arm of the immune network becomes severely compromised only at late stages of AIDS. However, NK and inducible LAK cells are perhaps the most readily amenable cells for immunomodulation. Inducible LAK cells of NK phenotype do not express HIV, are readily grown in the laboratory and have been already exploited in cancer immunotherapy. It will be challenging therefore, to investigate their potential use in AIDS management.

CONTRIBUTION TO KNOWLEDGE[1]

Our study represents to date the only thorough investigation on IL-2 inducible LAK cell responses in HIV-1 seropositive individuals. We have established that HIV-infection results in dramatic changes upon LAK cell number, cytotoxic function, effector cell phenotype and target cell repertoire of LAK activity.

We have shown that lymphocytes of HIV-seropositive individuals exhibit a decreased ability to generate LAK cells. In addition, LAK cell cytolysis in overt AIDS patients was demonstrated to be decreased relative to HIV-seropositive asymptomatics and healthy control subjects. Of interest we have also exhibited HIV-directed LAK cell responses in HIV-seropositive asymptomatic patients.

Employing exponential regression as well as flow cytometric analysis we have pointed out that CD56-expressing cells in CDC2 and CDC4 seropositive individuals, exhibit a decreased cytolytic ability on a per cell level.

The phenotype of the progenitors as well as effectors of LAK activity have been the object of intense studies as well as debates. We have shown that under our experimental conditions, CD56-expressing cells are by and large the progenitors as well as the mediators of LAK cell activity in all seropositive and seronegative groups. CD8-expressing lymphocytes play an inhibitory role on the generation of LAK cells in both healthy controls and overt AIDS patients. To our surprise, we have brought into light the recruitment of a novel CD8-expressing cell population in HIV-seropositive asymptomatics, that mediates non-MHC-restricted and antigen non-specific cytolysis

This study has described distinct differencies in overall cytotoxic function and the cellular basis of these responses between asymptomatic and overt AIDS groups. In addition interesting avenues that merit further investigation have been proposed.

[1]This section is a mandatory requirement of Ph. D. thesis submitted to the Faculty of Graduate Studies and Research, McGill University Montreal.

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