THE RELEVANCE OF APOPTOSIS IN THE PATHOGENESIS OF HUMAN IMMUNODEFICIENCY VIRUS-1 DISEASE

by

MARK FREDRIC COTTON

M.B.CH.B., M.Med(Paed), FCPaed(SA), DTM&H, DCH(SA).

Dissertation presented in fulfillment of the requirements for the degree of Doctor of Philosophy (Medical Microbiology) at the University of Stellenbosch.

PROMOTER: PROF. P.J.D. BOUIC

DECEMBER 2004

DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and has not previously, in its entirety or in part, been submitted at any university for a degree.

Signature:

Date:

SUMMARY

A simple and rapid scatter-based flow cytometric assay was developed to detect apoptosis in CD4+ and CD8+ T cells from a mixed population of cells. The assay was suitable for children.

Apoptotic PBMCs were confirmed by morphologic assessment in clinical samples *ex vivo* and after overnight culture. The scatter-based assay was validated in a number of ways. Firstly, PBMCs were irradiated with 500 rads and cultured overnight to induce apoptosis. Thereafter, PBMCs were labeled with a CD4 MAb. CD4+ cells were sorted into apoptotic and viable populations by scatter characteristics (diminished forward and increased side scatter). Morphology was assessed by fluorescence microscopy. The majority of cells with apoptotic scatter characteristics had apoptotic morphology (chromatin condensation) (80.6%). Ninety-two percent of cells from the viable region had normal morphology. CD4+ T cell apoptosis measured by scatter was then correlated with the TdT assay for DNA fragmentation. Lastly, CD4+ T cell apoptosis by scatter and annexin V uptake were also shown to correlate. In the latter experiments, PBMC morphology and cell death by trypan blue uptake were studied simultaneously and confirmed the two flow cytometric assays.

Apoptosis of CD4+ and CD8+ T cells has been shown in PBMCs from HIV-infected adults analyzed after overnight culture. Since cell death may be an artifact of *in vitro* culture, and because there is little information on apoptosis in paediatric HIV disease, I undertook a cross-sectional analysis in PBMCs analyzed immediately *ex vivo* from HIV-infected children and adults. Patients were studied in Denver, CO, USA. PBMCs from 21

children, 4 adolescents and 9 adults and seronegative age-matched controls were stained for CD4 and CD8 surface markers. Apoptotic cells were detected in a newly characterized flow cytometric assay by diminished forward and increased side scatter.

For the scatter assay, PBMCs had been labeled initially by an indirect method involving an intermediary incubation in the presence of biotinylated MAbs at 37°C for 30 minutes prior to incubating with streptavidin-FITC at 4°C for 20 minutes. Thereafter, the intermediary incubation step was removed and PBMCs were incubated with PE-conjugated CD4+ and CD8+ MAbs. Both CD4+ and CD8+ T cell apoptosis appeared enhanced in the indirect method. The significant differences were abolished after subtraction of data from simultaneously studied time-matched controls.

CD4+ and CD8+ T cell apoptosis were significantly higher in HIV-infected study subjects than in simultaneously studied seronegative controls. PBMCs were assayed immediately *ex vivo* and after overnight culture after stimulation by an anti-TCR MAb as well as spontaneously. There was a direct correlation between CD4+ and CD8+ T cell apoptosis and CD4+ T cell depletion. A significant correlation was also shown between apoptosis immediately *ex vivo* and after overnight culture.

I then studied apoptosis in a South African population comprising 18 symptomatic children and 4 seroreverters. CD4+ and CD8+ T cell apoptosis were significantly higher in symptomatic HIV-1-infected children than in seroreverters and seronegative controls. CD4+ T cell apoptosis correlated with depletion of CD4+ T cell percentage in symptomatic HIV-1-infected children. I also noted elevated CD4+ T cell apoptosis in patients recovering from intercurrent disease in comparison to those who were either acutely ill or relatively asymptomatic outpatient attendees.

Lastly, I compared CD4+ and CD8+ T cell apoptosis in cohorts from Denver, CO and Tygerberg Children's Hospital, South Africa. I selected only patients with moderate or severe HIV infection from both centers. South African patients were significantly younger, more malnourished, had higher gamma globulin levels and were less likely to receive ART. CD8+ T cell apoptosis was higher in North American patients suggesting a possible impairment in CD8+ activity in the South African study subjects.

OPSOMMING

'n Eenvoudige en vinnige vloei sitometriese toets is ontwikkel om apoptose aan te toon vanuit 'n gemengde populasie selle. Dit moes geskik wees vir kinders van wie net klein volumes bloed getrek kan word.

Die teenwoordigheid van apoptotiese perifere bloed mononuklêre selle (PBMS) was vasgestel deur morfologiese beoordeling in kliniese monsters *ex vivo* en na oornag kultuur. Die ondersoek is gebasseer op die verstrooings patroon van bestraalde PBMS wat apoptose induseer. PBMS is gemerk met a CD4 MAb. CD4+ selle is gesorteer in apoptotiese en lewensvatbare populasies deur verstrooings karakteristieke. Morfologie is beoordeel deur fluoreserende mikroskopie. Die meerderheid van selle met apoptotiese verstrooings karakteristieke (verminderde voorwaartse en verhoogde sywaartse verstrooings patroon) het apoptotiese karakteristieke gehad (80.6%). Twee-en-negentig persent van selle van die lewensvatbare area het normale morfologie gehad. Verstrooings patroon is ook gekorreleer met die TdT meting vir DNA fragmentasie in kliniese monsters van MIV-geinfekteerde kinders. Daarna is Annexin V gekorreleer met

verstrooings patroon, apoptotiese morfologie en trypan blou opname in selle wat blootgestel is na verskillende konsentrasies van beauvericin.

Apoptose van CD4+ en CD8+ T-selle is bewys in PBMS van MIV-geinfekteerde volwassenes na oornag kultuur. Omdat sel dood 'n artefak van *in vitro* kultuur kan wees, en omdat daar min inligting is oor apoptose in paediatriese MIV siekte, het ek onderneem om 'n deursnee analiese te doen in PBMS wat onmiddelik *ex vivo* geanaliseer is vanaf MIV-geinfekteerde kinders en volwassenes. Die pasiënte is bestudeer in Denver, Colorado, VSA.

PBMS van 22 kinders, 4 adolessente en 9 volwassenes en seronegatiewe ouderdoms-gepasde kontroles is gekleur vir CD4+ en CD8+ oppervlaksmerkers. Apoptotiese selle is vloeisitometries aangedui deur verandering in verstrooings patroon.

Vir die doeleindes van die verstrooings assay is die PBMS aanvanklik deur 'n indirekte metode gemerk, wat 'n intermediêre inkubasie in die teenwoordigheid van biogetinileerde MAbs by 37°C vir 30 minute voor dit geinkubeer is met streptavidin-FITC by 4°C vir 20 minute behels. Daarna is die intermediêre inkubasie stap verwyder en PBMC is geinkubeer met PE – gekonjugeerde CD4+ and CD8+ MAbs. Beide die CD4+ en CD8+ T-sel apoptose het verhoog voorgekom met die indirekte metode. Die betekenisvolle verskille het verdwyn na data van gelyktydige tyd – gepaarde kontroles afgetrek is.

CD4+ en CD8+ T-sel apoptose was betekenisvol hoër in MIV-geinfekteerde studie gevalle as in gelyktydig bestudeerde seronegatiewe kontroles. PBMS assays is gedoen onmiddelik *ex vivo* en na oornag inkubasie na stimulasie deur 'n anti-TCR MAb, sowel as spontaan. Daar was 'n direkte korrelasie tussen CD4+ en CD8+ T sel apoptosis

en CD4+ T sel vermindering. 'n Beduidende korrelasie is ook getoon tussen apoptose onmiddelik *ex vivo* en na oornag kultuur.

Daaropvolgend het ek apoptose in 'n Suid Afrikaanse populasie van 18 simptomatiese kinders en 4 serologies terukerende gevalle bestudeer. CD4+ en CD8+ T sel apoptose was aansienlik hoër in siptomatiese MIV – 1-geinfekteerde kinders as in die serologies terukerende gevalle en seronegatiewe kontroles. CD4+ T sel apoptose het gekorrelleer met vermindering van CD4+ T sel persentasie. Ek het ook opgemerk dat daar 'n tendens bestaan het tot verhoogde CD4+ T sel apoptose in pasiënte wat besig was om te herstel van bykomende siektes.

Ek het CD4+ en CD8+ T sel apoptose in kohorte van Denver, Colorado en Tygerberg, Suid Afrika vergelyk. Suid Afrikaanse pasiënte was jonger en meer wangevoed as hul Noord Amerikaanse ewekniëe. Suid Afrikaanse kinders het ook meer gevorderde siekte gehad. Wanneer pasiënte gepas is vir die graad van ernstigheid van siekte en slegs die minder ernstige (B) en ernstige siekte (C) vergelyk is, was CD8+ T sel apoptose beduidend hoër in Noord Amerikaanse pasiënte. Hierdie waarneming ondersteun die hipotese dat CD8+ T sel aktiwiteit moontlik onderdruk mag wees in in simptomatiese Suid Afrikaanse MIV-1-geinfekteerde kinders

TABLE OF CONTENTS

DECLARATION

SUMMARY

OPSOMMING

DEDICATION

ACKNOWLEDGMENTS

ABBREVIATIONS

PUBLICATIONS RELEVANT TO THESIS

CHAPTER 1

HIV INFECTION: VIROLOGY, EPIDEMIOLOGY AND PATHOGENESIS……..1

CHAPTER 2

APOPTOSIS……..14

CHAPTER 3

THE DEVELOPMENT OF A SCATTER-BASED ASSAY FOR THE SIMULTANEOUS DETECTION OF APOPTOSIS AND SURFACE PHENOTYPE IN A MIXED POPULATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCS)……..37

CHAPTER 4

DIRECT VERSUS INDIRECT LABELING OF PBMCS: EFFECT ON APOPTOSIS……82

CHAPTER 5

APOPTOSIS OF CD4+ AND CD8+ T CELLS ISOLATED IMMEDIATELY *EX VIVO* CORRELATES WITH DISEASE SEVERITY IN HIV INFECTION.....100

CHAPTER 6

CD4+ AND CD 8+ T CELL APOPTOSIS IN SYMPTOMATIC SOUTH AFRICAN HIV-1-INFECTED CHILDREN 133

CHAPTER 7

COMPARISON OF CD 4+ AND CD 8+ T CELL APOPTOSIS IN HIV-1-INFECTED CHILDREN FROM NORTH AMERICA AND SOUTH AFRICA: FURTHER EVIDENCE OF APOPTOSIS AS A CORRELATE OF PROTECTION.....159

CHAPTER 8

GENERAL CONCLUSIONS.....173

REFERENCES.....177

DEDICATION

To my wife, Reena, and children, Jonathan, Rozanne and Sonya, for their continued love and support.

To the patients and their families, whom I am privileged to have met and cared for, and whose sense of dignity has been an inspiration.

ACKNOWLEDGMENTS

National Jewish Center for Immunology and Respiratory Medicine and the Children's Hospital, University of Colorado Health Sciences Center, Denver, CO, USA

I thank Terri Finkel for her input and guidance. Elizabeth McFarland MD and staff of the Pediatric AIDS Clinical Trial Unit provided patient support. Carol Salbenblatt kindly provided data from the Denver cohort. Mary Schleicher and Richard Duke Ph.D. of the Immunology Core Facility UCHSC assisted with morphologic studies. Frank Accurso MD identified patients with cystic fibrosis and Nancy Madinger MD provided an adult patient. The Colorado AIDS Clinical Trial Unit gave permission for the use of their BL2+ facility. Myron J. Levin MD reviewed a portion of the manuscript. David Ikle Ph.D. gave valuable statistical advice. Rick Rapoport provided invaluable technical support and friendship. I was a Pediatric AIDS Foundation Scholar in 1995 and was also partially supported by the South African Medical Research Council.

Faculty of Health Sciences, Stellenbosch University and Tygerberg Hospital

I thank my promoter, Prof. Patrick Bouic for his constant support and guidance through difficult times. Anita Janse v Rensburg, my research assistant, provided loyal support. The nursing staff of the Family Clinic and C3A at Tygerberg Children's Hospital assisted with patient support. Dr. R Thompson of Tygerberg Hospital assisted with digital photography. Florette K Treurnicht from the department of Medical Virology helped with specimen preparation. Prof. Estrelita Janse v Rensburg, Head of Medical Virology, kindly made laboratory facilities and technical support available. Additional laboratory studies were performed by Wendy Brittle and Anel Clark from the department of Medical

Immunology. Chris Muller (M.Sc. Biostats) gave valuable advice. Anita Fourie kindly assisted with editorial aspects of the thesis and Elizabeth Wasserman with Afrikaans translation.

ABBREVIATIONS

Ab - antibody

AI - apoptotic index

AIDS - acquired immunodeficiency syndrome

ANOVA - analysis of variance

ART - antiretroviral therapy

ARV – antiretroviral

ATP - adenosine 5'-triphosphate

CBC - complete blood count

CDC -Center for Diseases Control

CMV - cytomegalovirus

CTL - cytotoxic lymphocyte

ddC - zalcytabine

ddI - didanosine

EIA - enzyme-linked immunosorbent assay

FADD - Fas-associated death domain

Fas-L - Fas-ligand

FITC - fluorescein isothiocyanate

HIV - human immunodeficiency virus

HSV - Herpes simplex virus

ICE - interleukin-1-converting enzyme

LIP - lymphoid interstitial pneumonitis

MAb - monoclonal antibody

MHC - major histocompatibility complex

N – number

NASBA - nucleic acid sequence-based amplification

NK - natural killer cells

NRTI - nucleoside reverse transcriptase inhibitor

NNRTI – non-nucleoside reverse transcriptase inhibitor

PBMCs - peripheral blood mononuclear cells

PBS - phosphobuffered saline

PCP - *Pneumocystis carinii* pneumonia

PCR - polymerase chain reaction

PE – phycoerythrin

PHA - phytohaemagglutinin

PTB - pulmonary tuberculosis

qcPCR - quantitative competitive polymerase chain reaction

RPM - revolutions per minute

RPR - rapid plasma reagin

RSA - Republic of South Africa

S - stimulated

SCID - severe combined immunodeficiency

SIV - simian immunodeficiency virus

TB - tuberculosis

TCR - T cell receptor

TdT - terminal deoxynucleotidyl transferase

Th - T helper cells

TNF - tumour necrosis factor

TRAIL - TNF-related apoptosis inducing ligand

TRADD - TNF receptor associated death domain

USA - United States of America

vs – versus

ZDV - zidovudine

PUBLICATIONS RELEVANT TO THESIS

Reviews

1. Cotton MF, Cassella C, Rapaport EL, Tseng P-O, Marschner S and Finkel TH. Apoptosis in HIV-1 infection. *Behring Inst. Mitt.* 1996, no. 97: 1 - 12.
2. Voss M, Cotton MF. Mechanisms and clinical implications of apoptosis. *Hosp Med* 1999; 59: 924 - 930.

Peer-reviewed articles

1. Finkel TH, Tudor-Williams G, Banda NK, Cotton MF, Curiel T, Monks C, Baba TW, Ruprecht RW, Kupfer A. Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV- and SIV-infected lymph nodes. *Nature Medicine*, 1995; 1: 129-134.

2. Cotton MF, Ikle D, Rapaport EL, Marschner S, Kurrle R, Finkel TH. $CD4^+$ and $CD8^+$ T cell apoptosis correlates with disease progression in HIV-1 infection. *Pediatr Res* 1997; 42: 656-664.
3. Finkel TH, Monks C, Casella C, Cotton MF, Banda NK, Kupfer A, Ikle D. HIV turnover and T Cell death. *Nature Medicine* 1995; 1: 386-387.

Congress proceedings (peer reviewed)

1. Cotton MF, Schleicher M, Duke R, Finkel THF. Apoptosis in pediatric HIV infection. Abstract J222. Journal of Cellular Biochemistry, 1994, Supplement 18b. Wiley Liss, Inc., NY. Presented at Keystone Symposium on Prevention and Treatment of AIDS. January 1994, Hilton Heads, South Carolina.
2. Banda NK, Tudor-Williams G, Kupfer A, Cotton MF, Finkel TH. HIV-1 infected cells in human lymph nodes are not apoptotic. Keystone Symposium, Denver, Co; April 17-23, 1995 HIV pathogenesis and sexually transmitted diseases in the HIV era. J Cell Biochem, 1995; Suppl 21B, Abstract # 201.
3. Cotton MF, Rapaport EL, Marschner S, Finkel TH. CD4 apoptosis in pediatric HIV infection. AIDS research and Human Retrovirology 1995; 11: #169. Presented at the 1995 annual meeting sponsored by the laboratory of tumor cell biology, Bethesda, Maryland; August 27-September 2, 1995
4. Cotton MF, McFarland E, Finkel THF, Bouic P. Comparison of CD4+ and CD8+ T cell apoptosis in HIV-1-infected children from North America and South Africa: further evidence of apoptosis as a correlate of protection . XIII World AIDS Congress, Durban South Africa, 9-14 July 2000.

5. Clarke A, Brittle W, Cotton MF and Bouic P. Measurement of CD4+ T cell apoptosis: comparison of annexin V binding to a scatter-based flow cytometric method. Joint congress HIV Clinicians, Infectious diseases, Travel Medicine and Sexually Transmitted Diseases Societies and Veterinary and Human Public Health. Music Conservatoire, Stellenbosch, South Africa 2 – 6 Dec 2001.

CHAPTER 1

HIV INFECTION: VIROLOGY, EPIDEMIOLOGY AND PATHOGENESIS

1. Introduction

The acquired immunodeficiency syndrome (AIDS) was first described in a cluster of 5 adult male homosexuals from Los Angeles, United States of America (USA). The patients presented with *Pneumocystis carinii* pneumonia (PCP) and oral candidiasis at three separate hospitals [1]. PCP, although known in immunocompromised patients, was a rare occurrence at that time. The patients had no obvious cause for immunodeficiency. Two years later, Barre-Sinoussi recovered a reverse transcriptase-containing virus from lymph node of a man with the persistent lymphadenopathy syndrome and provided the first evidence that AIDS could be due to a retrovirus. The retrovirus, initially termed "lymphadenopathy-associated virus", was subsequently renamed the "human immunodeficiency virus" (HIV-1) and has been conclusively shown to be the cause of AIDS [2]. Since then a second subtype, HIV-2, has been described in West Africa, causing less severe disease than HIV-1 [3].

1.2. Virology

HIV is a lentivirus from the *Retroviridae* family. Retroviruses are associated with many diseases, including malignancies, wasting diseases, neurological diseases, immunodeficiencies as well as asymptomatic lifelong viraemia. The virion is enveloped

and is about 100nm in diameter. Most retroviruses have only 3 genes; *gag*, *pol* and *env*. The *gag* and *env* genes encode the core nucleocapsid and surface-coat proteins, respectively. The internal nucleocapsid has several proteins with important catalytic roles during replication. These include a protease and two products of the *pol* gene: reverse transcriptase for converting single stranded viral RNA to double-stranded DNA and integrase, which covalently joins viral to cellular DNA [4].

Lentiviruses have complex viral genomes. They contain many more genes than the three outlined above. For example, HIV-1 has 6 additional genes (*vpu, vif, vpr, tat, rev and nef*) [5]. Lentiviruses cause a variety of neurological and immunological diseases and include the visna virus from sheep and equine infectious anaemia virus. Both are tropic for macrophages and cause encephalopathy, the latter also causing haemolytic anaemia [2]. HIV and simian immunodeficiency virus (SIV) are tropic for T lymphocytes and macrophages and are members of this family. Both cause immunodeficiency [2].

1.3. Modes of transmission

The four recognized routes of virus transmission are sexual contact with an HIV-infected individual, receipt of HIV-infected blood or blood products, parenteral exposure to HIV-contaminated equipment and vertical transmission from a HIV-infected pregnant woman. In adults, transmission is usually through heterosexual contact, transfusion or needle sharing. Children are almost exclusively infected through mother-to-child transmission either perinatally or through breast-feeding [6]. The incidence of perinatal transmission is approximately 30% [7] with a further 4% infected after five months of age through breast-feeding [8].

1.4. Epidemiology

In USA the number of new infections reached a peak of 150 000 per year in the mid 1980's but has since declined to 40 000 new infections per year [9]. Between 1996 and 2000, the CDC reported 264 405 persons with AIDS in USA [9]. The Joint United Nations Program on HIV/AIDS (UNAIDS) estimated that by June 2000, 24.5 million adults and children living with HIV / AIDS were in sub-Saharan Africa [10]. An illustration of the rapidity of spread is provided by the annual seroprevalence survey conducted in antenatal public clinics in the Republic of South Africa (RSA). The seroprevalence increased from 1 to 14.1% over a 7-year period [11]. By 2000, the overall seroprevalence rate had increased to 22.5% of antenatal clinic attendees in state-funded institutions [12]. In USA, 711 344 cases of AIDS had been reported between 1981 through to June 1999 [13].

1.5. Pathogenesis of HIV infection

1.5.1. Normal functioning of CD4+ and CD8+ T lymphocytes; acquired immune response to infectious agents

The vertebrate immune system encounters a large variety of pathogens. Specific recognition is by clonally distributed receptors expressed on lymphocytes, for example antibodies attached to B lymphocytes and T cell receptors (TCR) on surfaces of T lymphocytes. These receptors recognize antigenic subunits known as epitopes. T cells interact only with antigen presented by major histocompatibility complex (MHC) on the surface of antigen presenting cells [14].

CD4+ T cells recognize peptides presented by MHC class II-restricted antigen-presenting cells such as mononuclear phagocytes, B cells and dendritic cells. CD4+ T

cells are also termed T helper (Th) cells. After antigenic stimulation they produce cytokines, which induce the secretion of specific immunoglobulins by B cells or activate effector mechanisms in phagocytes. CD4+ T cells can be further subdivided into Th1 and Th2 subsets. Th1 cells produce γ interferron and interleukin-2 and activate macrophages and cytolytic CD8+ T cells. Th2 cells produce interleukin-4 and -5 and act mainly on B cells [14].

The most important task of CD8+ T cells is the lysis of target cells. CD8+ T cells recognise antigen presented on MHC class I molecules, expressed on all mammalian cells. Viral proteins are processed internally and bind to MHC I in the endoplasmic reticulum, before being expressed on the surface of the infected cell [14].

1.5.2. Pathogenesis

One of the earliest recognized immunological events of HIV infection was the progressive loss of CD4+ T cells with resultant immunodeficiency [15]. This defect is primarily the result of selective tropism of HIV for CD4+ cells. Klatzmann *et al* first demonstrated that HIV selectively replicates in CD4+ but not in other lymphocytes [16].

Enveloped viruses such as HIV enter cells following fusion with the cell membrane. The key step for viral entry is the interaction of viral gp120 with the CD4 molecule [5]. *In vivo*, macrophages are also susceptible to infection because of expression of CD4 [17]. Subsequently the co-receptors CCR5 and CXCR4 have been identified as important in viral entry. CCR5 is necessary for entry into macrophages and CXCR4 into quiescent CD4+ T cells [18].

1.5.3. Why does HIV infection progress to AIDS?

The main question in HIV pathogenesis is how the virus defeats the immune system. Genetic diversity is probably an important factor. HIV is one of the most variable viruses known. During each reverse transcription of viral RNA to DNA, the new DNA differs from its RNA template, on average, in one site; thus viral proteins may also vary [19]. Studies of viral kinetics have shown that between 10^8 and 10^9 virions are produced on a daily basis. Therefore there is much potential for antigenic variation and immunological escape [20, 21].

1.6. Immunology and clinical manifestations of HIV infection in children and adults

HIV infection affects the functioning and numbers of CD4+ T cells, B cells, monocyte/macrophage cells and neutrophils. The most important clinical effect of HIV infection is impaired cell-mediated immunity via decreased CD4+ T cell numbers and function. Ab function is dysregulated probably as a result of loss of CD4+ T cell assistance [22]. The immunopathologic consequences and associated laboratory Abnormalities are summarized in Table I [22]:

Table I. Immunopathological consequences of HIV infection

T cell Abnormalities

Clinical manifestations

- Impaired delayed hypersensitivity
- Opportunistic infections
- Chronic active viral infections (varicella, CMV)
- Neoplasms

Laboratory manifestations

- Lymphopaenia
- Selective deficiency of CD4+ T cells
- Impaired mitogen/antigen responses
- Impaired alloantigen activity
- Impaired cytokine production
- Impaired CD8+ cytotoxicity

B cell Abnormalities

Clinical manifestations

- Hypergammaglobulinaemia
- Impaired Ab responses

Laboratory manifestations

- Elevated B cells spontaneously secreting Ab
- Circulating immune complexes

Mononuclear phagocytes

Clinical manifestations

Impaired delayed-type hypersensitivity

Impaired granuloma formation

Opportunistic infections

? Pneumococcal bacteraemia

Laboratory manifestations

Impaired splenic clearance

Elevated tumour necrosis factor

Natural Killer Cells

Clinical manifestations

? Chronic herpes virus infections

? Neoplasms

Laboratory manifestations

Impaired cytotoxicity of large granular lymphocytes

Neutrophils

Clinical manifestations

? Candidiasis

Laboratory manifestations

Neutropaenia

Impaired chemotaxis

1.6.1. Clinical course

HIV infection is associated with significant mortality and morbidity. In a 5-year retrospective study of HIV-infected children seen at Tygerberg Children's Hospital in the Western Cape province of RSA, I showed a mortality rate of 40%. In the first year of the study, HIV-infected children accounted for 0.009% of total paediatric admission days. By the last year (1996), this had increased 3.4 fold to 0.32% ($p < 0.0001$). Most children were malnourished and the majority had multiple diagnoses, the most common of which were gastroenteritis and pneumonia [23].

Hussey *et al* has shown that the median survival of HIV-1-infected children in Cape Town RSA is 32 months from time of diagnosis, with the median age of diagnosis being 5 months [24]. In contrast, a North American child had a 75% chance of surviving to 5 years of age [25].

1.6.2. Clinical and Immunological Categories for Children with HIV

The clinical categories were outlined by the Center for Diseases Control (CDC), Atlanta, GA, USA and give a further indication of disease spectrum [26]. Patients are classified both for clinical appearance as well as the degree of CD4+ T cell depletion. Patients with major opportunistic infections are classified as "C". Clinical and immunological categories are shown in Tables II and III respectively.

Table II: Clinical categories for children with HIV infection

Category	Characteristics
N	No signs or symptoms considered to be the result of HIV infection or only 1 condition listed in A.
A (Mild)	2 or more conditions listed below but none from B or C. Lymphadenopathy (≥ 0.5cm at more than 2 sites; bilateral, 1 site). Hepatomegaly Splenomegaly Parotitis Dermatitis Recurrent or persistent upper respiratory tract infections, sinusitis, or otitis media
B (Moderate)	Symptomatic conditions other than from A or C and attributed to HIV infection; including but not limited to: Anaemia (< 8 g/L); neutropaenia (< 1 000/mm^3); thrombocytopaenia (< 100 000/mm^3) - persisting ≥ 30 days Bacterial meningitis, pneumonia or sepsis (single episode) Candidiasis, persisting > 2 months in children > 6 months of age Cardiomyopathy Cytomegalovirus (CMV) infection, onset < 1 month of age Diarrhoea - recurrent or chronic

	Hepatitis Herpes simplex virus (HSV) stomatitis > 2 episodes within a year HSV bronchitis, pneumonitis or oesophagitis with onset < 1 year of age Herpes zoster (shingles) ≥ 2 episodes or > 1 dermatome Leiomyosarcoma Lymphoid interstitial pneumonitis (LIP) or pulmonary lymphoid hyperplasia complex Nephropathy Nocardiosis Persistent fever (> 1 month) Toxoplasmosis, onset < 1 month of age Varicella, disseminated
C (Severe)	Any condition listed below: Serious bacterial infections, multiple or recurrent (at least 2 culture-confirmed episodes within a 3 year period) of: septicaemia, pneumonia, meningitis, bone or joint infection, or abscess of an internal organ or body cavity. Candidiasis (oesophageal or pulmonary). Coccidioidomycosis (disseminated). Cryptococcosis (disseminated). CMV disease with onset at age > 1 month (at site other than lymph

	nodes, spleen, liver). Encephalopathy. HSV causing mucocutaneous ulcer persisting > 1 month, or bronchitis, oesophagitis, pneumonitis, oesophagitis in a child older > 1 month. Histoplasmosis (disseminated). Kaposi sarcoma. Lymphoma, primary in brain, Burkitt's, immunoblastic, large cell, B cell or unknown. *Mycobacterium tuberculosis* (disseminated or extrapulmonary). *Mycobacterium avium* complex or *Mycobacterium kansasii* (disseminated). PCP Progressive multifocal leukoencephalopathy. Salmonella septicaemia (recurrent). Cerebral toxoplasmosis with onset > 1 month of age. Wasting syndrome in the absence of illness other than HIV that could explain the following: persistent weight loss > 10% of baseline, or downward crossing of at least 2 of the following percentiles on a weight for age chart (95th, 50th, 25th, 5th) in a child ≥ 1 year of age; or < 5th centile weight for height on 2 consecutive measurements ≥ 30 days apart plus 1) chronic diarrhoea (≥ 2 loose

	stools per day ≥ 30 days); or documented fever ≥ 30 days intermittent or constant.

Table III: Immunological categories

	Age of child					
	< 12 months		1 - 5 years		6 - 12 years	
Immunological category	CD4 µl	(%)	CD4 µl	(%)	CD4 µl	(%)
1.No immunosuppression	≥ 1500	(≥ 25)	≥ 1000	(≥ 25)	≥ 500	(≥ 25)
2.Moderate immunosupression	750 - 1499	(15 - 24)	500 - 999	(15 - 24)	200 - 499	(15 - 24)
3.Severe immunosuppression	< 750	(<15)	< 500	(<15)	< 200	(<15)

CHAPTER 2

APOPTOSIS

2.1. Apoptosis – a review

2.1.1. Historical overview

Virchow first noted the concept of cell death in the mid 19th century. He introduced the term "necrosis" which he referred to as a "passive degenerative process" to describe an advanced stage of tissue breakdown [27]. He was referring to "gangrene", which he described as follows: "in necrosis we conceive the mortified (gangrenous) part to be preserved more or less in its external form". In a later lecture, he noted focal bone necrosis in dental caries [28]. He also proposed using the term "necrobiosis", first introduced by Schultz, to describe gradual decay and death of tissue.

Carl Weigert introduced the term "coagulation necrosis" in 1877. Although today, this term implies a "white infarct" secondary to ischaemia, he referred to the combination of epithelial cell death, fibrin and leukocyte infiltration of diphtheritic pseudomembrane. He proposed that dead leukocytes caused fibrinogen to coagulate resulting in wedge-shaped "white infarcts" or "fibrin wedges" [29].

Spontaneous cell death was recognized soon after histological stains became available in the 1880's. Walther Flemming studied mammalian ovarian follicles and

noted that the epithelial lining of regressing follicles contained cells with fragmenting nuclei. His detailed drawings showed pyknotic chromatin characteristic of apoptotic nuclei, and also free nuclear fragments, now known as apoptotic bodies, in follicular cavities. He named the process "chromatolysis" because the fragmented nuclei later disappeared. Nissen, a German medical student [29], observed the same process one year later in lactating mammary glands.

In 1914, Graper published a paper entitled "A new point of view regarding elimination of cells" [29]. He hypothesized that "chromatolysis" counterbalanced ongoing mitosis and that neighbouring epithelial cells engulfed the resultant debris. This important paper was published at the start of World War 1 in a German journal on cellular investigation and was probably not widely read. His concepts were re-introduced 58 years later by Wyllie and Kerr. Kerr had induced liver atrophy in rats by tying off a large branch of the portal vein. He noted the loss of cells by a process he initially called "shrinkage necrosis" and "apoptosis" a year later [30-32].

In the intervening years, embryologists recognized the importance of "chromatolysis". In 1950, Glucksman identified "chromatopyknosis" (morphologically identical to chromatolysis) in embryological tissue and assumed that "controlled deletion" was unique to embryogenesis [33]. The concept that cells might have a suicide mechanism was also introduced in the 1950's shortly after the discovery of lysosomes. De Duve erroneously suggested that cells might be killed from within by lysosomes acting as "suicide bags" [29].

"Apoptosis" is derived from the Greek words "apo" (apart) and "ptosis" (to fall) and describes the "dropping off" of petals from flowers or leaves from trees. It implies

cell loss that is both physiological and part of the normal life cycle, as is petal and leaf loss in the plant kingdom [30, 31].

Kerr and Wyllie described the unique morphology of apoptosis and distinguished it from "accidental death" or "necrosis". They identified specific morphological features of apoptotic cells including loss of cell volume, zeiosis (blebbing of the cell membrane) and condensation and margination of nuclear chromatin. They noted that apoptosis usually occurred asynchronously.

More importantly, they conceptualized the significance of apoptosis including its role in normal tissue homeostasis, physiological involution and atrophy, and focal elimination of cells during embryogenesis. They noted its occurrence in neoplasms, both spontaneously and after chemotherapy. They observed that stimuli such as electromagnetic radiation and hepatotoxins could induce both necrosis and apoptosis [30, 31].

Some major differences between apoptosis and necrosis are set out in Table IV [34]. Phagocytosis of apoptotic cells with intact plasma membranes is a key feature of apoptosis as this avoids leakage of proinflammatory cytosolic components. Changes in cell surface proteins such as thrombospondin up regulation and the translocation of phosphatidyl serine (PS) from the inner to the outer surface membrane allow recognition by phagocytes [35, 36].

Table IV: Comparison of cardinal features of necrosis and apoptosis

Features:	**Necrosis:**	**Apoptosis:**
Stimuli	Toxins, severe hypoxia, massive insult, conditions of ATP depletion	Physiological and pathological conditions without ATP depletion
Energy requirement	None	ATP-dependent
Histology	Cellular swelling, disruption of organelles, death of patches of tissue	Chromatin condensation, apoptotic bodies, death of single isolated cells
DNA breakdown pattern	Randomly-sized fragments	Ladder of fragments in internucleosomal multiples of 185 base pairs
Plasma membrane	Lysed	Intact, blebbed with molecular alterations
Phagocytosis of dead cells	Immigrant phagocytes	Neighbouring cells
Tissue reaction	Inflammation	No inflammation

2.1.2. Apoptosis in immunology

Programmed cell death is important in normal immune function. Wyllie *et al* [31] recognized that "sensitisized" T cells could induce apoptosis in target cells. Cytotoxic T cells and NK cells induce apoptosis in targets such as tumor cells and virus-infected cells [37, 38]. Antibody-dependent cellular cytotoxicity is also associated with apoptosis [39]. Apoptosis of thymocytes occurs in normal thymic development and is responsible for the deletion of autoreactive thymocytes, which would, otherwise, cause autoimmune disease [40, 41].

Lymphocyte apoptosis has been demonstrated during viral infections in rodents, monkeys and in humans, for example, in mice infected with lymphocytic choriomeningitis virus [42]. In humans, CMV [43], Epstein-Barr virus [44], measles and varicella-zoster virus infections are all associated with lymphocyte apoptosis [45].

2.1.3. Mechanisms of apoptosis

The genetics and molecular mechanisms were first characterized in the nematode worm *Caenorhabditis elegans* in the 1980's and early 1990's. Programmed cell death was shown to be precise and predictable during development from larval to adult form. Specific genes were described that when expressed, killed 131 cells leaving 959 cells remaining in the adult worm [46]. Genes such as ced-3 and ced-4 were shown to be pro-apoptotic and others such as ced-9 anti-apoptotic. Human equivalents of these genes have been identified [47].

The following sequential steps were identified and conserved throughout animal evolution from worm to human [34].

- Commitment to death by extracellular or intracellular triggers.
- Cell killing by activation of intracellular proteases (caspases)
- Engulfment of the cell corpse by other cells
- Degradation of the cell corpse within the lysosomes of phagocytic cells

2.1.4. Signaling Mechanisms

Apoptosis involves several signaling systems. The signal to initiate apoptosis may come from within the cell, for example, after damage to the genome, or from binding of a cell surface "death receptor". In some situations, more than one signaling system has been implicated. For example, cytotoxic T lymphocytes may induce apoptosis via the Fas death receptor or perforin-granzyme pathway [48].

2.1.4.1. Death receptors

The most widely studied receptor is the Fas receptor (also known as CD95 or Apo 1). This is a transmembrane glycoprotein related to the tumour necrosis factor (TNF) receptor and is activated by binding of Fas-ligand (Fas-L). Fas receptor (Fas) is constitutively expressed in a variety of haemopoietic and epithelial tissues and in tumours. Fas expression may also be induced in tissues that do not normally express it [49].

The Fas pathway is important in controlling the immune response. Cytotoxic T lymphoctes expressing Fas-L target Fas-bearing cells and induce apoptosis. Activated T cells express Fas receptor and become susceptible to apoptosis. In this way the immune

response is regulated. Failure of this mechanism in a mouse model results in autoimmune disease [50]. Conversely, down regulation of Fas and over expression of Fas-L in the target cell may induce apoptosis of infiltrating T lymphocytes and result in immune evasion [51].

The TNF-TNF-receptor system is similar but utilizes different biochemical pathways. A TNF-related apoptosis-inducing ligand (TRAIL) has now been identified. Cancer cells are particularly susceptible to TRAIL-induced apoptosis [52].

Following binding of a ligand to its death receptor, the signal is transduced by intracellular adaptor molecules designated as "death domains." Both Fas- (FADD) and TNF receptor-associated death domains (TRADD) have been identified. Activated death domains then recruit the effector mechanism.

2.1.4.2. The Effector Mechanism

The central events in the apoptotic cell are proteolysis and mitochondrial inactivation. The disruption of cellular structure results from activation of a family of cysteine proteases called caspases. Caspases exist as proenzymes in the cytosol and form a biochemical pathway, which is highly conserved from worms to humans. The dominant specificity of the caspases is cleavage of peptide bonds following aspartate residues. This property is shared by granzyme B, an effector serine protease in cytotoxic lymphocytes. In mammals caspases have also evolved the function of activating proinflammatory cytokines. The prototype caspase is related to interleukin 1-converting enzyme (ICE/caspase 1) and ten have already been identified [53, 54].

2.2. Detection of apoptosis

2.2.1. Introduction

A number of assays have been developed for measuring programmed cell death. The percentage of apoptotic cells in a sample is usually referred to as the apoptotic index (AI). Although AI can often be quantified in histological specimens, flow cytometry has become the method of choice for cells in suspension. Some assays are suitable for both. Many assays rely on a single feature of apoptotic cells and should always be validated with a second method [55].

2.2.2. Methods of apoptosis detection

2.2.2.1. Microscopy

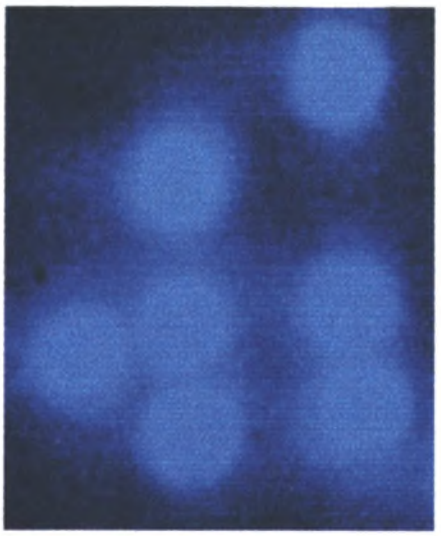
As apoptosis was originally defined by characteristic morphology [30], microscopy remains an important tool. Conventional, fluorescent and electron microscopy are used. The most recognizable characteristic is chromatin condensation. Microscopic methods are labour-intensive and often rely on subjective assessments, but are important to validate other assays [55].

Apoptotic events have been detected on histological sections stained with haemotoxylin and eosin. For example, apoptotic bodies, previously called Councilman bodies, are occasionally seen in biopsies from patients with chronic hepatitis [31]. Also, Cree *et al* have identified apoptotic cells in granulomas [56].

Fluorescence microscopy has facilitated the identification of apoptosis. Apoptotic cells stained with the Hoechst 33342 dye and viewed through an ultraviolet filter appear

bright blue because of intense nuclear chromatin condensation. "Viable" cells show an "open" chromatin pattern and take up the dye far less intensely [57]. Examples are shown in Figure 1.

A



B

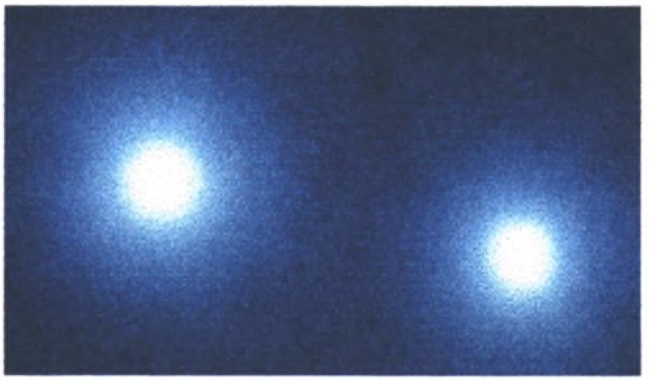


Figure 1. A. Viable cells showing "open" nuclear patterns. B. Apoptotic cells showing nuclear chromatin condensation. PBMCs were stained with the Hoechst 33342 dye (10µl of cell suspension mixed with 10µl of dye at 25 µg/ml in PBS) and examined by

fluorescence microscopy (Nikon Diaphot-TMD), using an UV filter. The characteristic increased density of nuclear chromatin identified apoptosis. Magnification X400.

Another useful strategy is the combination of the fluorochromes acridine orange and ethidium bromide, permitting the discrimination between live and dead apoptotic cells. “Viable” cells exclude ethidium bromide. Nuclei of "viable" cells take up acridine orange and have an "open" chromatin pattern. Live apoptotic cells show condensed nuclear chromatin, but exclude ethidium bromide. Cells with an "open" nuclear chromatin pattern, but which take up ethidium bromide are necrotic. Dead apoptotic cells have condensed nuclear chromatin and take up ethidium bromide [57].

2.2.2.2 Flow cytometry

Flow cytometry permits rapid and objective assessment of large numbers of cells. The principle of flow cytometry is the measurement of light emitted by individual cells traversing in single file through a laser beam. The intensity of light scattered in a forward direction (forward scatter) correlates with cell size and at right angle (side scatter) with granularity, refractiveness and the presence of intracellular structures [58]. Apoptotic cells are both small and refractile and are relatively easily detected by scatter characteristics [41, 55, 59].

Fluorescent dyes emit light of specific wavelengths that can be measured. For example, propidium iodide emits red light and can be used either for “live”/“dead” discrimination in “unfixed” cells or for cell cycle analysis in samples “fixed” in ethanol. Propidium iodide is excluded by viable cells and intercalates with DNA of dead or “fixed” samples. Apoptotic cells lose fragmented DNA after fixation with ethanol, resulting in a population with diminished DNA [60].

Flow cytometric assays have used Hoechst 33342 to stain chromatin from apoptotic cells more brightly than viable cells [61, 62]. A disadvantage of this dye is the need for an additional laser emitting in the region of ultraviolet wavelengths.

Other dyes such as fluoroisothiacyanate (FITC) and phycoerythrin (PE) can be attached to monoclonal antibodies (MAb) or specific molecules with receptors on or within cells. When excited by 488 nm light, FITC emits fluorescence in the green part of the spectrum and PE in the orange / yellow part. Both can be simultaneously detected with the most commonly used argon laser flow cytometers.

2.2.2.3. DNA fragmentation

Extensive DNA cleavage was the first recognizable molecular characteristic of apoptosis and preceded other advances by approximately ten years. It occurs as a result of activation of endogenous endonucleases that cleave double-stranded DNA at internucleosomal sites resulting in integer multimers of 180 base pairs. A characteristic "ladder" pattern is seen on agarose gel electophoresis [32]. Disadvantages of this assay are its labour intensiveness and unsuitability for studying mixed populations of cells.

DNA fragmentation may also be detected by *in situ* end labelling techniques (ISEL), a technique suitable for histology [63] and flow cytometry [64]. Nucleotides tagged to fluorochromes are incorporated onto terminal ends of fragmented DNA by means of terminal deoxynucleotidyl transferase (TdT). When used for flow cytometry, apoptosis can be detected in specific cell types identified by MAbs attached to a second fluorochrome.

2.2.2.4. Annexin V affinity assay

Apoptotic cell death is accompanied by a change in plasma membrane structure. Phosphatidyl serine (PS) translocates from the internal to the external surface. PS has a high affinity for Annexin V, a recently described molecule with anti-phospholipase, anticoagulant and anti-kinase activity. By attaching fluorochromes to Annexin V, one has a further tool for detection of apoptotic cells. This technique is suitable both for flow cytometry and histochemistry [65].

2.2.3. Problems with measuring apoptosis

Many technical issues have not been fully resolved [66]. Apoptotic cells are rapidly phagocytosed and rapidly removed *in vivo* [30, 31, 67, 68]. Consequently, apoptotic events may be missed. Unless pathological specimens are immediately fixed after removal, apoptosis may continue for some time *ex vivo*.

Apoptotic cells fragment into apoptotic bodies. Although not usually seen *in vivo* because of rapid clearance, they occur in cell cultures and may interfere with flow cytometric assessments, especially those based on scatter or detecting cells with diminished DNA content.

Both necrotic and late apoptotic cells permit entry of dyes such as propidium iodide. Similarly, Annexin V can reach PS on the inner surface of cell membranes of necrotic cells. DNA fragmentation occurs in necrotic cells but is probably less marked than in apoptotic cells [64].

It is likely that most cell death measured in biological systems is, in fact, apoptotic and not necrotic. For example, when validating a scatter-based assay of apoptosis, I sorted peripheral blood mononuclear cells (PBMCs), previously irradiated to

induce apoptosis. PBMCs were sorted by scatter criteria into viable and apoptotic populations. Morphological assessment with acridine orange and ethidium bromide confirmed the validity of the assay and showed that an insignificant number of cells from both populations were necrotic [69].

Cell necrosis is associated with an initial increase and then rapid decrease in forward and side scatter, reflecting initial swelling followed by membrane rupture and resultant leakage of cell contents. During apoptosis, increased side scatter, rather than the decrease seen in necrosis accompanies decreased forward scatter. The light scatter changes are not specific to apoptosis. Mechanically broken cells, isolated nuclei, and necrotic cells all have diminished Ability to scatter light [55]. Carbonari *et al* demonstrated that cells lysed as a result of complement activation also accumulate in the apoptotic region, but then after approximately 30 minutes, disperse widely throughout the scattergram [70].

2.3. Apoptosis in HIV infection

2.3.1. Introduction

One of the first immunological events recognized in HIV infection was that immunodeficiency was accompanied by a progressive loss of CD4+ T cells [15]. Schnittman *et al* showed that HIV RNA synthesis, a marker for productive infection, occurred in only 1/1000 CD4+ T cells from patients with AIDS [71]. At the same time, others demonstrated CD8+ and CD4+ T cell dysfunction in clinically stable individuals even prior to a decline in CD4+ T cell numbers [72, 73].

In April 1991 Ameisen and Capron hypothesized that excessive apoptosis might explain both the relative lack of productively infected CD4+ T cells and early T cell dysfunction seen in HIV infection [74]. Two years later, Embretson *et al* and Pantaleo *et al* showed that lymph nodes and not PBMCs were the major source of productive infection [75, 76]. Nevertheless, within months of publication of the "apoptosis hypothesis", investigators had demonstrated apoptosis in HIV-infected lymphoid cell lines [77, 78].

The first demonstration of apoptosis in HIV-infected patients was by Groux and colleagues. CD4+ and CD8+ T cells were identified by negative selection. They showed activation-induced apoptosis in CD4+ T cells from asymptomatic HIV-1-infected patients. Cell death was assayed by trypan blue exclusion, and apoptosis was confirmed by DNA ladder formation and electron microscopy. Cells were activated by pokeweed mitogen and staphylococcal enterotoxin B. PBMCs undergoing apoptosis were anergic thus providing an explanation for immunological dysfunction in HIV infection [79].

Thereafter, Meyaard *et al* showed both elevated CD4+ and CD8+ T lymphocyte apoptosis after overnight incubation. Although spontaneous apoptosis was noted, it was enhanced by activation with anti-CD3 antibodies (Ab) [80]. Since then, many studies have confirmed apoptosis in PBMCs from HIV-infected individuals [70, 81-83].

2.3.2. Mechanisms of apoptosis in HIV-1 infection

A number of mechanisms may account for the increased levels of apoptotic CD4+ and CD8+ T cells in HIV infection. As both CTL and NK activity occur in HIV infection [68, 84] they are likely to induce apoptosis of virus-infected cells.

2.3.2.1. CD4+ T cell apoptosis

The CD4+ T lymphocyte is the main target of HIV [85]. A critical step in viral entry is the binding of gp120 to the CD4 molecule. Banda *et al* [86] first showed a mechanism for apoptosis of uninfected CD4+ T cells in HIV infection. The stimulus for his work was the observation of Newell *et al* that murine CD4+ T cells die by apoptosis after sequential ligation with monoclonal anti-CD4 MAb, cross-linking and then stimulating through the T cell receptor (TCR) [87].

After confirming this mechanism in human CD4+ T cells, Banda *et al* applied the model to HIV [86]. He induced apoptosis in uninfected human cells after sequentially incubating with gp120, cross-linking with anti-gp120 antibodies and then stimulating through the TCR. Apoptosis could be triggered by gp120 levels as low as 10ng per ml corresponding to clinically detectable levels in patients with AIDS [88]. Because anti-gp120 antibodies also occur *in vivo*, a mechanism was provided whereby uninfected CD4+ T cells, exposed to gp120 and cross-linked with anti-gp120, would be primed to

undergo apoptosis upon stimulation of the TCR, as would occur with an intercurrent infection. Tat protein (a HIV-1 regulatory protein) enhances apoptosis by this mechanism [89]. Aceituno and colleagues confirmed Banda's work by inducing apoptosis in uninfected CD4+ T cells by first incubating with gp120 / anti-gp120 immune complexes from HIV-1-infected patients and then stimulating with PHA (phytohaemagglutinin) [90].

Others have confirmed that uninfected CD4+ T cells die by apoptosis. For example, in HIV-infected human thymus explanted into mice with severe combined immunodeficieny (SCID), most of the dying cells are uninfected [91]. *In vitro* experiments showed that gp120 expressed on either infected or transfected T cells can interact with CD4 on uninfected CD4+ T cells, resulting in apoptotic death of the latter [88].

Indirect evidence for apoptosis of uninfected CD4+ T cells in patients comes from kinetic studies by Wei *et al* [21] and Ho *et al* [20]. They described a daily turnover of 2×10^9 CD4+ T cells and 1.1×10^8 virions, implying that over 10-fold more CD4+ T cells are lost when compared to the number of virions produced. As a CD4+ T cell releases approximately 174 virions during a replicative burst [92], it is reasonable to assume that most CD4+ T cells that die are uninfected.

The Fas/Fas-ligand interaction may also be important in HIV disease. Fas antigen expression is up regulated on lymphocytes from HIV-infected patients and correlates directly with CD4+ T cell depletion [93]. Katsikis *et al* showed a positive correlation between anti-Fas Ab-induced CD4+ T cell apoptosis and CD4+ T cell depletion in HIV-infected adults [62]. Bohler and colleagues have confirmed Fas-induced apoptosis of memory T cells in children [94].

2.3.2.2. CD8+ T cell apoptosis

Meyaard and colleagues first noted elevated CD8+ T cell apoptosis in HIV-infected adults [80]. A number of mechanisms may account for CD8+ apoptosis. Baumler and colleagues have implicated the Fas/Fas-L mechanism for both CD4+ and CD8+ T cells [95]. Herbein *et al* have implicated the interaction of gp120 and the chemokine receptor CXCR4 in macrophage-induced CD8+ T cell apoptosis; thus providing a specific HIV-related mechanism for CD8+ T cell depletion also seen in HIV-infected patients [96].

2.3.3. Apoptosis in lymph nodes

The lymph nodes are a major site of HIV disease [75, 76]. It was therefore logical to examine the relationship between infection and apoptosis in lymphoid tissue. Our team, led by Terri Finkel, studied lymph node material from four HIV-infected children and a SIV-infected Rhesus macaque [97]. Other members of this team were G Tudor-Williams, NK Banda, MF Cotton, T Curie, C Monks, TW Baba, RM Ruprecht and Kupfer A. Viral mRNA indicative of productive infection was detected by *in situ* hybridization and apoptosis by the *in situ* TdT assay for DNA fragmentation (Figure 2).

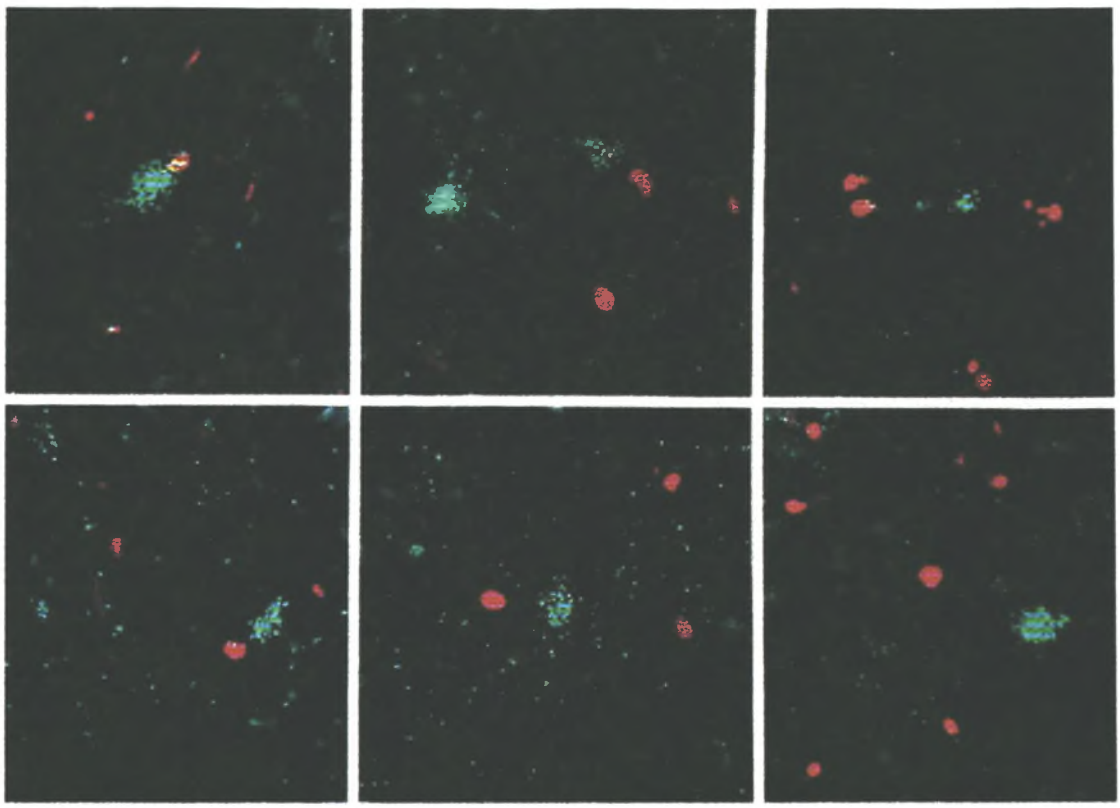


Figure 2. Apoptosis and productive infection are not detected simultaneously in cells from lymph node of HIV-infected children. Apoptosis (red) detected by *in situ* end labelling and productive infection (green) by *in situ* hybridization of viral mRNA. (Reproduced with permission from Nature Medicine)

During the analysis of 219 fields, each measuring 200μm, 1,031 apoptotic and 696 infected cells were counted. Actin mRNA was detected in apoptotic cells, suggesting that if HIV mRNA were present, it would also be detected. Goebel *et al* had previously shown that mRNA from vesicular stomatitis virus, another RNA virus, is not degraded in apoptotic cells, despite rapid degradation of host DNA [98].

It was immediately apparent that by far the majority of productively infected cells were not apoptotic and that apoptotic cells were not productively infected. Less than 0.10% of apoptotic cells were productively infected and less than 0.14% of productively infected cells were apoptotic [97].

A possible explanation for these observations is provided by McCloskey and colleagues who showed that Tat protein induced apoptosis in T cells exposed to anti-CD3 antibodies, but Tat-expressing cells were resistant to the cytopathic effects of Tat and other inducers of apoptosis [99].

The phenotypes of the apoptotic cells were not fully identified in our study [97], as surface epitopes may be lost during fixation and paraffin embedding. The majority of apoptotic cells were localized to the secondary follicles, specifically to the apical light zone of the follicles, a region rich in B cells, activated CD4+ T cells, CD8+ T cells and follicular dendritic cells.

A recent study of lymph node-derived lymphocytes (LNMCs) by Sunila *et al* supports a role for gp120 inducing apoptosis in uninfected cells. In lymph node from HIV-1-infected patients, gp120 was found mainly on apoptotic and to a lesser extent on

non-apoptotic CD4+T cells, thus possibly marking them for death. HIV particles were not associated with or budding from either normal or apoptotic lymphocytes [100].

Muro-Cacho *et al* studied apoptosis in lymph nodes from HIV-1-infected adults and found increased levels in all areas of nodes as opposed to only in germinal centers of seronegative controls. They correlated apoptosis with the degree of cellular activation and in cells derived from fresh lymph node showed that not only CD4+ T cells, but also CD8+ T cells and B cells were also apoptotic [101].

Progressive lymph node destruction could facilitate the escape of apoptotic lymphocytes into the periphery and provide an alternative explanation for the increased levels of PBMC apoptosis in advanced disease. Support for this hypothesis comes from our own lymph node study [97]. In three children with mild or no CD4+ T cell depletion, there was a direct correlation between productive infection and apoptosis. However, the child with the most severe CD4+T cell depletion had productive infection only and no apoptosis [97]. Huang *et al* have shown in the murine system that apoptotic infected cells leave the lymph nodes for other reticulo-endothelial organs such as the liver [102].

2.3.4. Is apoptosis a correlate of protection or pathogenicity?

Several studies argue for apoptosis being a correlate of pathogenicity. Banda *et al* described the mechanism whereby uninfected CD4+ T cells might be primed for apoptosis by binding with viral gp120 [86]. A number of investigators have correlated apoptosis with CD4+ T cell depletion [70, 82]. Lastly, Finkel and colleagues have shown, not only a positive correlation between apoptosis and productive infection in lymph node, but also the absence of productive infection in apoptotic cells [97].

In contrast, data from elsewhere suggested that apoptosis might be a correlate of protection. Mittler and colleagues have shown that plants use programmed cell death as a mechanism of isolating and destroying pathogens [103]. Also, *in vitro* studies have shown increased HIV production in tissue culture after inhibition of apoptosis [104, 105].

CHAPTER 3

THE DEVELOPMENT OF A SCATTER-BASED ASSAY FOR THE SIMULTANEOUS DETECTION OF APOPTOSIS AND SURFACE PHENOTYPE IN A MIXED POPULATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCS)

3.1. Introduction

The majority of assays for apoptosis are either very labour-intensive or involve purchasing expensive reagents, mitigating against its introduction in clinical laboratories. Should measuring apoptosis provide pivotal data of the same magnitude as measuring plasma HIV RNA or CD4+ T cell numbers, the added expense will probably not be insurmountable and the cost of the assay of choice will be reduced with increased use. If,measuring apoptosis provides useful, but not essential information, it will probably not gain acceptance in the majority of laboratories, unless an inexpensive assay is used.

3.2. Summary

A simple flow cytometric technique was developed based on the light scatter characteristics of apoptotic compared to live cells. Apoptotic cells have diminished forward scatter (decreased size) and increased side scatter (increased granularity) [40].

A number of strategies were employed to validate the scatter-based assay. CD4+T cells were sorted into viable and apoptotic populations by scatter criteria. Thereafter nuclear morphology was examined for characteristics of apoptosis by fluorescence microscopy using acridine orange and ethidium bromide. The reproducibility of the scatter assay was confirmed in a limited number of specimens after accumulating 10^4 and then 10^5 scatter events. CD4+ and CD8+ T cell apoptosis were compared by scatter and the TdT method for beauvericin-induced apoptosis. Annexin V expression was compared with scatter in samples where apoptosis was induced by beauvericin. Simultaneous assays of PBMC death by trypan blue uptake and PBMC apoptotic morphology by fluorescence microscopy were undertaken in the same samples.

In clinical samples, cell death was confirmed by trypan blue uptake and compared with the scatter assay after overnight incubation. Apoptotic morphology was confirmed in PBMCs isolated *ex vivo* and after overnight culture. Apoptotic PBMCs were identified by fluorescence microscopy. CD4+ T cell apoptosis measured by scatter was evaluated in the same specimens. The scatter-based assay and TdT method for CD4+ and CD8+ T cell apoptosis were also compared in HIV-infected patients and seronegative controls.

3.3. Reagents

3.3.1. Denver, CO, USA

Foetal calf serum was purchased from Hyclone Laboratories Inc, Logan, UT; penicillin-streptomycin and Dulbecco's phosphate-buffered saline (PBS) from Gibco, Grand Island, N Y. RPMI 1640 with glutamine and sodium bicarbonate, Histopaque, acridine orange, ethidium bromide and beauvericin were purchased from Sigma, St.

Louis, MO. Cells were cultured in RPMI 1640 supplemented with 10% foetal calf serum and penicillin at 50 U/ml and streptomycin at 50 μg/ml. Goat anti-mouse Ab was obtained from Jackson Immunoresearch, West Grove, PA. Ficoll-Paque was obtained from Pharmacia Biotechnica, Upsala, Sweden. Twenty-four well plates were from Costar, Cambridge, MA. Hoechst 33342 dye was the generous gift of Richard Duke, Immunology Core Laboratory, and University of Colorado Health Science Center. Biotin-16-dUTP and terminal deoxynucleotidyl transferase (TdT) with buffers was purchased from Boehringer Mannheim Corporation, Indianapolis, IN, USA.

BMA-031 (pan anti-TCR MAb) was the generous gift of Roland Kurrle, Behringwerke AG, Marburg, Federal Republic of Germany. Leu 3a MAb was the generous gift of the Sloan-Kettering Institute. OKT8 and OKT3 MAbs were derived from cell lines obtained from the ATCC. All were of IgG1 isotype. Biotinylation and FITC conjugation were performed in the laboratory according to established protocols. Titering of MAbs was done on PBMCs from HIV-seronegative controls. Streptavidin-PE and FITC were purchased from Tago Pharmaceuticals, Burlingame, CA. Leu 3a-PE and Leu 2a-PE antibodies were purchased from Becton Dickinson, San Jose, CA. Murine isotype control Ab (IgG2a-FITC; IgG1-PE) was purchased from Olympus, Lake Success, NY.

3.3.2. Tygerberg Hospital, RSA

Foetal calf serum was purchased from Delta Bioproducts, Kempton Park, RSA. Penicillin-streptomycin, phosphate-buffered saline (PBS) and RPMI 1640 with glutamine and sodium bicarbonate were purchased from Bio-Whittaker, Walkerville, MD, USA. Cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum and penicillin at 50 U/ml and streptomycin at 50 μg/ml. Histopaque and beauvericin were purchased

from Sigma, St. Louis, MO, USA. Twenty-four well plates were obtained from Cel-Cult, Sterilin Ltd, Feltham, England. FITC-Annexin V was purchased from Sigma Diagnostics, St Louis, MO, USA.

3.4. Methods

3.4.1. Sample processing

Heparinized blood samples were collected from patients and seronegative controls. PBMCs were isolated by Ficoll-Paque or Histopaque density gradient centrifugation. Briefly, whole blood was diluted 1:1 with PBS and layered carefully over 30 ml of Histopaque or Ficoll-Paque. Centrifugation at room temperature was at 1400 rpm for 30 minutes. PBMCs were removed from the buffy interface between plasma and either Histopaque or Ficoll-Paque and washed twice in PBS. Cells from patients and controls were stimulated by incubating with BMA-031 (pan anti-TCR MAb) at 100 μg/ml in PBS for 40 minutes on ice. Unstimulated and stimulated PBMCs were cultured overnight at 37°C and 5% CO_2 in 24-well plates, previously coated with goat anti-mouse Ab. PBMCs exposed to 500 rads of γ-irradiation were used as a positive control. Aliquots of PBMCs were fixed in 1% paraformaldehyde for morphological assessment at time zero and after overnight incubation.

3.4.2. Phenotypic labeling of cells for flow cytometry

PBMCs were labelled for flow cytometry immediately after isolation *ex vivo* and overnight incubation. Non-specific staining was first excluded by incubating cells for 10 minutes in staining solution containing human gamma globulin at 1 mg/ml. Representative samples were also stained with an isotype control Ab to exclude non-

specific uptake of MAb by dying or dead cells. CD4 and CD8 surface markers were labelled with biotinylated MAbs by incubating at 37°C for 30 minutes. Cells were then counterstained with streptavidin-PE or FITC and incubated at 4°C in the dark for 20 minutes. In some experiments, cells were stained using PE-conjugated CD4 and CD8 MAbs (direct staining protocol). After washing, samples were fixed in 1% paraformaldehyde in PBS. They were protected from light at room temperature for 30 minutes for HIV inactivation and then at 4°C until flow cytometric analysis, usually within 24 hours.

3.4.3. Flow cytometry

A FACScan (Becton Dickinson) was used for flow cytometric analysis. Ten thousand scatter events were recorded per sample. Standard compensation techniques were used. Events were accumulated at the same flow rate for each patient and control pair in order to decrease intra-assay variability. Data were stored in list mode and analyzed using Lysys software (Becton Dickinson).

The initial step for analysis of CD4+ T cell apoptosis was to draw a gate for CD4+ T cells only from the FL2 histogram, thus electronically excluding debris and CD4- cells. The forward and side scatter of CD4+ T cells was then further analyzed on a dot plot diagram. Region "R1" was drawn around the major lymphocyte population and a second region "R2" to the left, representing cells with diminished forward and side scatter [40, 62, 70].

3.4.4. Statistics

Statistical analyses were performed using JMP v 4.04, SAS Institute, NC, USA. The paired t test was used for comparisons between HIV-infected patients and seronegative controls. Each patient assay was performed simultaneously with a control sample, and each "patient and control pair" was studied at a separate time point. Hence, because each "patient and control pair" was *time-matched*, the paired t test is appropriate and valid [106]. The p-value for observed significance of a two-tailed test is reported. Data are presented as median and range.

For regression analyses, the method of least squares was used. R^2 measures the proportion of variation around the mean explained by the linear model. The remaining variation is attributed to random error. R^2 is 1 if the model fits perfectly. An R^2 of zero indicates that the fit is no better than the simple mean model. By analysis of variance, the total variance of a sample is partitioned into components. These are used to compute an F ratio that evaluates the effectiveness of the model. The ratio measures difference from a horizontal line at the mean.

3.5. Validation of flow cytometric scatter assay for CD4+ T cell apoptosis: identification of cell morphology in "viable" and "apoptotic" regions.

3.5.1. Aim

The aim of the experiment was to confirm that CD4+ T cells designated as "viable" had the morphology of viable cells and those in the "apoptotic" region of apoptotic cells. Apoptotic cells are smaller and denser than viable cells, hence have diminished forward and increased side scatter [40, 55, 59, 70, 107].

3.5.2. Method

PBMCs from a HIV-seronegative individual were exposed to 500 rads of γ-irradiation were incubated for 48 hours at 37°C and in 5% CO_2. Thereafter, cells were labelled with an anti-CD4 (Leu 3a-PE) or isotype control MAb. A region for CD4+ T cells was drawn from a FL2 histogram. The forward and side scatter of electronically gated CD4+ T cells was then studied. The main population of electronically gated CD4+ PBMCs was placed in R1, the region for "viable" PBMCs. A second region (R2) was drawn to the left, signifying cells with diminished forward and increased side scatter.

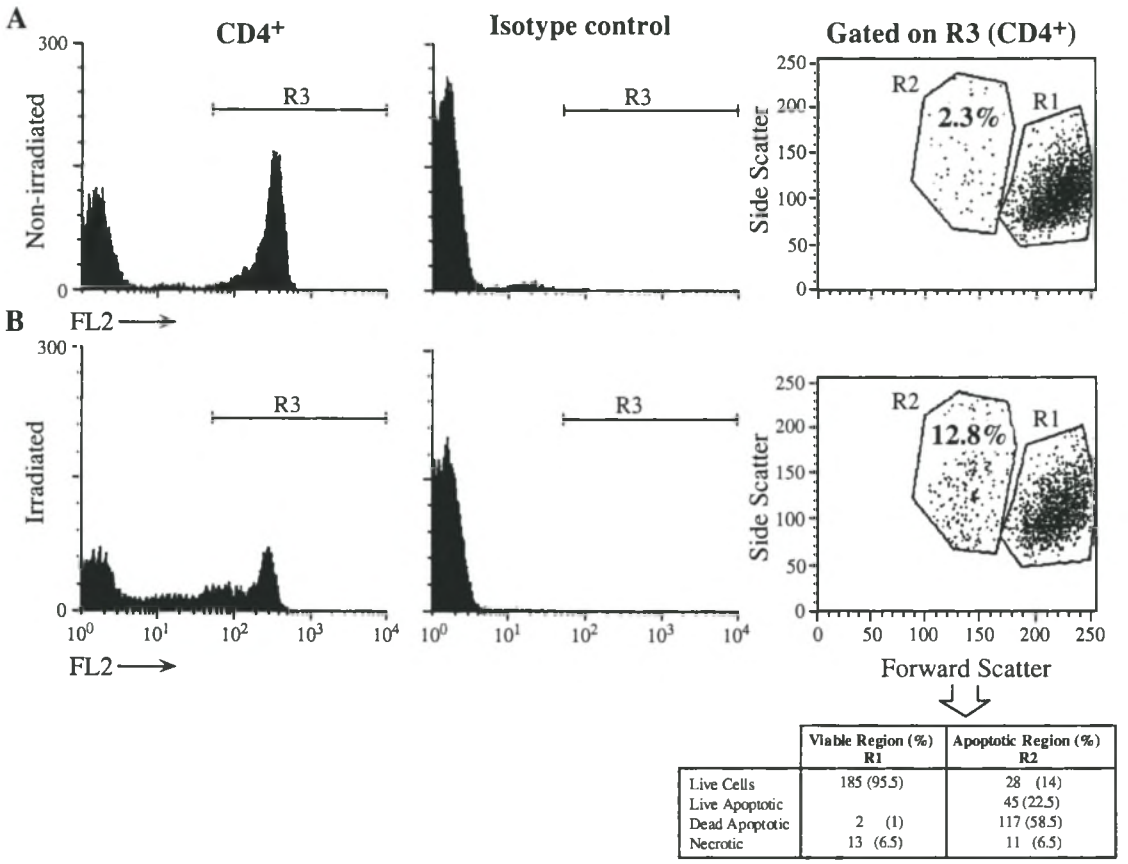
CD4+ T cells were then sorted into R1 (viable) and R2 (apoptotic) populations by scatter characteristics, using a Coulter EPICS 700 cell sorter, and analyzed by fluorescence microscopy for apoptotic morphology using ethidium bromide and acridine orange.PBMCs were immediately suspended in an equal volume of PBS with acridine

orange (3 μg/mL) and ethidium bromide (10 μg/mL). Microscopy was performed on cells using a Nikon Diaphot-TMD fluorescent microscope at 400X magnification. Nuclei of "viable" cells took up acridine orange only and had an "open" chromatin pattern. Live apoptotic cells had condensed nuclear chromatin, but excluded ethidium bromide. Cells with an "open" chromatin pattern, but with ethidium bromide uptake were regarded as necrotic. Dead apoptotic cells had condensed chromatin but took up ethidium bromide [57]. I performed the microscopy.

3.5.3. Results

The results of sorting γ–irradiated $CD4^+$ T cells into "apoptotic" and "viable" populations by scatter are shown in Figure 3. In the irradiated sample, normal morphology was found in 95% of cells from **R**1 (the viable region) and in only 14% from R2 (the apoptotic region). In contrast, 1% of cells in R1 were apoptotic as opposed to 71% in R2.

Before and after sorting, approximately 75% of apoptotic cells were dead, illustrating the importance of a flow cytometric assay that detects both live and dead apoptotic cells. Only 6% of cells from either the "viable" or "apoptotic" regions were necrotic, suggesting that necrotic cells are unlikely to confound the quantification of apoptotic cells.



	Viable Region (%) R1	Apoptotic Region (%) R2
Live Cells	185 (95.5)	28 (14)
Live Apoptotic		45 (22.5)
Dead Apoptotic	2 (1)	117 (58.5)
Necrotic	13 (6.5)	11 (6.5)

Figure 3. Scatter characteristics of irradiated and non-irradiated CD4+ T cells· (A) non-irradiated PBMCs; (B) irradiated PBMCs. Irradiated PBMCs were stained with PE-conjugated anti-CD4 MAb and positively stained cells were then sorted by scatter characteristics into R1 (viable) and R2 (apoptotic) subpopulations. Apoptotic cells have decreased forward and increased side scatter. Cells from each region were examined by fluorescence microscopy for apo ptotic morphology after staining with acridine orange and ethidium bromide. PBMCs were obtained from a HIV-uninfected individual.

3.5.4. Discussion

The sorting experiment confirmed the validity of the scatter assay as CD4+ T cells were sorted by scatter characteristics into apoptotic and viable cells. Cells with apoptotic morphology were confined to the "low forward scatter / high side scatter" region and viable cells to the "high forward scatter / low side scatter" region. [1] The sorting experiment also confirmed that apoptotic CD4+ T cells express the CD4 receptor on the cell surface. Also of interest were the lack of contribution of necrotic cells to overall percentages and the presence of equal numbers in "apoptotic" and "viable" regions. The latter finding suggests that necrotic cells should not influence the assay as they were found in both regions and in low numbers.

---

[1] The sorting experiment was also performed on irradiated CD8+ cells. PBMCs were subjected to 500rads of γ irradiation and incubated for 48 hours. PBMCs were stained with PE-conjugated leu 2A CD8 MAbs and sorted by scatter criteria into viable (R1) and apoptotic (R2) regions as illustrated in figure 4. Sorted populations were stained with Hoechst 33342 dye and examined by fluorescence microscopy with an ultraviolet filter (Nikon Diaphot-TMD fluorescent microscope at 400X magnification, 10µl of cell suspension mixed with 10µl of dye at 25 µg/ml in PBS). In an unsorted sample, 70% of cells were viable and 30% apoptotic. After sorting, 94% of cells in R1 were viable and 94% in R2 apoptotic.

3.6. Clinical examples of CD8+ T cell apoptosis in HIV-infected patients measured by the scatter assay.

An example of CD8+ T cell apoptosis is illustrated in Figure 4 where two HIV-infected children (stages N2 and C3) were studied simultaneously with a seronegative control both immediately *ex vivo* and after overnight incubation. PBMCs irradiated (500 rads) and cultured overnight served as a positive control for the experiment.

The two seropositive patients showed a higher percentage of apoptosis than the seronegative controls. The more severely affected "C3" patient (time zero - 3.5%; overnight incubation - 7%) had a higher percentage of apoptotic cells than the simultaneously studied "N2" patient (time zero - and overnight incubation - 1.5%). In the seronegative control, CD8+ T cell apoptosis was 0.5% at time zero and 0.7% after overnight incubation.

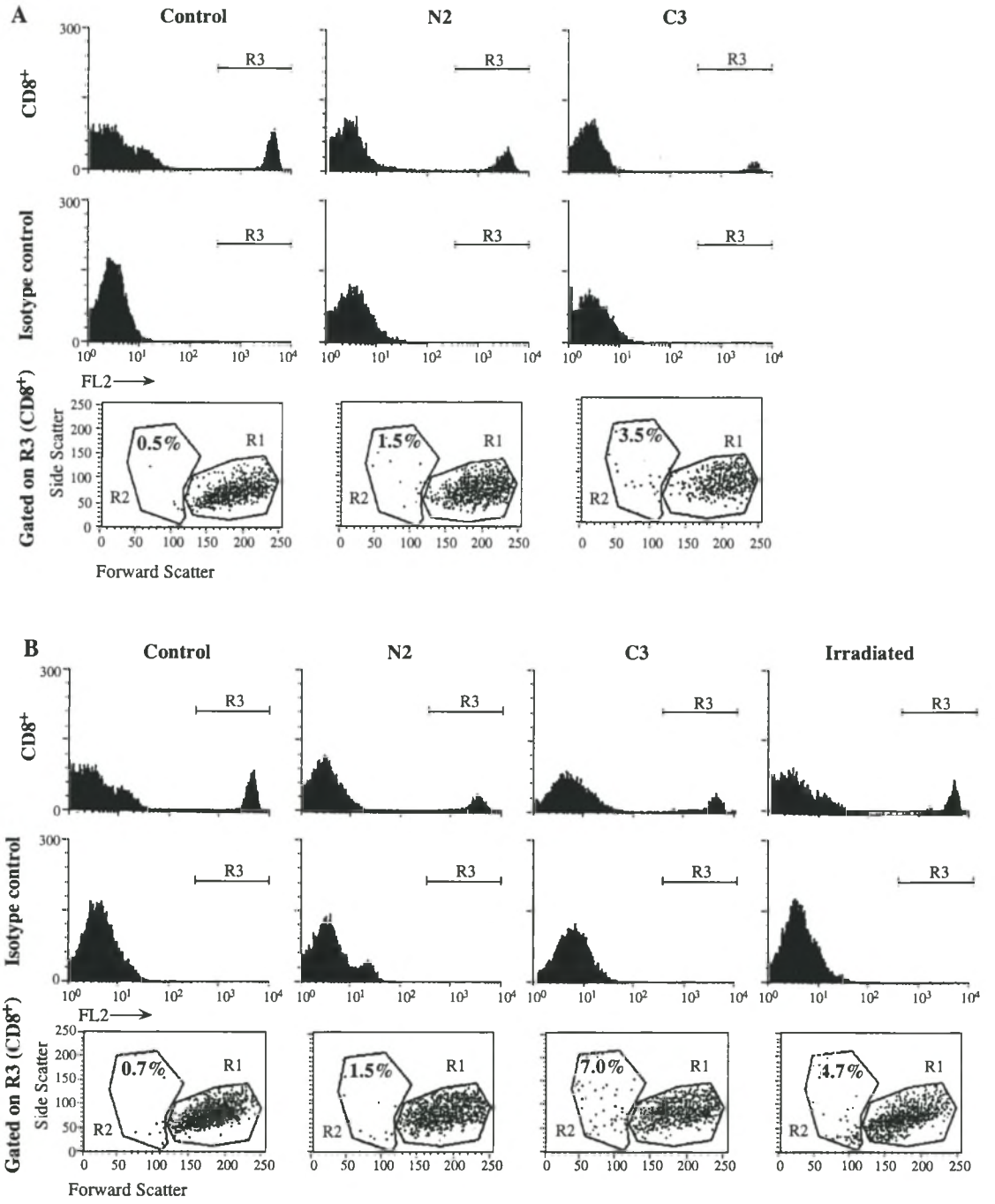


Figure 4. Increased percentages of CD8+ T cell apoptosis (A) *ex vivo* and (B) after overnight incubation in HIV-infected children. CD8+ T cell apoptosis was assayed by the scatter assay immediately *ex vivo* in a seronegative control and two HIV-infected patients,

one with moderate disease and moderate CD4+ T cell depletion (B2) and another with both severe disease and CD4+ T cell depletion (C3). PBMCs irradiated with 500 rads and incubated overnight serve as a positive control for apoptosis.

3.7. Reproducibility of scatter assay

3.7.1. Introduction and aims

The scatter assay relies on differences in light scattering characteristics of apoptotic and viable cells. In order to explore the reproducibility of this assay, I undertook an evaluation of variability in percentage CD4+ and CD8+ T cell apoptosis by repeat evaluation of specimens, but after accumulation of more scatter events. I considered this especially important for patients with severe CD4+ T cell depletion (hence low numbers of cells).

3.7.2. Methods

In clinical samples submitted for flow cytometric analysis, 10^4 scatter events were collected for analysis. Immediately thereafter, 10^5 events were accumulated under the same conditions and flow rate. Apoptosis was measured as previously described. Briefly, PBMCs were labelled for flow cytometry immediately after isolation *ex vivo* or after overnight culture. Only the direct labelling protocol was followed. Cells were stained using PE-conjugated CD4 and CD8 MAbs PBMCs were incubated with MAbs in the dark at 4°C in the dark for 20 minutes. The flow cytometric assay for apoptosis by scatter consisted of drawing a gate for CD4+ or CD8+ T cells from the FL2 histogram, thus electronically excluding debris. The forward and side scatter of labelled T cells was then further analyzed on a dot plot diagram. Region "R1" was drawn around the major lymphocyte population and a second region "R2" to the left, representing cells with

diminished forward and increased side scatter. Statistical analysis was by linear regression and R^2 and p values are reported.

3.7.3. Results

Twelve samples were studied, 9 from HIV-1-infected study subjects and three from seronegative controls; nine of which were immediately *ex vivo* and three after overnight incubation. The median CD4% was 5% (0.9% – 20%). The seronegative samples included two assayed immediately and one after overnight incubation.

In linear regression analysis shown in Figure 5, there was a high degree of correlation for 10^4 and 10^5 scatter events (CD4+: $R^2 = 0.99$; $p = 0.0000$; $n = 10$) and (CD8+: $R^2 = 0.99$; $p = 0.0000$; $n = 8$).

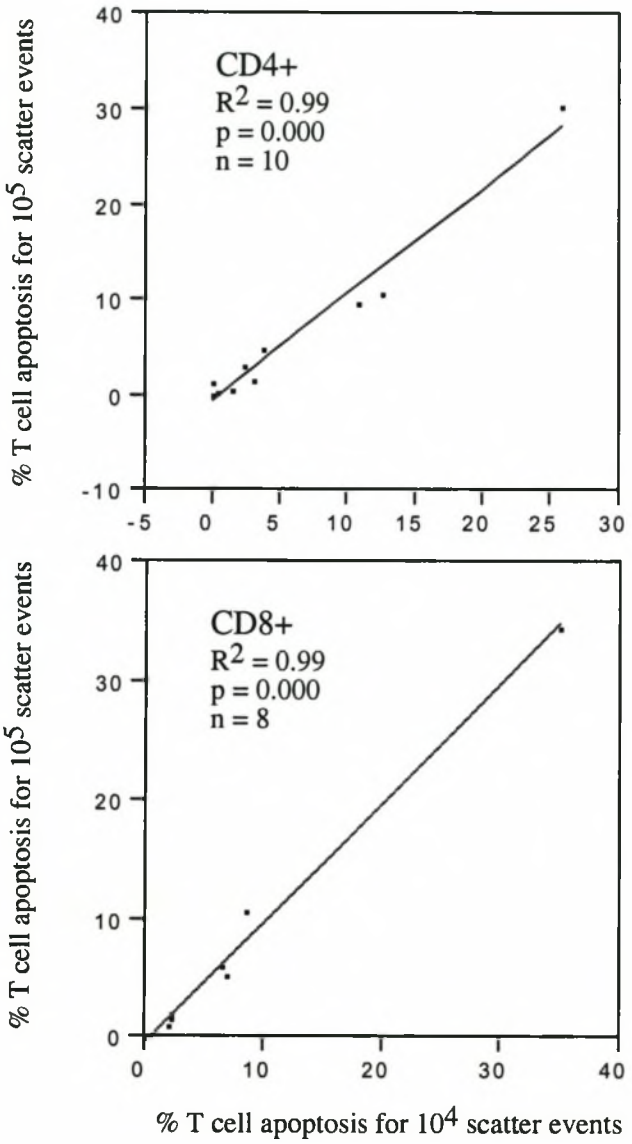


Figure 5: Comparison of 10^4 and 10^5 scatter events in identical samples after measurement of CD4+ and CD8+ T cell apoptosis by the scatter assay

3.7.4. Discussion and conclusion

A significant correlation was found when 10^4 and 10^5 scatter events were compared. The analysis was performed in order to confirm the reliability and reproducibility of the scatter assay and supports its use for measurement of CD4+ and CD8+ T cell apoptosis in clinical samples, including those with severe CD4+ T cell depletion.

3.8. Comparison between trypan blue uptake for PBMC cell death and CD4+ T cell apoptosis measured by the scatter assay

3.8.1. Introduction and aims

Trypan blue is excluded by viable cells but is taken up by dead cells that have lost their membrane integrity. The trypan blue assay does not address the mechanism of cell death but has been used as an indirect marker of apoptosis [79, 86].

In this analysis I sought to demonstrate cell death by trypan blue uptake in PBMCs from HIV-infected patients. A second aim was to compare cell death as measured by trypan blue uptake and CD4+ T cell apoptosis as measured by scatter and to show that both were elevated in HIV-infected patients in comparison with seronegative controls.

3.8.2. Method

3.8.2.1. Trypan blue

Unfixed samples were examined by light microscopy (Nikon TMS-F, inverted phase contrast microscope at 100X magnification; 10X ocular and 10X objective lens). Dead cells appear blue as loss of membrane integrity permits the entry of trypan blue whereas viable cells exclude the dye. Cell death after overnight incubation was assayed by trypan blue uptake. Unfixed aliquots from seronegative controls and HIV-infected patients were suspended in 0.2% trypan blue and counted after harvesting.

Briefly, the following method was employed: note was taken of the volume of cell suspension from which 10 µl were added to 50 µl PBS and 40 µl of 0.2% trypan blue, thus creating a 1/10 dilution. Cells were counted at 10X magnification on four squares of a Neubauer haemocytometer. The total and percentage of live and dead cells were calculated according to the following formula:

Cell count/4 X 10^4 X 10 (dilution factor) X volume of cell suspension

3.8.2.2. CD4+ T cell apoptosis

CD4+ T cell apoptosis was measured flow cytometrically by the scatter method described above. Briefly, the scatter characteristics of MAb-labelled CD4+ T cells were studied. The main population of cells was gated in region 1 (R1) and labelled as "viable". An apoptotic gate, region 2 ("R2") was drawn for CD4+ T cells with diminished forward and increased side scatter and the percentage of cells in R2 was calculated.

3.8.2.3. Patient selection

Patients and seronegative controls were from Denver, where trypan blue and scatter data were available for analysis. A full description of subject demographics is presented in chapter 5.

3.8.3. Results

There was significantly more cell death measured by trypan blue uptake in HIV+ patients than controls as shown in Table V. Similarly, CD4+T cell apoptosis was also elevated in HIV-infected study subjects. For most comparisons p-values of similar magnitude were obtained. Of note is that in both assays higher values were obtained for PBMCs from HIV-infected patients than seronegative controls (stimulated and unstimulated). When seronegative controls were compared to irradiated samples, a significant increase in CD4+ T cell apoptosis was seen by scatter; in contrast, no differences in cell death were evident by trypan blue uptake.

Table V. Percentages cell death measured by trypan blue uptake and CD4+ T cell apoptosis measured by scatter technique; both after overnight incubation

	Control n = 20	Control stimulated n = 20	HIV-infected n = 20	HIV-infected stimulated n = 20	Irradiated n = 16
% trypan blue uptake	6.6 (1.1 - 22.1)	9.27 (1.8 - 25)	10.71 (0.7 - 25.5)	14.36 (6.6 - 33)	6.67 (2.8 - 64.5)
% CD4+T cell apoptosis	3.35 (0.7 - 11.2)	4.2 (1 - 11.4)	7.45 (0.6 - 25.5)	8.8 (2.2 - 34.6)	8.8 (2.2 - 34.6)

Data presented as median (range). Comparisons by paired t test.

P values (paired t test)

		Trypan blue:	CD4+ T cell apoptosis
Control vs:	HIV:	0.055	0.0013
	Control(s)	0.025	0.23
	Irradiated	0.28	0.008
HIV(s) vs:	Control(s)	0.001	0.002
	HIV	0.03	0.014

PBMCs were stimulated by incubating with BMA-031 (pan anti-TCR MAb) at 100 μg/ml in PBS for 40 minutes on ice. PBMCs exposed to 500 rads of γ-irradiation were used as a positive control. Irradiated PBMCs were from a seronegative control.

Stimulated (s)

3.8.4. Discussion

The above data confirms that cell death as assayed by trypan blue uptake in clinical samples from HIV-infected patients is significantly higher than in seronegative controls. Similarly, CD4+ T cell apoptosis is also higher in stimulated and unstimulated samples from HIV-infected patients in comparison to seronegative controls. Therefore, the comparisons between HIV-infected and uninfected study subjects which show significantly increased cell death by both methods, supports the scatter method for measuring CD4+ or CD8+ T cell apoptosis.

Trypan blue uptake measures death of all PBMCs including CD8+ T cells and B cells. Both contribute to cell death. In irradiated samples, CD4+ T cell apoptosis appeared higher than cell death measured by trypan blue uptake suggesting that scatter changes might precede loss of membrane integrity and might thus be a more sensitive marker for cell death.

3.9. Fluorescence microscopy for detection of PBMC apoptotic morphology in clinical samples from HIV-infected and seronegative controls

3.9.1. Introduction and aims

Morphological changes of apoptotic cells have been well characterized by Duke [57]. Apoptosis of PMBCs is accompanied by chromatin condensation and formation of apoptotic bodies. Chromatin condensation is easily visualized by fluorescence microscopy using fluorescent dyes that stain nuclear material.

The aim of this analysis is to demonstrate that apoptotic cells were present in clinical samples from HIV-infected patients and seronegative controls both immediately *ex vivo* and after overnight incubation. Secondary aims were to compare apoptotic cell death by fluorescence microscopy and CD4+ T cell apoptosis measured by scatter in the same group of HIV-infected and uninfected subjects.

3.9.2. Method

PBMCs, previously fixed in 1% paraformaldehyde, were suspended in Hoechst 33342 dye (10µl of cell suspension mixed with 10µl of dye at 25 µg/ml in PBS) and examined by fluorescence microscopy (Nikon Diaphot-TMD; 400X magnification), using an UV filter. Apoptosis was identified by the characteristic increased density of nuclear chromatin [57]. Mary Schleicher, Immunology Core Facility, University of Colorado Health Sciences, Denver, CO performed the fluorescence microscopy. She was unaware of the identity of the specimens. Two hundred cells were counted for each specimen. Statistical analysis was by the paired t test as samples were time-matched. Scatter methodology was

as described in the previous two sections. All patients and seronegative controls were derived from the Denver clinic and demographic data are presented in the next chapter.

3.9.3. Results

Morphological assessment confirmed the presence of apoptotic cells in clinical samples, but revealed no significant difference between patients and controls immediately *ex vivo*. After overnight culture, however, significantly more apoptosis was seen in HIV-infected patients than in controls. Again, differences were more marked in BMA031-stimulated samples (Table VI).

Comparisons of CD4+ T cell apoptosis by the scatter assay are shown in Table VII. Significantly higher levels were found in HIV-infected study subjects at all time points, including immediately *ex vivo*.

Table VI. PBMCs with apoptotic morphology in HIV-infected and seronegative patients

Time point	Number of time-matched pairs	HIV+ve (%)	Control (%)	p-value
Ex vivo	9	6 (1 - 10)	2 (0 - 14)	0.28
Day 1 unstimulated	9	13 (0 – 21)	9 (1.5 – 18)	0.085
Day 1 stimulated	9	12 (2 – 24)	6 (0 – 8)	0.003

Data presented as median (range).

Morphology was assessed by fluorescence microscopy (Nikon Diaphot-TMD; 400X magnification) with a UV filter, using the Hoechst 33342 stain (10µl of cell suspension mixed with 10µl of dye at 25 µg/ml in PBS). Cells were stimulated with BMA031. Apoptotic cells were identified by the characteristic nuclear morphology. Two hundred cells were counted for each condition. Statistical analysis was by the paired t test.

Table VII. CD4+ T cell apoptosis measured by scatter in HIV-infected and seronegative patients

Time point	Number of time-matched pairs	HIV+ve (%)	Control (%)	P-value
Ex vivo	7	5.9 (0.2 - 16.7)	2.9 (0.7 - 5.8)	0.02
Day 1 unstimulated	8	8.4 (2.2 – 36.9)	4.4 (0.7 – 12.4)	0.04
Day 1 stimulated	9	15.8 (4.8 - 70)	4.5 (1.4 – 16.9)	0.08

Data presented as median (range).

Statistical analysis was by the paired t test for time-matched pairs. CD4 surface markers were labelled with biotinylated MAbs and counterstained with streptavidin-PE.

3.9.4. Discussion

The above data demonstrates that apoptotic PBMCs as measured by fluorescence microscopy were present in clinical samples studied immediately *ex vivo* and after overnight incubation. No significant differences were detected between infected and uninfected patients immediately *ex vivo*. Yet, in this small sample, increased CD4+ T cell apoptosis was evident in HIV-infected patients when determined by the flow cytometric scatter method. Possible explanations for this divergence *ex vivo* are that a) for morphological assessment, CD4+ T cells, CD8+ T cells and B cells are counted b) for microscopy, only 200 cells were counted compared to 10 000 events with flow cytometry c) a decrease in cell size precedes DNA fragmentation [108]. DNA fragmentation is most likely to be associated with changes in nuclear morphology. Some clumping of chromatin does occurs at the time of cell shrinkage but, in my opinion, is hard to differentiate from normal nuclear morphology. It is, therefore, likely that the early morphological changes of apoptosis will not be easily detected by fluorescence microscopy, while flow cytometry will detect these early changes.

3.10. TdT assay for DNA fragmentation

3.10.1. Introduction and aims

DNA fragmentation, characteristic of apoptosis may also be detected by ISEL, a technique suitable for histology [63] and flow cytometry [64]. Nucleotides tagged to fluorochromes are incorporated onto terminal ends of fragmented DNA by means of TdT.

When used for flow cytometry, apoptosis can be detected in specific cell types identified by MAbs attached to a second fluorochrome.

The main aim was to compare CD4+ T cell apoptosis as measured by both the scatter and TdT methods. Comparisons were made firstly for HIV-negative samples in which apoptosis was induced by the ionophore beauvericin. Thereafter specimens from HIV-infected patients and seronegative controls were compared. Demographic data of patients and seronegative controls are presented in the next chapter.

3.10.2. Methods

PBMCs were stained with Leu 3a-PE and isotype control MAbs, fixed in 1% paraformaldehyde and analyzed by the scatter assay. Representative samples were stored in 75% ethanol for 12 - 36 hours. The TdT assay was performed as described by Gorczyca *et al* [64] and adapted by Su *et al* [91]. The TdT reaction was carried out in the presence of biotin-16-dUTP. FITC-avidin was used to detect biotin-16-dUTP incorporated onto terminal portions of DNA. Flow cytometric analysis was by FACScan. Ten thousand events were recorded for each specimen. Correction was made for non-specific uptake of biotin-16-dUTP and streptavidin-FITC background staining in CD4+ PBMCs.

In order to compare the two assays for CD4+ T cell apoptosis, 4 samples were studied on separate occasions. On each occasion, PBMCs were incubated overnight in cell culture medium with or without beauvericin at 6μmol/L. Thereafter, CD4+ T cell apoptosis was compared immediately *ex vivo* in nine patient-control pairs (demographics presented in next chapter) by the scatter and TdT methods.

3.10.3. Statistics

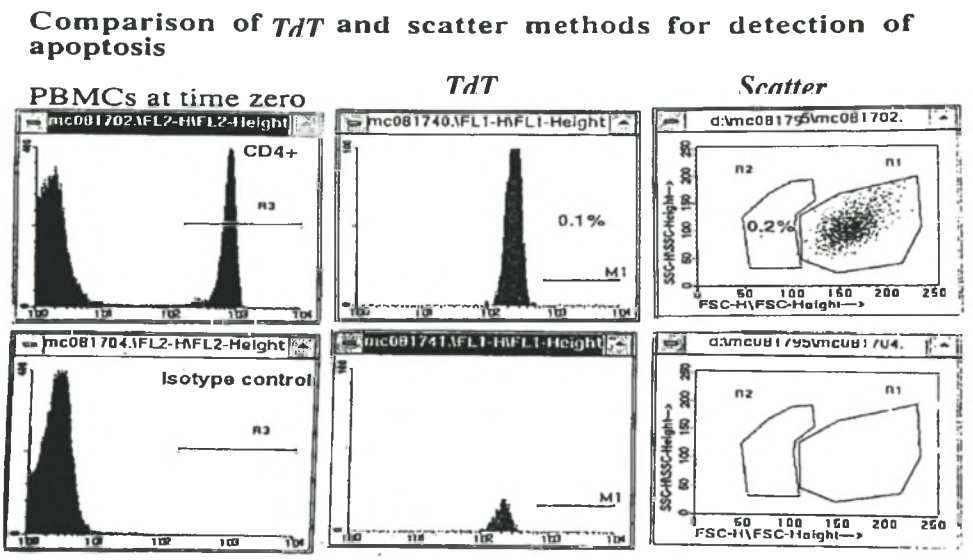
Statistical analysis was by paired t test for time-matched specimens and linear regression for comparison of the scatter and TdT methods.

3.10.4. Results

3.10.4.1. Beauvericin

The TdT assay was compared to the scatter assay where apoptosis was induced by the ionophore, beauvericin in healthy HIV-seronegative study subjects. A representative experiment is shown in Figure 6. Here 4.7% of CD4+ T cells were apoptotic by the TdT and 7.4% by the scatter method. Although the scatter assay tended to show higher levels of apoptosis, the two methods correlated well (R^2 = 0.89; p = 0.06; n = 4). In the untreated samples, both the TdT and scatter methods showed few apoptotic events (0.1% and 0.2% respectively).

A



B

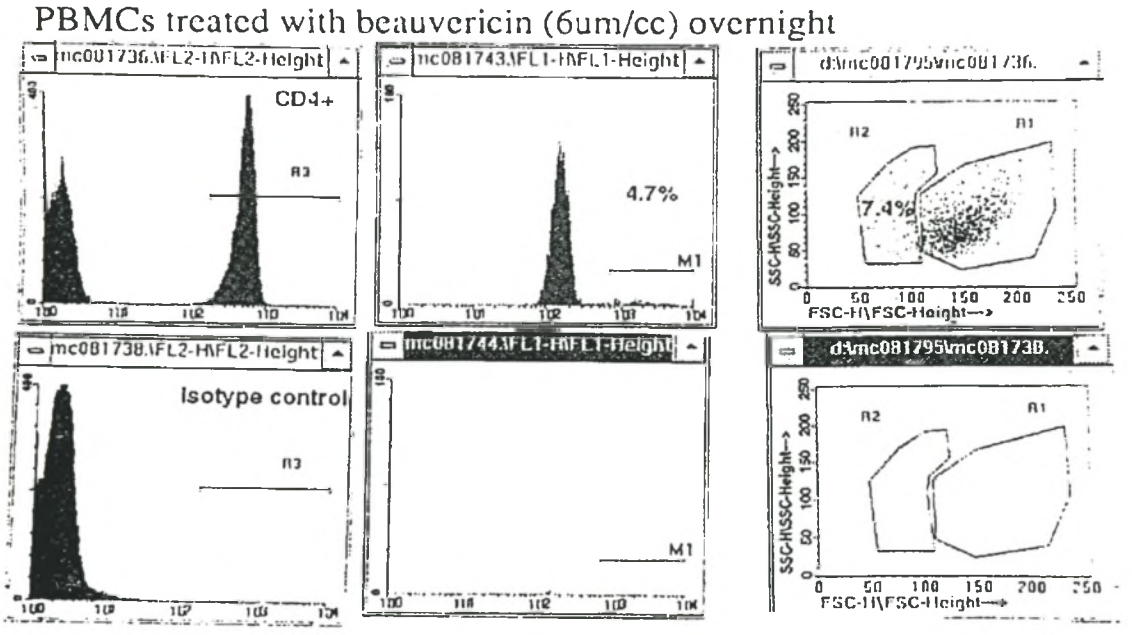


Figure 6. Comparison of scatter and TdT assays for apoptosis A) immediately *ex vivo* and B) after overnight incubation with beauvericin at 6µmol/l. PBMCs from a HIV-

seronegative subject were assayed for CD4+ T cell apoptosis immediately *ex vivo* by both the scatter and TdT assays. The assays were repeated after overnight incubation in beauvericin at 6 µmol/l to induce apoptosis. An isotype control MAb was used to exclude non-specific staining.

3.10.4.2. HIV-infected patients versus seronegative controls

The scatter and TdT assays were also compared in PBMCs isolated immediately *ex vivo* from nine patients and nine seronegative controls. Each pair was studied individually. Individual percentages of CD4+ T cell apoptosis obtained by both the scatter method and the TdT method are shown in Table VIII. There was a good correlation for both corrected (HIV-infected patient minus seronegative control) CD4+ T cell apoptosis within each pair ($R^2 = 0.73$; $p = 0.0034$) and for uncorrected apoptosis ($R^2 = 0.59$; $p = 0.015$).

There was no difference by the paired t test between percentages of CD4+ T cell apoptosis obtained by the scatter and TdT assay when compared in controls ($p = 0.79$; paired t test) and when compared in HIV-infected study subjects ($p = 0.56$; paired t test) suggesting that the two assays gave similar percentages of apoptosis. Similarly for corrected apoptosis (HIV minus control), there was no difference between the assays ($p = 0.64$; paired t test).

Control data was subtracted from patient data as region sizes were drawn for each time-matched pair. The amount of scatter in a region can be influenced by region size. In a larger the region size, there is a strong possibility that a higher percentage of scatted data would be captured. Thus there is potentially interassay variability in region sizes. By subtracting control data from HIV-infected, one corrects for potential inter-assay variations in region size.

Table VIII. Comparison of percentage of CD4+ T cell apoptosis measured by both the scatter method and TdT assay in HIV-infected study subjects and seronegative controls immediately *ex vivo*

Percentage CD4 T cell apoptosis					
TdT assay			Scatter assay		
Seronegative control	HIV+	HIV minus control	Seronegative control	HIV+	HIV minus control
1	2.6	1.6	1.6	3.4	1.8
0.25	0.47	0.22	0.5	1.3	0.8
0.4	0.83	0.43	3.3	5.5	2.2
0.58	1.98	1.4	3.7	4.2	0.5
0.93	1.84	0.91	3.4	1.6	-1.8
3.7	28	24.3	2.1	9.1	7
1.27	1.38	0.11	0.5	3.6	3.1
3.38	7.68	4.3	0.8	2	1.2
12.83	2.27	-10.56	5.8	3.5	-2.3
Median (range) 1 (0.25 – 12.83)	Median (range) 1.98 (0.47 – 28)	Median (range) 0.91 (-10.56 – 24.3)	Median (range) 2.1 (0.5 – 5.8)	Median (range) 3.5 (1.3 - 9.1)	Median (range) 1.2 (-2.3 – 7)

Each pair (HIV-infected patient and seronegative control) was assayed by both methods but at a different time points.

Regression analyses:

1. Scatter vs TdT for "corrected" CD4+ T cell apoptosis (HIV minus control) - $R^2 = 0.73$; $p = 0.0034$

2. Scatter vs TdT for "uncorrected" CD4+ T cell apoptosis (HIV) - $R^2 = 0.59$; $p = 0.015$.

3.10.5. Discussion

DNA fragmentation measured by the TdT assay correlated with the scatter method for beauvericin-treated apoptotic CD4+ T cells and in clinical samples *ex vivo* from HIV-1-infected children, thus providing further evidence that the scatter-based assay has validity for measuring T cell apoptosis in clinical samples. The scatter-based assay is far less expensive than the TdT method as neither additional reagents nor laboratory time are necessary.

3.11. Annexin V assay

3.11.1. Introduction and aims

Fluorochrome-labelled Annexin V has been used for flow cytometric detection of apoptotic PBMCs [65]. I compared the annexin V uptake to the scatter technique for evaluation of CD4+ T cell apoptosis in PBMCs cultured overnight in increasing concentrations of beauvericin. In addition, cell death measured both by trypan blue uptake and fluorescence microscopy (apoptotic morphology) was correlated with the two flow cytometric methods.

3.11.2. Methods

3.11.2.1. General

PBMCs were obtained from two separate seronegative individuals on different occasions (termed experiment 1 and 2). PBMCs were isolated by Histopaque density centrifugation and cultured overnight as previously described. Samples were also prepared for analysis immediately *ex vivo*. PBMCs were cultured overnight in beauvericin at the following concentrations: 0.25, 0.5, 0.75, 1 and 2 μmol/L (concentration 1 μmol/μL in 100% ethanol). Control specimens without beauvericin were also cultured overnight. PBMCs were cultured at a cell density of 10^6/ml.

After overnight incubation, samples were harvested. Unfixed aliquots were examined immediately for trypan blue exclusion (method previously described). Additional samples were fixed in 1% paraformaldehyde and stored overnight for

fluorescence microscopy. Acridine orange at a concentration of 3μg/mL was mixed with an equal volume of cell suspension and examined by fluorescence microscopy as previously described. Wendy Brittle and Anel Clark from the department of Medical Microbiology, Tygerberg Hospital performed the microscopy. Staining with PE-conjugated CD4 MAb was as previously described.

3.11.2.2. Annexin V assay

The assay was performed according to manufacturer's instructions. Briefly, after washing in PBS, cells were resuspended in 100μL Hepes buffer and incubated with 2μL of annexin V at room temperature in the dark for 15 minutes. Cells were washed at 1500 rpm, resuspended in Hepes buffer and after a further wash, were fixed in 1% paraformaldehyde and stored at 4°C in the dark until flow cytometry was performed (usually on the same day).

3.11.2.3. Flow cytometry

Flow cytometry was performed on a FacsCalibur (BD). The scatter assay was performed as previously described. Briefly, CD4+ T cells were gated on a FL2 channel histogram. Forward and side scatter of CD4+ T cells were then viewed on a "dot-plot" scattergram. "Region 1" was drawn around the main population of cells and an apoptotic region termed "region 2" was drawn in the adjacent area of low forward scatter.

For annexin V analysis, CD4+ T cells were displayed on a FL1 vs FL2 dot plot. Gates for annexin v uptake were drawn using unstained PBMCs as controls. The percentage of PE+ve annexin V +ve cells were then calculated using appropriate software.

3.11.2.4. Statistics

Data are presented as median and range. Linear regression analysis was performed. R^2 measures the proportion of variation around the mean explained by the linear model. The remaining variation is attributed to random error. R^2 is 1 if the model fits perfectly and 0 if the fit is no better than the simple mean model. Observed significance probabilities of 0.05 or less are considered as evidence of a regression effect [109]. The same regions were used throughout for both experiments, and flow cytometry was performed at the same time for each experiment. For this reason, it was not necessary to subtract control data from beauvericin treated-samples.

3.11.3 Results

The data from the two experiments are shown in Table IX. Trypan blue uptake and apoptotic morphology were not assessed in the *ex vivo* samples from experiment 1. CD4+ T cell apoptosis measured by annexin V uptake gave higher values than the scatter method. The results of linear regression analysis are shown in Table X. CD4+ T cell apoptosis measured by scatter and annexin V showed a good correlation ($R^2 = 0.68$; $p = 0.0003$). PBMC cell death measured by trypan blue uptake and apoptosis measured by characteristic morphology in acridine orange-stained cells also showed a strong correlation ($R^2 = 0.54$; $p = 0.004$). PBMC apoptosis (fluorescence microscopy) correlated well with CD4+ T cell apoptosis measured by scatter but not to annexin V uptake. PBMC cell death, however, correlated poorly with CD4+ T cell apoptosis as measured by scatter and annexin V uptake.

Table IX. Comparison of beauvericin-induced CD4+ T cell apoptosis measured by annexin V uptake and scatter and cell death by apoptotic morphology and trypan blue uptake.

Beauvericin (µmol/L)	% CD4+ T cell apoptosis (annexin V)	% CD4+ T cell apoptosis (scatter)	% PBMC apoptosis (acridine orange)	% PBMC death (trypan blue uptake)
Experiment 1				
0 (*ex vivo*)	5.8	1.95	Not done	Not done
0 (overnight)	6.49	6.04	0	2.56
0.25	18	7.8	2	2.7
0.5	19.71	8.5	4	20
0.75	16.47	9.24	14	43.24
1	36.66	16.23	30	42.85
2	75.2	21.76	70	48.57
Experiment 2				
0 (*ex vivo*)	10.03	0.53	6	3.51
0 (overnight)	6.21	0.05	4	5.26
0.25	80.11	14.05	18	10.53
0.5	82.16	14.14	10	11.11
0.75	31.33	5	14	5.26

1	10.58	0.51	20	31.56
2	9.28	0.42	22	38.89

PBMCs from a healthy HIV seronegative study subject were exposed to increasing concentrations of beauvericin and incubated overnight. This experiment was repeated on two occasions.

Table X. Linear regression analysis comparing beauvericin-induced CD4+ T cell apoptosis measured by annexin V uptake and scatter and cell death by apoptotic morphology and trypan blue uptake

	Pairs	R^2	p value
CD 4+ T cell apoptosis (annexin V) vs:			
• CD 4+ T cell apoptosis (scatter)	14	0.68	0.0003
• PBMC death	13	0.02	0.66
• PBMC apoptosis	13	0. 27	0.07
CD 4+ T cell apoptosis (scatter) vs:			
• PBMC death	13	0.16	0.18
• PBMC apoptosis	13	0.39	0.02
PBMC apoptosis vs:			
• PBMC death	13	0.54	0.004

PBMC death measured by trypan blue uptake. PBMC apoptosis measured by fluorescence microscopy in PBMCs fixed in 1% paraformaldehyde and stained with acridine orange at 3μg/mL.

3.11.4. Discussion

The scatter assay correlated well with annexin V uptake for measurement of CD4+ T cell apoptosis. There was also a significant correlation between the scatter method and PBMC apoptosis as measured by acridine orange stainining. Comparison of CD4+ T cell apoptosis as measured by annexin V uptake with morphology assessed by acridine uptake was not significant.

The absence of a correlation between PBMC death as measured by trypan blue uptake and the two flow cytometric techniques can be explained by the different parameters being measured. Trypan blue uptake measures cell death in all PBMCs including B lymphocytes and CD8+ T cells. It will not detect apoptotic cells where membrane integrity is still intact. Apoptosis can be detected in cells with intact membranes by the scatter method [108] (and see section 5.4) and annexin V uptake [65].

3.12. General conclusions

The scatter-based flow cytometric assay provides a simple method for quantifying apoptosis in phenotypically identified subpopulations of PBMCs, and correlates well with apoptotic morphology and DNA fragmentation. Although used in the present studies to detect apoptosis in CD4+ and CD8+ T cells, by extension, any cell population labelled with fluorochrome-tagged MAb can be studied. By measuring the scatter characteristics of only those cells stained for a specific surface marker, one electronically excludes debris and other cells not identified by the surface marker and can quantify apoptosis in defined cell populations.

Although many other flow cytometric techniques have been described [60, 110, 111], advantages of the scatter assay include low cost, simplicity and safety, since cells are fixed in paraformaldehyde. The scatter assay does not involve the purchase of expensive reagents nor additional laboratory time.

Diminished forward scatter and increased side scatter reflecting decreased cell size and increased granularity were among the earliest flow cytometric characteristics of apoptosis recognized [41, 59, 61, 107]. Wesselborg and Kabelitz have shown that a decrease in forward scatter precedes DNA fragmentation [108] and might therefore be a sensitive marker of apoptosis. DNA fragmentation is most likely to be associated with changes in nuclear morphology. Although some chromatin clumping occurs at the time of cell shrinkage it is difficult to differentiate from normal nuclear morphology. Therefore, early morphological changes will not be easily detected by fluorescence microscopy, in contrast to flow cytometry.

Carbonari *et al* confirmed the sensitivity of a scatter-based approach. They detected apoptosis by a combination of scatter changes and reduced CD45 expression [70]. These authors showed that after irradiating cells to induce apoptosis, many cells with decreased forward scatter still expressed a high level of CD45, suggesting that scatter changes might precede loss of CD45 expression in apoptotic PBMCs.

Morphological assessment of fluorescent cells confirmed that apoptosis was present in PBMCs immediately *ex vivo*. Significantly elevated apoptosis and cell death (by trypan blue uptake) were shown after overnight incubation in unstimulated and stimulated PBMCs. The extent of apoptosis by fluorescence microscopy and cell death by trypan blue uptake was of a similar magnitude to CD4+ T cell apoptosis by the scatter assay.

All PBMCs were included in the microscopic assays, including B cells and CD8+ T cells. For this reason, subtle differences in CD4+ and CD8+ apoptosis might have been missed in samples assayed immediately *ex vivo* and after overnight incubation. Also of note is the higher percentages of apoptosis detected by scatter than cell death by membrane permeability, suggesting greater sensitivity of the scatter method. This was especially evident in irradiated cells.

As a further validation, DNA fragmentation measured by the TdT assay correlated with the scatter method for beauvericin-treated apoptotic CD4+ T cells and in clinical samples from HIV-1-infected children. The annexin V assay also showed an excellent correlation with scatter for CD4+ T cell apoptosis. Therefore, since I have shown that the scatter-based flow cytometric measurement of apoptosis correlated with published

techniques and that this method has been well validated (as presented in the current chapter), for the remainder of the thesis apoptosis was measured by the scatter method.

CHAPTER 4

DIRECT VERSUS INDIRECT MONOCLONAL ANTIBODY LABELLING OF PBMCS: EFFECT ON APOPTOSIS

4.1. Introduction

In the study undertaken to measure CD4+ and CD8+ T cell apoptosis in HIV-infected children and adults in Denver, CO and presented in the next chapter, two cell labelling methods were used for detecting CD4+ and CD8+ T cells prior to flow cytometry. Initially an indirect method was used. PBMCs were first incubated with biotinylated MAbs at 37°C for 30 minutes and then, with streptavidin-PE or FITC for 20 minutes at 4°C. In the course of the study, biotinylated MAbs were replaced by PE-conjugated MAbs, thus eliminating the need for an intermediate incubation period of 30 minutes at 37°C, and also the accompanying "washing" procedures. An analysis is presented in this chapter of the influence of the intermediary incubation step on CD4+ and CD8+ T cell apoptosis.

4.2. Aims

I compared CD4+ and CD8+ T cell apoptosis by the two labelling procedures to determine whether the intermediate incubation of PBMCs with biotinylated MAb at 37°C for 30 minutes might have enhanced apoptosis.

4.3. Materials and Methods

4.3.1. Patient selection

All study subjects were attending the Colorado Pediatric AIDS Clinical Trials Unit and were recruited between July 1993 and October 1994. HIV serology was performed on all pediatric controls to confirm seronegativity. Patients and controls were matched for age and time of blood draw.

4.3.2. Reagents

Ficoll-Paque was obtained from Pharmacia Biotechnica, Upsala, Sweden. BMA-031 (pan anti-TCR MAb) was the generous gift of Roland Kurrle, Behringwerke AG, Marburg, FRG. Leu 3a MAb was the generous gift of the Sloan-Kettering Institute. OKT8 and OKT3 MAbs were derived from cell lines obtained from the ATCC. All were of IgG1 isotype. Biotinylation and FITC conjugation were performed in the laboratory according to established protocols. Titering of MAbs was done on PBMCs from HIV-seronegative controls. Streptavidin-PE and FITC were purchased from Tago Pharmaceuticals, Burlingame, CA. Leu 3a-PE and Leu 2a-PE antibodies were purchased from Becton Dickinson, San Jose, CA. Murine isotype control Ab (IgG2a-FITC; IgG1-PE) was purchased from Olympus, Lake Success, NY.

4.3.3. Sample processing

Heparinized blood samples were collected from patients and seronegative controls. PBMCs were isolated by Ficoll-Paque or Histopaque density gradient centrifugation. Briefly, whole blood was diluted 1:1 with PBS and layered carefully over 30 ml of Histopaque or Ficoll-Paque. Centrifugation was performed at room temperature

at 1400 rpm for 30 minutes. PBMCs were removed from the buffy interface between plasma and either Histopaque or Ficoll-Paque and were washed twice in PBS. PBMCs exposed to 500 rads of γ-irradiation to induce apoptosis were used as a positive control.

4.3.4. Phenotypic labelling of cells for flow cytometry

PBMCs were labelled for flow cytometry immediately after isolation *ex vivo* . Non-specific staining was first excluded by incubating cells for 10 minutes in staining solution containing human gamma globulin at 1 mg/ml. Representative samples were also stained with an isotype control Ab to exclude non-specific uptake of MAb by dying or dead cells. Two labelling protocols were followed, initially an indirect method, in which CD4 and CD8 surface markers were labelled with biotinylated MAbs by incubating at 37°C for 30 minutes. Cells were then counterstained with streptavidin-PE or FITC and incubated at 4°C in the dark for 20 minutes (indirect method).

The method was changed to using PE-conjugated CD4 and CD8 MAbs (direct staining protocol) whereby PBMCs were incubated with MAbs in the dark at 4°C for 20 minutes, thus eliminating a 30-minute incubation period at 37°C in the presence of biotinylated MAbs.

After the labelling procedure, samples were fixed in PBS containing 1% paraformaldehyde. Samples were protected from light at room temperature for 30 minutes for HIV inactivation and then at 4°C until flow cytometric analysis, usually within 24 hours.

4.3.5. Flow cytometry

A FACScan (Becton Dickinson) was used for flow cytometric analysis. Ten thousand scatter events were recorded per sample. Standard compensation techniques were used. Events were accumulated at the same flow rate for each patient and control pair in order to decrease intra-assay variability. Data were stored in list mode and analyzed using Lysys software (Becton Dickinson).

The initial step for analysis of CD4+ and CD8+ T cell apoptosis was to draw a gate for CD4+ or CD8+ T cells only from the FL2 histogram, thus electronically excluding debris. The forward and side scatter of CD4+ T cells was then further analyzed on a dot plot diagram. Region "R1" was drawn around the major lymphocyte population and a second region "R2" to the left, representing apoptotic cells with diminished forward and increased side scatter [40, 62, 70].

4.3.6. Statistical analysis

Three analyses were undertaken. A) First, percentages of CD4+ and CD8+ T cell apoptosis were compared for samples labelled either by the indirect or direct procedure. Comparisons were undertaken for samples from HIV-infected study subjects and seronegative controls. B) Thereafter, comparisons were undertaken for HIV-infected study subjects after subtraction of apoptosis from the simultaneously assayed seronegative control patient. C) Lastly, data from individual patients evaluated by both labelling methods, were compared.

Statistical analyses were performed using JMP v 4.04, SAS Institute, NC, USA. Comparisons were by the Wilcoxon rank sums test for continuous and Chi-squares for

discrete variables. For comparison of individual patients studied by both labelling methods, the paired t test was used. A p-value is regarded as significant at or below 0.05. Data are presented, from time zero (i.e. immediately *ex vivo*), as median and range.

Graphical data are presented as quantile boxes. The lowest line represents 10th and uppermost the 90th centile. The box represents the 25th to 75th centiles and the horizontal line in each box represents the median for that sample.

For regression analyses, the method of least squares was used. R^2 measures the proportion of variation around the mean explained by the linear model. The remaining variation is attributed to random error. R^2 is 1 if the model fits perfectly. An R^2 of zero indicates that there is no correlation between the variables (no better than the simple mean model). By analysis of variance (ANOVA), the total variance of a sample is partitioned into components. These are used to compute an F ratio that evaluates the effectiveness of the model. The ratio measures the difference from a horizontal line at the mean and is reported as a p-value.

4.3.7. Results

4.3.7.1. Comparison of apoptosis in subjects where PBMCs were labelled either by direct or indirect method

Twenty-three samples were labelled by the indirect and 11 by the direct method. There were no significant differences in ages between the two groups either for HIV-infected study subjects or seronegative controls. CD4+ T cell percentages and antiretroviral therapy (ART) were also not significantly different (Table XI).

Table XI. Demographic data of study subjects and seronegative controls where PBMCs were labelled either by the indirect or direct method

	Indirect Labelling n = 23	Direct Labelling n = 11	p value
Age (years)			
HIV-infected	12.6 (0.1 - 40.9)	9.6 (0.5 - 38.7)	0.96
Control	16.3 (1 - 40.2)	13.8 (0.3 - 54.2)	0.65
Median CD4% of group	24 (0 - 44) [2]	25 (2 - 39)	0.89
Antiretroviral agents			0.89
1	10	5	
2	3	2	

Data presented as median (range). Analysis by Wilcoxon rank sums test.

[2] Two study subjects with 0 CD4+ T cell percentage by "conventional flow" cytometry were included as sufficient scatter events were considered to be accumulated in both R1 (viable) and R2 (apoptotic regions): one had 48 and 20 events and the other 151 and 283 events respectively in R1 and R2.

The comparison of CD4+ T cell apoptosis is shown in Table XII and CD8+ T cell apoptosis in Table XIII. Also shown in both tables is the effect of subtracting data of the simultaneously studied seronegative control from each study subject.

In seronegative controls and study subjects, CD4+ T cell apoptosis was significantly higher by the indirect labelling method ($p = 0.005$ and 0.004) respectively. However, when control values were subtracted from those of study subjects, the significant differences disappeared ($p = 0.18$).

The same significant differences were observed for CD8+ T cell apoptosis ($p = 0.003$ and 0.002), for controls and study subjects, respectively. Again, after correction by subtraction of control data from that of study subjects, the significance disappeared.

Table XII: Percentage CD4+ T cell apoptosis *ex vivo* in HIV-1-infected and seronegative study subjects by direct and indirect labelling

	% CD4+ T cell apoptosis		
	Indirect Labelling n = 23	Direct Labelling n = 11	p value
Control	4 (0.7 - 12.3)	1.5 (0 - 3.9)	0.005
HIV+	5.6 (0.2 - 29.4)	2. 6 (1.3 - 13.3)	0.004
HIV pos minus control	2.8 (-2.7 - 25.4)	1.2 (-1.8 - 11.8)	0.18

Median (range). Analysis by Wilcoxon rank sums test.

Table XIII: Percentage CD8+ T cell apoptosis *ex vivo* in HIV-1-infected and seronegative study subjects by direct and indirect labelling

	% CD8+ T cell apoptosis		
	Indirect Labelling n = 22	Direct Labelling n = 10	p value
Control	3.3 (0.3 - 18.4)	1.3 (0.4 - 4.6)	0.003
HIV+	8.1 (0.2 - 21.2	2.6 (1.6 - 6.6)	0.002
HIV+ minus control	2.4 (-8.3 - 9.2)	1.3 (-0.8 - 2.2)	0.35

Median (range). Analysis by Wilcoxon rank sums test.

4.3.7.2. Comparison of apoptosis in subjects where PBMCs labelled both by direct and indirect method

Both indirect and direct labelling was performed on PBMCs from a small group of HIV-infected patients. Demographic details of nine study subjects and seronegative controls are shown in Table XIV and XV respectively. No patient showed progression of disease during the interval between the two assays. No new ARV agents were commenced during the study period. The median time interval between the two assays was 13.6 (3.5 - 22.1) months. Seronegative controls were significantly older ($p = 0.008$) than study subjects where the direct labelling method was used. [3]

[3] The effect of age on CD4+ and CD8+ T cell apoptosis is addressed in the next chapter.

Table XIV. Demographic features of HIV-infected patients in whom apoptosis was assayed both by the indirect and direct labelling techniques

Number of study subjects	9
Time interval between 2 assays (months)	13.6 (3.5 - 22.1)
Median CD4%	19 (0.03 - 32)
Class: N A C	 1 3 5

Table XV. Comparison of demographic features in seropositive patients in whom CD4+ and CD8+ T cell apoptosis was measured by both indirect and direct labelling methods

	Seropositive n = 9	Seronegative n = 9	p value
Indirect Labelling			
male: female	1.25	3.5	0.34
age (years)	11.8 (4.8 - 18)	10 (4.1 - 37.7)	0.17
Direct Labelling			
male: female	1.25	2	1
age (years)[4]	13.1 (6.1 - 19.1)	38.4 (1.4 - 40.1)	0.008

[4] The patients were older when studied by the direct method.

The results of comparison are shown in Tables XVI and XVII. Again, apoptosis, when measured by the indirect labelling method, was significantly higher than after direct labelling for all comparisons except for percentage of CD4+ T cell apoptosis in HIV-infected patients ($p = 0.14$). After subtracting data from the simultaneously studied seronegative control, the differences were no longer significant for either CD4+ or CD8+ T cell apoptosis.

Table XVI: Comparison of percentage CD4+ T cell apoptosis *ex vivo* in the same HIV-1-infected patients after direct and indirect labelling

	% CD4+ T cell apoptosis		
	Indirect Labelling n = 8*	Direct Labelling n = 8	p value
Seronegative control	4.1 (1.4 - 12.3)	2.1 (0.3 - 3.9)	0.03
HIV+	10.9 (2.4 - 28.4)	5.2 (0 - 14.7)	0.14
HIV+ minus seronegative control	9.1 (-0.1 - 22.2)	3.5 (-0.6 - 11.8)	0.25

Median (range). Analysis by paired t test.

* no data by direct labelling for one study subject as non-specific labelling by isotype control Ab in excess of labelling by PE-conjugated CD4 MAb

Table XVII: Comparison of percentage CD8+ T cell apoptosis ex vivo in HIV-1-infected and seronegative study subjects after direct and indirect labelling

	% CD8+ T cell apoptosis		
	Indirect Labelling n = 9	Direct Labelling n = 9	p value
Control	7.4 (2.7 - 16.8)	1 (0.2 - 4.4)	0.001
HIV+	9.4 (7.4 - 19.5)	3.2 (0.3 - 10.5)	0.003
HIV+ minus control	3.6 (-8.3 - 7.8)	2.2 (-0.9 - 7.5)	0.93

Median (range). Analysis by paired t test.

4.3.8. Discussion

For both CD4+ and CD8+ T cell apoptosis, significantly higher values were obtained by the indirect method of CD4 and CD8 cell labelling in seronegative controls and HIV-infected patients. After subtracting control values from those of the simultaneously studied HIV-infected study subjects, the significant differences between the two labelling methods disappeared.

Absolute percentages of apoptosis were compared. Although regions were slightly different for each time-matched pair, the same gating strategy was used throughout; therefore it is unlikely that small differences in region size would have impacted significantly. In addition, this effect would occur in directly and indirectly labelled specimens in an equivalent manner.

A likely explanation for the increased levels of apoptosis after indirect labelling is that the incubation period at 37°C for 30 minutes, in the presence of MAbs to CD4 and CD8, induced apoptosis. Evidence for this comes from Oyaizu *et al.* [83] who investigated the contribution of CD4 ligation to CD4+ T cell apoptosis and CD4+ T cell depletion in HIV infection. They had demonstrated that when PBMCs were incubated with anti-CD4 MAb previously cross-linked through prolonged incubation in goat-antimouse coated 24-well plates, CD4+ T cell apoptosis was enhanced. However, even in PBMCs from seronegative controls, incubation in the presence of CD4 MAb for an hour at 4°C, was sufficient to increase the percentage of PMBC apoptosis after prolonged culture in the absence of cross-linking with goat anti-mouse Ab. Hence, although cross-linking with goat-antimouse Ab enhanced apoptosis, incubation in the presence of MAb alone was

sufficient to induce apoptosis. Oyaizu did not comment on the significance of this finding nor was the possible contribution of other MAbs (such as CD8) explored. An effect evident after incubation at 4° C is even more likely to occur after incubation at 37°C.

A number of years earlier, Mittler *et al* had shown that FITC-labelled OKT3, OKT4 and OKT8 induced expression of PBMC activation markers OKIa1, OKT9 and OKT10, after cross-linking with horse-antimouse Ab. All incubation steps were conducted on ice or at 4°C for 30 minutes [112]. In my indirect labelling procedure, PBMCs were incubated with biotinylated antibodies against CD4 and CD8 at 37°C for 30 minutes and then incubated with streptavidin at 4°C for 20 minutes. The presence of activation markers induced by the labelling procedure was not sought in the present study. Both the incubation step at 37°C and cross-linking with streptavidin may have contributed to the excessive cell death in the indirectly labelled samples.

The comparison of direct versus indirect labelling of PBMCs implies that by reporting percentage apoptosis above control, one is correcting for differences in the staining methods and supports this approach for analysis of the Denver cohort. However, one could argue that individual analysis by labelling methodology should be undertaken as after correction, as the p value for CD4+ T cell apoptosis after correction was 0.18, 3.6-fold higher than 0.05 for comparison of subjects studied either by the indirect or direct methods.

There are, however, two additional reasons for reporting apoptosis "above control". Firstly, in many biological assays, this is standard practice. An example is in the measurement of cytotoxic lymphocyte activity. Secondly, when performing flow cytometry, the regions drawn may need adjustment from time to time. Thus, there may be

small differences in region size between assays. By subtracting the percentage of apoptosis of the simultaneously measured seronegative control from the study sample, one is correcting for this small difference. Merely by drawing the region closer to the main PBMC population, one can increase the number of events measured.

CHAPTER 5

APOPTOSIS OF CD4+ AND CD8+ T CELLS ISOLATED IMMEDIATELY *EX VIVO* CORRELATES WITH DISEASE SEVERITY IN HIV-1 INFECTION

5.1. Introduction

Both spontaneous and activation-induced apoptosis of PBMCs, CD4+ and CD8+ T lymphocytes have been shown in HIV-infected adults after *in vitro* culture [79-81, 83]. Studies in adults have shown a direct relationship between PBMC apoptosis and CD4+ T cell depletion after overnight or more prolonged *in vitro* culture [70, 82].

A number of studies have subsequently been performed in HIV-infected children, the first of which was the documentation of high levels of CD95 expression in lymphocytes, thus an indirect measure of activation-induced apoptosis [113]. Baumler *et al* have also reported increased spontaneous CD4+ and CD8+ T cell apoptosis in children, but did not correlate it with CD4+ T cell depletion [95]. More recently, Bohler and colleagues described increased spontaneous and anti-CD95 (Fas)-associated apoptosis in HIV-1-infected children [114].

5.2. Aims

The main aim of the present study was to compare both CD4+ and CD8+ T cell apoptosis in HIV-1 infected study subjects and seronegative controls immediately *ex vivo*, using a simple flow cytometric assay based upon the light scatter characteristics of apoptotic cells. This assay was validated as described in Chapter 3. I also sought to compare apoptosis immediately *ex vivo* and after overnight culture. Furthermore, I sought to examine the relationship between CD4+ and CD8+ T cell apoptosis and consequences of HIV disease such as severity of HIV infection, CD4+ T cell depletion and response to antiretroviral (ARV) medication. My patients were mainly infants and children, in whom there are few data [95]. A secondary aim was to evaluate the influence of age on apoptosis as in some cases time-matched controls were older than study subjects (see results section).

5.3. Materials and Methods

5.3.1. Patient selection

All children attending the Colorado Pediatric AIDS Clinical Trials Unit were eligible for study. Patients were recruited between July 1993 and October 1994. They were selected at random, based on availability and whether blood was being collected for clinically indicated laboratory tests. Informed consent was obtained from parents or guardian. HIV serology was performed on all pediatric controls to confirm seronegativity. Patients and controls were matched for age and time of blood draw. Seronegative controls were recruited from patients undergoing elective outpatient-based surgery. If there were no suitable control patients or if the parent/guardian refused consent, a seronegative adult

was used as a control in order to have a time-matched comparison to the study patients. Under these circumstances, there were no other alternatives to obtaining time-matched specimens from other seronegative children. Children for elective day surgery received general anaesthesia and required intravenous cannulation as part of clinical care. For these reasons they were ideal candidates for providing blood specimens. The Colorado Multiple Institutional Review Board approved the study.

Clinical information was recorded prospectively and patients were classified for disease severity with the assistance of a physician who was unaware of apoptosis data. Disease classification followed CDC guidelines for paediatric and adult patients [26, 115]. Patients were defined as paediatric if below 13 years of age, adolescent if between 13 and 18 years and adult if above 18 years of age.

A small group of children with cystic fibrosis was also studied as disease-matched controls for HIV-1-infected patients in category "C". All were HIV-seronegative with bronchiectasis and chronic sinusitis, although none had acute exacerbations at the time of the assay. None were receiving therapy for intercurrent infection.

5.3.2. Reagents

Foetal calf serum was purchased from Hyclone Laboratories Inc., Logan, UT; penicillin-streptomycin and Dulbecco's phosphate-buffered saline (PBS) from Gibco, Grand Island, N Y. RPMI 1640 with glutamine and sodium bicarbonate, Histopaque, acridine orange and ethidium bromide were purchased from Sigma, St. Louis, MO. Cells were cultured in RPMI 1640 supplemented with 10% foetal calf serum and penicillin at 50 U/ml and streptomycin at 50 μg/ml. Goat anti-mouse Ab was obtained from Jackson Immunoresearch, West Grove, PA. Ficoll-Paque was obtained from Pharmacia

Biotechnica, Upsala, Sweden. Twenty-four well plates were from Costar, Cambridge, MA. Biotin-16-dUTP and terminal deoxynucleotidyl transferase (TdT) was purchased from Boehringer Mannheim Corporation, Indianapolis, IN, USA.

5.3.3. Antibodies

BMA-031 (pan anti-TCR MAb) was the generous gift of Roland Kurrle, Behringwerke AG, Marburg, FRG. Leu 3a MAb was the generous gift of the Sloan-Kettering Institute. OKT8 and OKT3 MAbs were derived from cell lines obtained from the ATCC. All were of IgG1 isotype. Biotinylation and FITC conjugation were performed in the laboratory according to established protocols. Titering of MAbs was done on PBMCs from HIV-seronegative controls. Streptavidin-PE and FITC were purchased from Tago Pharmaceuticals, Burlingame, CA. Leu 3a-PE and Leu 2a-PE antibodies were purchased from Becton Dickinson, San Jose, CA. Murine isotype control Ab (IgG2a-FITC; IgG1-PE) was purchased from Olympus, Lake Success, NY.

5.3.4. Sample processing

Heparinized blood samples were collected from patients and seronegative controls. PBMCs were isolated by Ficoll-Paque or Histopaque density gradient centrifugation. Briefly, whole blood was diluted 1:1 with PBS and layered carefully over 30 ml of Histopaque or Ficoll-Paque. Centrifugation was performed at room temperature at 1400 rpm for 30 minutes. PBMCs were removed from the buffy interface between plasma and either Histopaque or Ficoll-Paque and washed twice in PBS. Cells incubated with BMA-031 (pan anti-TCR MAb) at 100 μg/ml in PBS for 40 minutes on ice. Unstimulated and BMA031-stimulated PBMCs were cultured overnight at 37°C and 5%

CO_2 in 24-well plates, previously coated with goat anti-mouse Ab. PBMCs exposed to 500 rads of γ-irradiation were used as a positive control.

5.3.5. Phenotypic labelling of cells for flow cytometry

PBMCs were labelled for flow cytometry immediately after isolation *ex vivo* and again after overnight incubation. Non-specific staining was first excluded by incubating cells for 10 minutes in staining solution containing human gamma globulin at 1 mg/ml. Representative samples were also stained with an isotype control Ab to exclude non-specific uptake of MAb by dying or dead cells. Two labelling protocols were followed, initially an indirect method, in which CD4 and CD8 surface markers were labelled with biotinylated MAbs by incubating at 37°C for 30 minutes. Cells were then counterstained with streptavidin-PE or FITC and incubated at 4°C in the dark for 20 minutes (indirect method).

The method was changed to using PE-conjugated CD4 and CD8 MAbs (direct staining protocol) whereby PBMCs were incubated with MAbs in the dark at 4°C for 20 minutes. The reason for the switch from the indirect to direct protocol was to simplify the apoptosis assay by eliminating an incubation step and washing procedure.

After the labelling procedure, samples were fixed in PBS containing 1% paraformaldehyde. Samples were protected from light at room temperature for 30 minutes for HIV inactivation and then at 4°C until flow cytometric analysis, usually within 24 hours.

5.3.6. Flow cytometry

A FACScan (Becton Dickinson) was used for flow cytometric analysis. Ten thousand scatter events were recorded per sample. Standard compensation techniques were used. Events were accumulated at the same flow rate for each patient and control pair in order to decrease intra-assay variability. Data were stored in list mode and analyzed using Lysys software (Becton Dickinson).

The initial step for analysis of CD4+ and CD8+ T cell apoptosis was to draw a gate for CD4+ or CD8+ T cells only from the FL2 histogram, thus electronically excluding debris. The forward and side scatter of CD4+ T cells was then further analyzed on a dot plot diagram. Region "R1" was drawn around the major lymphocyte population and a second region "R2" to the left, representing apoptotic cells with diminished forward and increased side scatter [40, 62, 70].

5.3.7. Statistical analysis

Statistical analyses were performed using JMP v 4.04, SAS Institute, NC, USA. The paired t test was used for comparisons between HIV-infected patients and seronegative controls. Each patient assay was performed simultaneously with a control, and each "patients and control pair" was studied at a separate time point. The p-value for observed significance of a two-tailed test is reported. Hence, because each "*patients and control pair*" was *time-matched*, the paired t test is appropriate [106]. Statistical analyses of continuous variables were by Wilcoxon one-way analysis for comparison of two groups

and Kruskal-Wallis for three or more groups [5]. Discrete variables were compared using Chi-squares analysis.

Graphical data are presented as quantile boxes. The lowest line represents 10th and uppermost the 90th centile. The box represents the 25th to 75th centile line through both boxes and the horizontal line in each box represents the median for that sample. The continuous line through both boxes represents the median of the entire study sample.

For regression analyses, the method of least squares was used. R^2 measures the proportion of variation around the mean explained by the linear model. The remaining variation is attributed to random error. R^2 is 1 if the model fits perfectly. An R^2 of zero indicates that there is no correlation between the variables (no better than the simple mean model). By analysis of variance (ANOVA), the total variance of a sample is partitioned into components. These are used to compute an F ratio that evaluates the effectiveness of the model. The ratio measures differences from a horizontal line at the mean and is reported as a p-value. A p-value is regarded as significant at or below 0.05.

Percentage of apoptotic cells in controls was subtracted from that in HIV-positive patients to compensate for small amounts of inter-assay variability in gating. As the scatter assay had been developed and validated specifically for this study, the addition of a "correction factor" was considered valid. Analyses for labelling by direct and indirect methodology are also presented. Data are presented as median and range.

---

[5] The Shapiro-Wilks test was applied to data sets of continuous variables. The majority had a non-normal distribution, hence the selection of a non-parametric test.

5.4. Results

5.4.1. Patients

Twenty-one children, four adolescents and nine adults were enrolled in this cross-sectional study. The demographic characteristics of patients and controls are shown in Tables XVIII and XIX. Nineteen patients were vertically infected. Study subjects tended to be younger than controls. On four occasions, seronegative adults served as controls for seropositive children. In all cases, the patients were over six years of age, when adult CD4+ T cell counts are seen [26]. One paediatric patient, classified as "N2" was excluded from time zero analysis because of poor staining in both patient and control samples. For the paediatric group, the seronegative controls were significantly older. In the adolescent group there was also a trend towards increased age in seronegative controls that was not significant.

Eight patients had experienced an intercurrent infection within 30 days, but none within a week of being studied. One patient, classified as "N2", had a lower respiratory tract infection at the time of study. Six patients in subgroup "C3" had chronic sinusitis and two also had bronchiectasis. None were overtly symptomatic at the time of study.

Table XVIII. Demographic characteristics of HIV-infected patients and seronegative controls

Adults (≥18 years)			
	Seropositive	Seronegative controls	p value
Number	9	9	
Male: female	1:0.7	1:2.3	1
Age (years)	34.6 (23.6 - 40.9)	35 (25.5 - 54.2)	0.34
Adolescents (≥13 - 18 years)			
Number	4	4	
male: female	2:1	3:0	0.75*
Age (years)	17.4 (13.3 - 17.9)	34.7 (16.3 - 37.4)	0.08
Children (0 - <13 years)			
Number	21	21	
Male: female	1:1.1	1:1.3	0.53*
Age (years)	8.2 (0.1 - 12.9)	7.7 (0.3 - 37.4)	0.05

Median (range) Comparisons by paired t test for matched pairs of continuous variables and Chi-squares for discrete variables

* Fisher's exact.

Table XIX. Disease Classification for HIV-infected patient study group

Children (N = 21)				
Symptoms	**N**	**A**	**B**	**C**
CD4+ T cell depletion				
1	1	3		1
2	3	4	1	
3			1	7
Adults & Adolescents (N = 13)				
Symptoms		**A**	**B**	**C**
CD4+ T cell depletion				
1 (≥500/µl)		3		
2 (200-499/µl)		6	1	
3 (<200/µl)			3	

Adult classification: A: asymptomatic, acute HIV disease or persistent generalized lymphadenopathy [26, 115].

5.4.1.1. Seronegative children with cystic fibrosis

Four children (one male and three females) were studied. Their median age was 9.1 (6.1 - 13) years. All had bronchiectasis and chronic sinusitis. None were overtly symptomatic at the time of blood draw and none had been hospitalized within the preceding month.

5.4.2. HIV-seropositive patients have significantly more CD4+ and CD8+ T cell apoptosis than seronegative controls

HIV-infected patients had a significantly higher percentage of CD4+ and CD8+ T cell apoptosis than seronegative controls immediately *ex vivo* and after overnight culture, both for stimulated and unstimulated samples. A comparison of each HIV-infected patient and simultaneously studied seronegative control is shown in Figure 7. The magnitude of the p value for stimulated samples appeared greater than for unstimulated cells suggesting an augmentation of apoptosis. Data from direct and indirectly labelled PBMCs are combined. The identical labelling method was used for each simultaneously assayed seronegative control and study patient pair. In addition, the same regions were set for each "patient-control" pair, thus minimizing inter-assay variability.

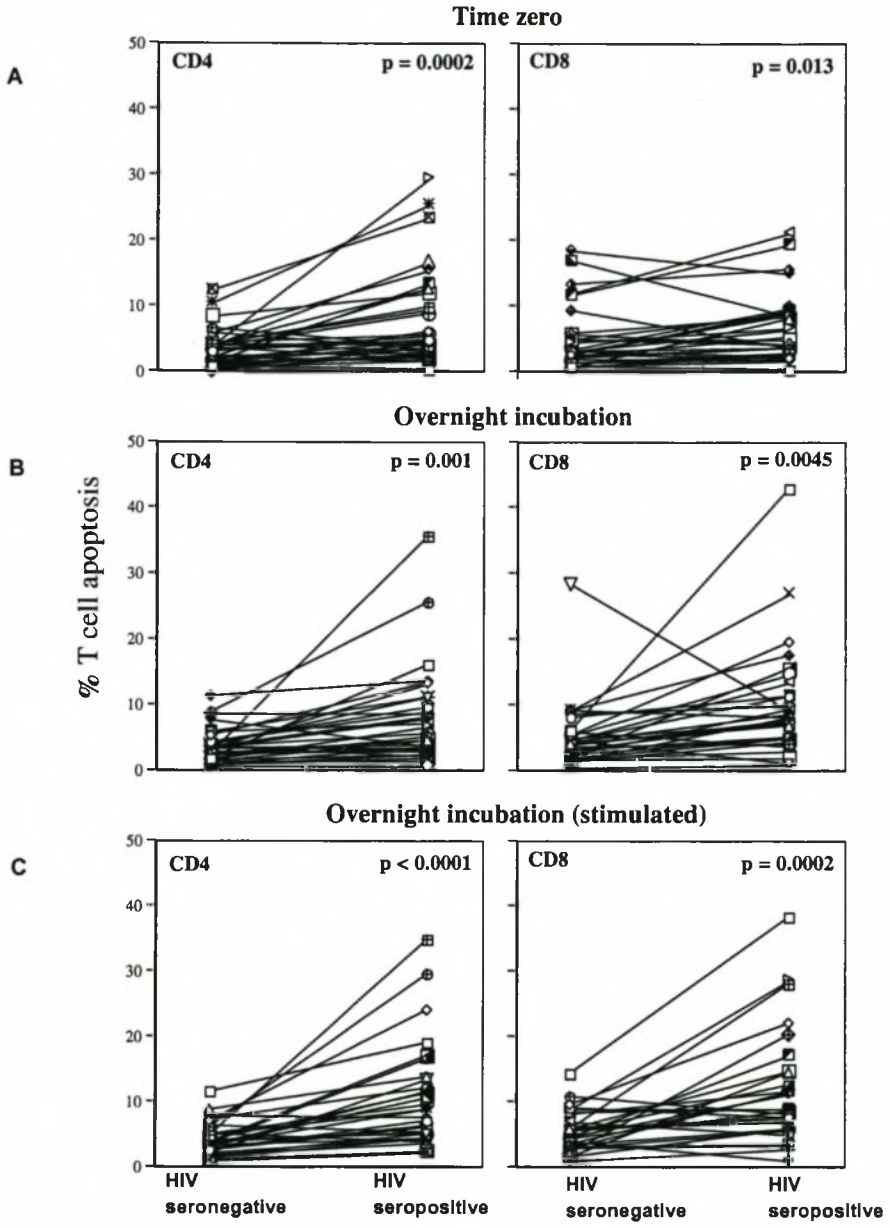


Figure 7. CD4+ and CD8+ T cell apoptosis is significantly higher in HIV- seropositive patients than in seronegative controls *ex vivo* and after overnight culture. Analyses were by paired t test.

5.4.3. CD4+ T cell apoptosis in HIV-infected children and comparison with severity of disease

5.4.3.1. Combined indirect and direct labelling

There was no significant difference between the groups by rank sums analysis, although the median percentage apoptosis in children with the most advanced disease ("C") was above that of those children with milder disease ($p = 0.1$). The four disease-matched children with cystic fibrosis were included in this analysis and had similar percentages of apoptosis to HIV-infected children not in stage "C" suggesting that the low-grade persistent bacterial infection occurring in "C3" patients with chronic sinusitis and bronchiectasis does not contribute to the increased percentage of CD4+ and CD8+ T cell apoptosis (Figure 8). Similarly, there was no significant relationship between CD8+ T cell apoptosis and progression of disease ($p = 0.2$)

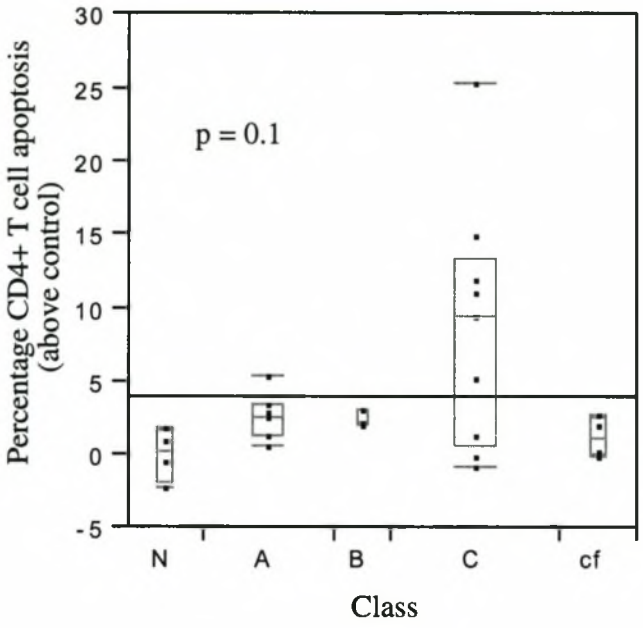


Figure 8. Comparison of percentage CD4+ T cell apoptosis immediately *ex vivo* and severity of disease in HIV-seropositive children. N - asymptomatic; A - mild symptoms; B - moderate symptoms; C - severe symptoms; cf - seronegative children with cystic fibrosis. Apoptosis from seronegative controls was subtracted from that of HIV-seropositive children. Analysis was by Kruskal-Wallis (rank sums). Graphical data are presented as quantile boxes. The lowest line represents 10th and uppermost the 90th centile. The box represents the 25th to 75th centiles and the horizontal line in each box represents the median for that sample. The dotted line represents the mean value of the total sample. Numbers: N - 4; A - 7; B - 3; C - 9; cf - 4.

5.4.3.2. Separate analysis by indirect labelling

The data was re-analyzed by taking into consideration the two labelling methods. Repeat analysis, stratified by labelling method, was performed. There was no significant relationship for samples labelled by the indirect method (Figure 9). No patients with cystic fibrosis had T cells labelled by the indirect method. Similarly, for CD8+ T cell apoptosis, there was no correlation with disease stage ($p = 0.37$).

For children in whom PBMCs were labelled by the direct method, no significant correlation was found for either CD4+ ($p = 0.94$) or CD8+ T cell apoptosis ($p = 0.89$) and disease stage.

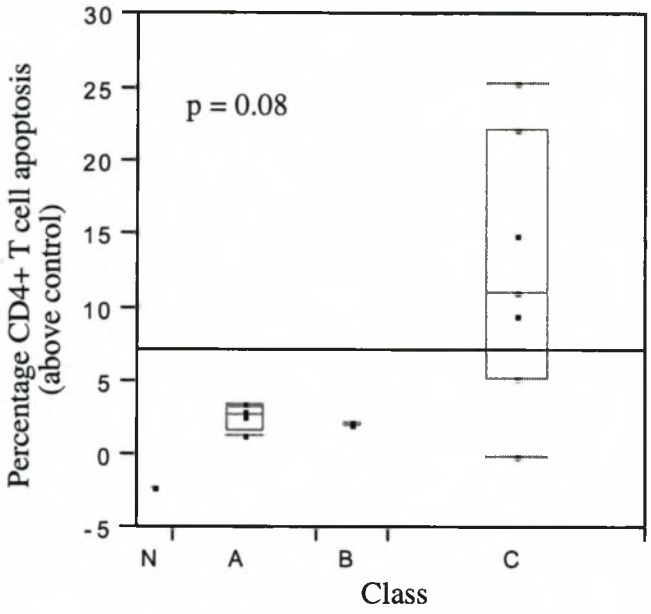


Figure 9. Percentage of CD4+ T cell apoptosis measured after indirect labelling and immediately *ex vivo* in HIV-seropositive children with different stages of disease. N - asymptomatic; A - mild symptoms; B - moderate symptoms; C - severe symptoms; cf - seronegative children with cystic fibrosis. Apoptosis from seronegative controls was subtracted from that of HIV-seropositive children. Analysis was by Kruskal-Wallis (rank sums). Graphical data are presented as quantile boxes. The lowest line represents 10th and uppermost the 90th centile. The box represents the 25th to 75th centiles and the horizontal line in each box represents the median for that sample. Numbers: N - 1; A - 4; B - 2; C - 7.

5.4.4. Comparison of adult and paediatric apoptosis data

5.4.4.1. Introduction and aim

Apoptosis data from adult, adolescent and paediatric were combined for analysis of the relationship between apoptosis, ARV therapy (ART) and lymphocyte subsets. The aim of this section is to present data that supports combining data, irrespective of age group because of a lack of evidence that age influences the analyses.

5.4.4.2. Methods

For the purposes of this analysis, adult and adolescent data were combined to reduce the number of subgroups for analysis. CD4 percentages, CD4/8 ratios and ART were first compared to show that the two age groups were "immunologically equivalent" for these parameters. CD4+ T cell numbers were not compared because of the normal decline with age [26]. Both CD4+ and CD8+ T cell apoptosis were compared for adult and paediatric study groups. Analysis was by Wilcoxon rank sums for continuous variables and Chi-squares for discrete variables. For seronegative controls, regression analyses were performed for CD4+ and CD8+ T cell apoptosis and age. Seronegative controls were stratified by direct and indirect labelling of PBMCs.

5.4.4.3. Results

Comparisons of CD4%, CD4/8 ratio and ARV use are shown in Table XX. There were no significant differences for CD4% and CD4/8 ratio. More paediatric patients

tended to receive ARVs than the adult/adolescence group but when re-analyzed by number of ARVs per patient, this was less evident.

Table XX. No difference between CD4%, and CD4/8 ratio in adults, adolescents and paediatric study patients

	Adult/adolescent (N = 13)	Paediatric (N = 21)	P value
Median CD4%	19 (6 - 39)	26 (0 - 44)	0.75
Median CD4/8	0.27 (0.1 -1.2)	0.58 (0 - 2)	0.62
N on ARVsψ	5	15	0.06*
ARVs per patient: ψ			0.16*
0	8	6	
1	4	11	
2	1	4	

Median (range); *Chi-squares analysis

ψ 7 on ZDV; 6 on ddI and 1 on lamivudine. Of 5 patients on dual therapy, all received ZDV; 2 also received ddI and 3 ddC.

Comparisons between paediatric and adult/adolescent CD4+ and CD8+ T cell apoptosis corrected for control values are shown in Table XXI. Of note is that there were no differences between the two groups. The analysis was repeated for samples labelled by the indirect method only (directly labelled samples were excluded as only two adult samples were assayed). No age differences were noted either when absolute percentages or percentage above control were measured. The analysis was repeated for samples labelled by the indirect and direct methods alone and no significant differences were found for the two age groups.

Table XXI. No difference between CD4+ and CD8+ T cell apoptosis when adult/adolescent group compared with paediatric group

	CD4+ T cell apoptosis (patient minus seronegative control)	CD8+ T cell apoptosis (patient minus seronegative control)
Adult/adolescent (N = 13)	1.7 (-2.7 - 12.7)	0.25 (-5.8 - 7.8)
Paediatric (N = 21)	2.6 (-2.3 - 25.4)	2.1 (-8.3 - 9.2)
p value	0.61	0.88

Data presented as median (range).

5.4.4.4. Discussion

The adult and adolescent groups were combined because of the small number of adolescents (n = 4). Adult/adolescent (referred to as adult) and paediatric groups were matched for CD4% and number of ARV agents. The two groups were not compared by disease stage because of differences in classification. Asymptomatic adults are classified as "A" and children as "N". Mildly asymptomatic children are classified as "A" [26, 115].

There were no significant differences in either CD4+ or CD8+ T cell apoptosis immediately *ex vivo*. The above analyses support the combination of paediatric and adult data for comparison with CD4% and to evaluate for a possible ARV response.

5.4.5. CD4+ and CD8+ T cell apoptosis correlate with CD4+ T cell depletion in HIV infection

The percentages of CD4+ and CD8+ T cell apoptosis above control were inversely related to CD4+ T cell percentage (p = 0.006 and 0.012, respectively, Figure. 10). Similar results were seen after overnight incubation and when adult and pediatric study patients were analyzed separately (data not shown).

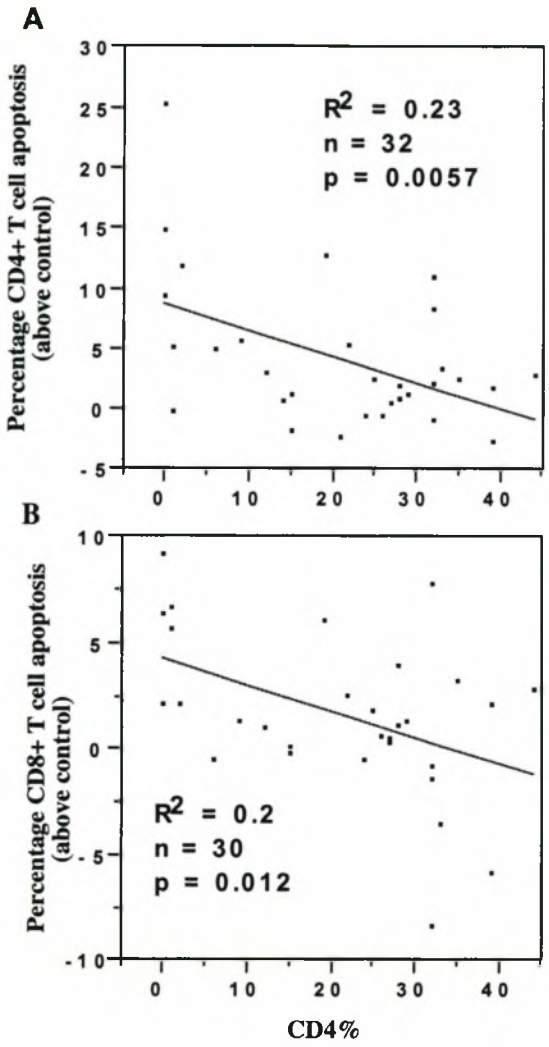


Figure 10. Percentages of CD4+ and CD8+ T cell apoptosis immediately *ex vivo* correlate with CD4+ T cell depletion in HIV-infected adult and paediatric patients. (A) %CD4+ T cell apoptosis; (B) %CD8+ T cell apoptosis. [6]

---

[6] The control data was subtracted from that of study subjects for the following reasons: a) both direct and indirect labelling was used (in chapter 6, apoptosis was higher after indirect labelling but was not significant only when controls were subtracted from patient values) b) subtraction corrected for small interassay differences in region sizes and c) subtraction of control data is commonly performed in some biological assays such as cytotoxic lymphocyte activity.

Apoptosis from seronegative controls was subtracted from HIV-seropositive patients (hence corrected values are shown). Adult and paediatric values were combined for analysis. Linear regression analysis was by ANOVA.

5.4.6. Correlations between CD4+ and CD8+ T cell apoptosis ex vivo and after overnight incubation

There was a strong correlation for both CD4+ and CD8+ T cell apoptosis when values obtained immediately *ex vivo* and after overnight incubation were compared. Percentages of apoptosis were not corrected for seronegative controls as the same regions were drawn for the two time points and are therefore comparable. Data from each study subject was compared immediately *ex vivo* and after overnight culture, but were analyzed at the same time, therefore using identical flow cytometric regions. Under these circumstances it was not necessary to subtract percentage apoptosis of seronegative controls from study subjects. Furthermore, the identical labelling methodology for CD4+ and CD8+ T cells were used for each specimen at the two time-points. The results of linear regression analysis are illustrated in Figure 11 and show a strong correlation.

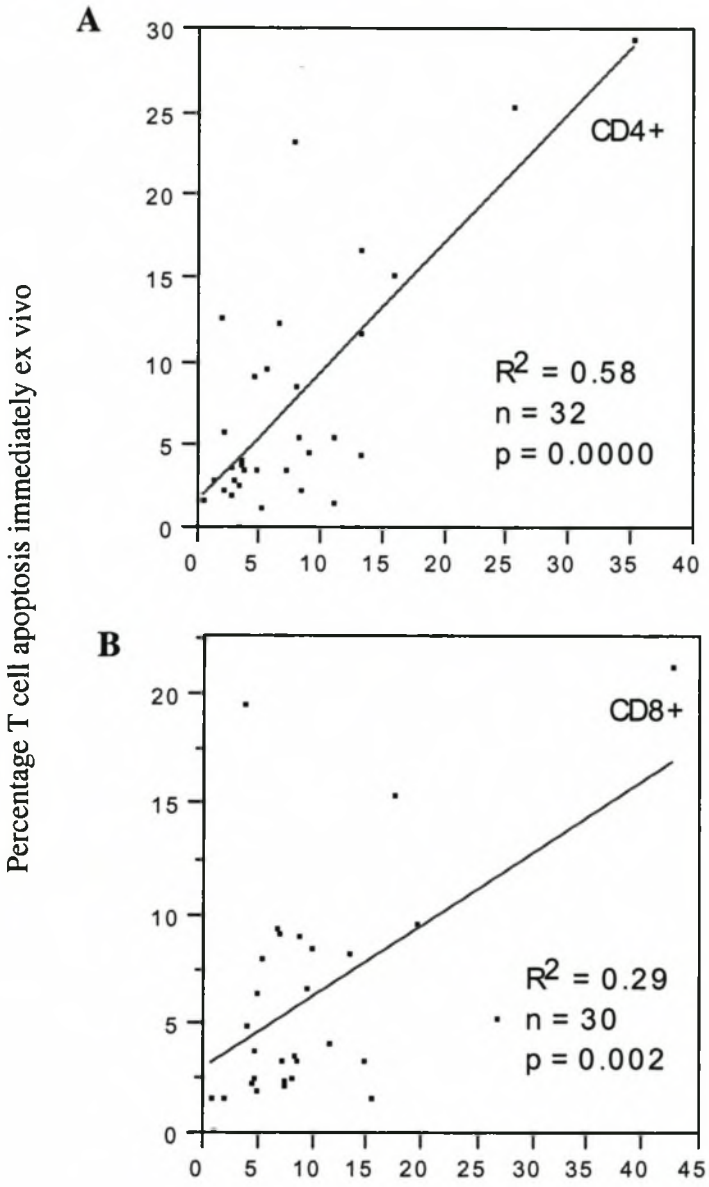


Figure 11. Correlation between (A) CD4+ and (B) CD8+ T cell apoptosis immediately *ex vivo* and after overnight incubation. Adult and paediatric values and direct and indirect

labelling methods were combined for analysis. Linear regression analysis was by ANOVA.

5.4.6. Antiretroviral therapy and apoptosis: correlation with response to treatment?

Fourteen patients were on monotherapy at time of analysis; 7 on ZDV; 6 on ddI and 1 on lamivudine. Of 5 patients receiving ZDV as part of dual therapy, two also received ddI and three ddC. At the time of study, no other forms of ART were available.

There was no correlation between CD4+ T cell apoptosis and time on medication, ($R^2 = 0.12$; $p = 0.13$; $n = 19$) (data not shown). Patients who responded to ART with the greatest increase in CD4+ T cell percentage tended to have the lowest percentage of CD4+ T cell apoptosis ($R^2 = 0.15$; $p = 0.1$; Figure12), although this was not significant at the 0.05 level. Similarly, there was no relationship between change in CD4+ T cell percentage on ART and CD8+ T cell apoptosis ($R^2 = 0.053$; $p = 0.34$). The median time on an unchanged regimen was 0.7 (0.1 - 2.4) years. No patients were receiving protease inhibitors or non-nucleoside reverse transcriptase inhibitors (NNRTIs). Percentages of CD4+ or CD8+ T cell apoptosis were similar when compared for number of ARVs taken simultaneously by study subjects.

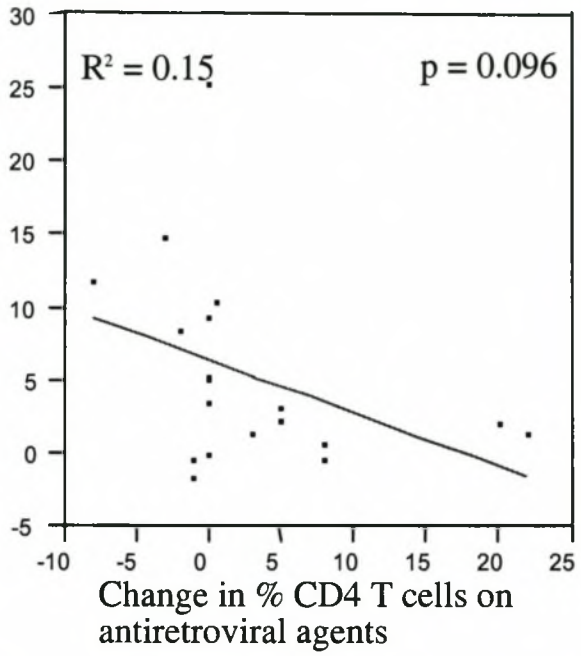


Figure 12. Comparison of CD4+ T cell apoptosis *ex vivo* and CD4+ T cell percentage in response to antiretroviral therapy. N = 19. 7 on ZDV; 6 on ddI and 1 on lamivudine. Of 5 patients receiving ZDV as part of dual therapy, two also received ddI and three received ddC. Mean duration of unchanged antiretroviral therapy was 0.7 (0.1 - 2.4) years. Control apoptosis was subtracted from patient apoptosis. Regression analysis was by ANOVA.

5.5. Discussion

5.5.1. Apoptosis in HIV infection

The present study makes a number of observations relevant to HIV disease. The most significant finding is that the rate or extent of T cell death by apoptosis is significantly increased in HIV-infected children and is detectable immediately *ex vivo*. This finding is in agreement with data previously reported for HIV-infected adults after overnight or more prolonged incubation [70, 79, 81, 82, 116]. My demonstration of a direct correlation between CD4+ and CD8+ T cell apoptosis measured immediately *ex vivo* and after overnight incubation strongly supports the validity of the *ex vivo* measurement.

Debatin and colleagues first reported a high level of CD95/Fas expression in HIV-1-infected children thus indirectly inferring increased levels of activation-induced apoptosis [113]. Baumler *et al* have also reported increased spontaneous CD4+ and CD8+ T cell apoptosis in children, but did not correlate it with CD4+ T cell depletion [95]. More recently, Bohler and colleagues described increased spontaneous and anti-CD95 (Fas)-associated apoptosis in HIV-1-infected children [114]. They measured apoptosis by the method of Carbonari *et al* [70], which characterizes apoptotic cells as having reduced CD45 fluorescence and reduced size (forward scatter). Bohler demonstrated a negative correlation between anti-CD95 induced apoptosis and CD4+ T cell percentage. They found no correlation between CD8+ anti-CD95-induced apoptosis and C4+T cell percentage. Although CD95 expression was higher in children from category B than A, no difference in spontaneous CD4+ T cell apoptosis was noted between the two groups.

In a separate study, they showed that most CD95-induced apoptosis occurred in memory cells [94].

My work also extends prior studies in demonstrating increased percentages of CD4+ and CD8+ T cell apoptosis in PBMCs analyzed immediately *ex vivo* without the need for overnight incubation or stimulation, thus providing strong evidence that apoptosis occurs *in vivo.* In addition, as noted previously, both CD4+ and CD8+ T cell apoptosis obtained immediately *ex vivo* correlated with values after overnight incubation. Unlike Bohler *et al*, I found a significant correlation between spontaneous CD8+ T cell apoptosis and CD4+ T cell depletion. Possibly, mechanisms other than CD95 expression may contribute to CD8+ T cell apoptosis.

A number of questions arise from these observations. The first is how HIV infection leads to increased apoptosis of CD4+ and CD8+ T cells. While this study does not address mechanism, it is clear from prior work and that of others that a number of mechanisms are possible which could induce death of both infected and uninfected T cells [117]. The second question, addressed in part by these studies, is whether apoptosis plays a role in the progression of asymptomatic HIV infection to AIDS.

The central question is why there is an increased percentage of CD4+ and CD8+ T cell apoptosis with advanced HIV disease. CD4+ T cell depletion is a poor prognostic sign. Numerous paediatric studies have correlated low CD4+ T cell counts with more severe manifestations of HIV disease [118]. CD4+ T cell depletion is also associated with elevated plasma HIV-1 RNA, which is a poor prognostic marker. An adult study suggests that the CD4+ T cell count and plasma HIV RNA levels are *independent* predictors of

severe clinical disease [119]. It is, therefore, relevant to examine the relationship of both with apoptosis.

One of the hallmarks of HIV disease is the progressive loss of CTL activity, both HIV-specific and for other common antigens, including Epstein-Barr virus and *Mycobacterium tuberculosis* [120, 121]. Possibly, continued loss of CD8+ T cells via an apoptotic mechanism contributes to this process.

5.5.2. Apoptosis as a marker of disease activity

My study shows a correlation between CD4+ and CD8+ T cell apoptosis and CD4+ T cell depletion, suggesting that apoptosis plays a role in the pathogenesis of HIV disease. Earlier adult studies have yielded conflicting results. Meyaard *et al* found no correlation between apoptosis and either CD4+ and CD8+ T cell counts or a change from non-syncytium-inducing to syncytium-inducing viral phenotype [81]. However, their study, made use of cryopreserved cells from a limited number of patients. Carbonari *et al* found significantly more apoptosis after overnight culture of PBMCs from four patients with CD4+ T cell counts less than 400 per μl than in nine with higher counts [70]. The relationship between PBMC death after prolonged *in vitro* culture and CD4+ T cell depletion was confirmed by Pandolfi and colleagues [82]. Both Carbonari *et al* and Pandolfi *et al* [70, 82] found increased PBMC apoptosis in patients who subsequently showed clinical progression, suggesting that apoptosis may be a useful clinical marker for patients at risk. More recently, Prati *et al* confirmed these observations. They studied spontaneous lymphocyte apoptosis and noted elevated levels in asymptomatic individuals prior to clinical progression. Apoptosis was lower in long-term non-progressors than in symptomatic patients [122].

In addition to linking CD4+ T cell apoptosis with CD4+ T cell depletion, I found the same correlation for CD8+ T cells, possibly reflecting increased CD8+ immunological activity in response to progression of disease. However, a mechanism for gp120-mediated CD8+ T cell apoptosis has been described and could also explain the correlation with CD4+ T cell depletion [96]. CD8+ T cell depletion, although less commented upon than CD4+ T cell depletion, is nevertheless a feature of HIV infection.

5.5.3. Apoptosis as a marker of therapeutic benefit

Although my study of ARV therapy was retrospective and involved a small sample size, it showed a trend towards less CD4+ T cell apoptosis in those with the highest elevation of CD4+ T cell percentage on ART. This observation is in agreement with Andrieu *et al* who demonstrated a sustained increase in CD4+ T cell counts accompanied by diminished PBMC apoptosis in response to prednisone [123].

More recently, Bohler *et al* evaluated the influence of triple ARV therapy that included two NRTIs in combination with either protease inhibitors or NNRTIs in children [124]. They observed a significant reduction in the percentage of CD95+ CD4+ T cells, implying reduced activation-induced apoptosis. Resting and naive cells contributed significantly to the rise in CD4+ T cells seen in response to therapy. Pitrak *et al* recently showed significantly increased levels of PBMC apoptosis measured *ex vivo* in patients with the poorest response to antiretroviral therapy [125].

5.5.4. Comments concerning labelling methodology

The indirect method of PBMC labelling appeared to enhance apoptosis as trends towards significance were noted for comparison with staging of HIV disease. In contrast, for the direct method, the p values approached 1, although the sample size in the latter group was smaller. Possibly, with increased samples labelled by the indirect method, a significant relationship with disease progression may be found, although this remains speculative. This phenomenon is worthy of further study as it may increase the sensitivity of the apoptosis assay.

5.5.5. Conclusion

I have shown a correlation between CD4+ T cell apoptosis and CD4+ T cell depletion in HIV-infected children immediately *ex vivo*. The occurrence of this phenomenon directly after PBMC isolation and its correlation with apoptosis after overnight culture suggests that it occurs *in vivo* and reflects disease activity.

The absence of elevated percentages of CD4+ and CD8+ T cell apoptosis in disease-matched seronegative patients with cystic fibrosis suggests that "low-level" inflammation that may occur in patients with bronchiectasis and chronic sinusitis is not a significant factor, although apoptosis should be studied after indirect labelling of CD4+ and CD8+ T cells.

The trend towards less CD4+ and CD8+ T cell apoptosis in patients with the best response to ART, as measured by increased CD4+ T cell percentage, supports a role for the quantification of apoptosis in the monitoring of therapeutic efficacy. This would best be determined prospectively and with a larger sample size.

CHAPTER 6

CD4+ AND CD8+ T CELL APOPTOSIS IN SYMPTOMATIC SOUTH AFRICAN HIV-1-INFECTED CHILDREN

6.1. Introduction

CD4+ T cell apoptosis correlates with CD4+ T cell depletion in HIV-1-infected children and adults [69, 70, 82]. To date, most studies have been conducted in developed countries where patients have access to ART, better nutrition, better sanitation and also less exposure to many pathogens. There are few data on apoptosis in HIV-1-infected patients from developing countries and none, to my knowledge, in children. Children from developing countries have a more rapid rate of progression than those from developed countries, even in the absence of ART [24, 126].

6.2. Aims

I sought to study CD4+ and CD8+ T cell apoptosis in symptomatic HIV-1 infected children from RSA, a resource-poor country. My primary objectives were to document the extent of CD4+ and CD8+ T cell apoptosis in HIV-infected children, to compare with seroreverters and to correlate apoptosis with CD4+ T cell depletion.

Because of the absence of antenatal screening and perinatal prevention programs, HIV-infected children present for medical care only when symptomatic. The majority have multi-system disease, the most common of which are malnutrition, recurrent pneumonia, chronic gastroenteritis and oral candidiasis [23, 127]. Most patients receive treatment for tuberculosis, although direct evidence for infection is often lacking [128].

6.3. Methods

6.3.1. Patients selection

Patients attending Tygerberg Children's Hospital, a tertiary care institution in the Western Cape province, were recruited mainly from in-patients hospitalized for intercurrent illness. In a few instances, specimens were obtained from relatively asymptomatic children in the outpatient clinic. Between 3 and 5 ml of heparinized blood was drawn for apoptosis assays in patients requiring other clinically indicated laboratory investigations. Where possible, a small aliquot of plasma was stored at -70°C for viral load assays. Clinical information was recorded prospectively. Disease classification followed CDC guidelines [26]. Patients were studied between August 1 1996 and 29 July 1997.

Note was taken of the status of the study subjects at the time of the apoptosis assay. This was defined as (a) attending the outpatient clinic (and not requiring hospitalization for intercurrent disease), (b) hospitalized and ill due to intercurrent disease such as pneumonia and / or gastroenteritis and (c) recovering from intercurrent disease. These classifications were made according to clinical judgment and prior to analysis of apoptosis data.

Seronegative paediatric control patients were attending the endocrine, asthma or nephrology clinic and required laboratory tests for clinically indicated reasons simultaneously to the study patients. All were clinically well and none were receiving immunosuppressive therapy. HIV-seronegative adults were used as controls if a suitable time-matched seronegative paediatric control patient was unavailable. Informed consent was obtained for HIV serology and participation in the study. A small number of patients were studied on more than one occasion, usually during the same admission. The ethics committee of Stellenbosch University approved the study.

6.3.2. Laboratory investigations:

Sera were screened for antibodies to HIV by either the HIV-1/HIV-2 3rd generation $IM_X^®$ or AXSYM® (Abbott, Delkenheim, Germany) assays. Positive serology was confirmed by 2 EIAs for antibodies to HIV (ICE HIV-1.0.2, Murex, Dartford, England and Vironostika HIV Uni-form II plus O, Organon-Teknika, Boxtel, NL) [129]. p24 antigenaemia was also detected by EIA (Vironostika Organon-Teknica, Boxtel, NL). The polymerase chain reaction (PCR) for HIV-1 DNA was performed using primers for *env* and *gag* sequences [130, 131]. Virological studies were performed in the department of Medical Virology, Tygerberg Hospital, University of Stellenbosch.

6.3.3. Sample processing.

PBMCs were isolated from heparinized blood samples by Histopaque density gradient centrifugation (see chapter 5). Cells from patients and controls were cultured overnight in 24-well plates at 37°C and in 5% CO2. Beauvericin (6μmol/ml) was added to induce apoptosis and this served as a positive control for each assay. Staining

methodology was as previously described [69]. Cells were stained with directly PE-conjugated CD4 and CD8 MAbs. Apoptosis was measured by the previously characterized scatter-based flow cytometric technique [69].

6.3.4. Virus load measurement.

Plasma was stored at -70°C until viral load determination. Viral load was detected by either the Amplicor HIV-1 monitor test (Roche Diagnostic Systems, Neuilly, France) or nucleic acid sequence-based amplification (NASBA) (Organon Teknica, Fresnes, France). Results are given as RNA copies per ml of plasma. The highest reading for the Amplicor method was ">750 000" while the NASBA was Able to quantify higher values. For purposes of analysis, all NASBA values above 750 000 copies per ml were regarded as ">750 000" copies per ml. An excellent correlation has been shown for the two methods [132]. Plasma HIV-1 RNA was measured in the department of Medical Virology, Tygerberg Hospital and University of Stellenbosch.

6.3.5. Statistical analysis

Statistical analyses were performed using JMP v 4.04, SAS Institute, NC, USA. The paired t test was used for comparisons between HIV-infected patients and seronegative controls. Each patient assay was performed simultaneously with a control, and each "patients and control pair" was studied at a separate time point. The p-value for observed significance of a two-tailed test is reported. Hence, because each "*patients and control pair*" was *time-matched*, the paired t test is appropriate [106]. Statistical analyses of continuous variables were by Wilcoxon one-way analysis for comparison of two groups

and Kruskal-Wallis for three or more groups. Discrete variables were compared using Chi-squares analysis. Data are presented as median and range.

Graphical data are presented as quantile boxes. The lowest line represents 10th and uppermost the 90th centile. The box represents the 25th to 75th centiles and the horizontal line in each box represents the median for that sample.

For regression analyses, the method of least squares was used. R^2 measures the proportion of variation around the mean explained by the linear model. The remaining variation is attributed to random error. R^2 is 1 if the model fits perfectly. An R^2 of zero indicates that there is no correlation between the variables (no better than the simple mean model). By analysis of variance (ANOVA), the total variance of a sample is partitioned into components. These are used to compute an F ratio that evaluates the effectiveness of the model. The ratio measures difference from a horizontal line at the mean and is reported as a p-value. A p-value is regarded as significant at or below the 0.05 level.

Percentage of apoptotic cells in controls was not subtracted from that in HIV-positive patients as the regions set at the start of the study were used throughout.

Weight for age Z scores were derived from the software package Epi info 2000 (CDC, Atlanta GA, USA). Weight for age Z score represents the amount of deviation from mean weight for age and sex and is represented by the difference from the mean divided by the standard error of the difference [133]. The standard error, in turn, estimates the standard deviation of a distribution of means, each calculated from a sample size of N and is computed by dividing the sample standard deviation by the square root of N [109].

6.4. Results

6.4.1. Patients

Eighteen HIV-1 infected children and four seroreverters were studied. HIV-1 infection was confirmed by PCR in seven, p24 antigenaemia in three and persistence of antibodies to HIV-1 beyond 18 months of age in the remainder. Seroreversion was confirmed in four subjects by disappearance of antibodies by 12 months of age and absence of clinical disease suggestive of immunodeficiency. HIV-1 DNA was not detected by PCR in three of the seroreverters. Demographics of study patients are shown in Table XXII.

Control subjects were significantly older and had higher weight for age Z-scores than study subjects. The reason for selecting older seronegative controls was the difficulty experienced in recruiting age- and time-matched children requiring clinically indicated blood tests. Under these circumstances, a seronegative adult or older child served as the control.

Table XXII. Demographic data of HIV-infected patients, seroreverters and seronegative control patients

	HIV-infected patients (N = 18)	Seronegative controls (N = 13)	p value
Age (years)	0.8 (0.22 - 10.4)	8 (2.1 - 41.3)	0.002
Weight for age Z-score	-3.8 (-6.6 - 0.6)	0.6 (-4.5 - 0.7)	0.005
Classification B C	 6 12		
	Sereroverters N = 4	Seronegative controls N = 4	
Age (years)	0.5 (0.3 - 0.8)	24.1 (7.1 - 41.3)	0.13
Weight for age Z-score	-1.1 (-4 - 0.1)	*	

Analysis by paired t test for time-matched study subject and seronegative control pairs. Data presented as median (range).

* Weight for age Z score available for 2 paediatric controls but not for 2 time-matched adult controls.

Clinical and diagnostic characteristics of HIV-1-infected patients are shown in Table XXIII. All patients had manifestations of multi-system pathology, illustrating the complexity of their illness. Only one patient (patient no. 17) was on ART. ZDV had been commenced at 180 mg/m^2 6 hourly six weeks prior to the apoptosis study. At that stage she had been hospitalized for 116 days because of chronic refractory diarrhoea. She died one year later.

Eleven patients and one seroreverter were treated for suspected TB. In nine, therapy was commenced 0.7 (0 - 19.2) months prior to the apoptosis assay. Anti-TB therapy was commenced after the assay in two patients, one after 1.8 months and the other after three months. None had reactive Mantoux skin tests and all were culture negative for *Mycobacterium tuberculosis.* All were diagnosed on the basis of suspicious chest radiographs, although three patients had contact with TB-infected persons.

The majority of patients were in "B" or "C". The classification proved artificial in some cases. For example, patient 15 was classified as "B" despite being "severely" symptomatic. He presented at 2.6 months of age with failure to thrive, oral thrush and pneumonia. The weight for age Z-score was -4.3 standard deviations below the mean but absence of chronic diarrhoea or persistent fever precluded placement in "C".

Table XXIII. Clinical characteristics and classification of HIV-1-infected children at time of apoptosis assays

Subject	Age (m)	Stage (CDC)	Criteria for staging	Other intercurrent illness	Status
1	6.4 14.4	C3	PCP, suspected PTB, LIP	Hepatitis due to anti-TB therapy and CMV. Otitis media, oral thrush	recovering outpatient
2	3.3 3.5	C3	PCP, oral thrush		recovering recovering
3	7.8 8 8.5	C3	Laryngotracheal candidiasis, chronic diarrhoea		sick sick sick
4	57.6	C2	bronchiectasis, bacterial pneumonia, PTB		recovering
5	6.1	B2	LIP, chronic diarrhoea	wheezy chest	outpatient
6	29	C2	LIP, kwashiorkor		outpatient
7	22.1	B1	LIP, chronic diarrhoea	otitis media	outpatient

8	125.3	B1	LIP		outpatient
9	51.4	C3	LIP, chronic otorrhoea, recurrent bacterial pneumonia, miliary TB		recovering
10	15.1	C3	LIP, failure to thrive, chronic diarrhoea		
11	38.1 38.4 38.6	C3	PCP, recurrent bacterial pneumonia, lung Abscess, kwashiorkor		sick recovering recovering
12	15.9	C3	septicaemia, septic arthritis, LIP, oral thrush		outpatient
13	6.1	C1	miliary TB, oral thrush, severe failure to thrive, gastroenteritis	pneumonia, urinary tract infection	recovering
14	9.9	C	PTB, pneumonia, chronic diarrhoea, otorrhoea		sick
15	2.7	C3	oral candidiasis, pneumonia		recovering

	2.9				recovering
16	28.8 29.7	B3	pneumonia, severe chicken pox, PTB		recovering outpatients
17	11.1	C3	oral candidiasis, chronic diarrhoea, severe failure to thrive	prophylaxis for TB exposure, primary CMV disease	sick
18	57.8	B2	Bacterial meningitis, pneumonia, PTB	chronic otorrhoea	outpatient

Of 13 seronegative controls, one was studied on three occasions and another on two separate occasions. None were acutely ill at the time. Two were on hormone replacement therapy for hypopituitarism post therapy for craniopharyngioma. One had been followed for six years and the other for one year after intracranial irradiation. Two control subjects had adrenogenital syndrome for which they were receiving physiological amounts of corticosteroids. One subject had diabetes mellitus and another was being evaluated for short stature. Of the remainder, two had nephrotic syndrome but were not receiving immunosuppressive agents, and another was asthmatic, receiving only intermittent B_2 bronchodilator therapy. One patient had been treated for syphilis after sexual molestation. Her RPR titer had been 1/64 two months previously, after which she was appropriately treated. Two seronegative adults were controls on three separate occasions. For one patient, there was no seronegative control.

6.4.2. Percentage CD4+ and CD8+ T cell apoptosis is higher in HIV-1-infected patients than seroreverters and seronegative controls.

Both CD4+ and CD8+ T cell apoptosis were significantly elevated in HIV-1-infected patients. For this analysis, only initial apoptosis data were analyzed from patients

in whom multiple samples were measured. [7] Data are shown in Figure 13. HIV-infected patients had significantly higher percentages of CD4+ and CD8+ T cell apoptosis than seroreverters and seronegative controls. The percentages apoptosis in seroreverters was similar to that of seronegative controls. One seroreverter (patient no. 21) was studied during an admission for bronchiolitis.

---

[7] When data from patients with multiple assays was included, significant elevations in both CD4+ and CD8+ T cell apoptosis were seen and at a higher level of significance (CD4+: $p = 0.0006$; CD8+: $p = 0.0043$) (Statistical analysis by Kruskal-Wallis rank sums test).

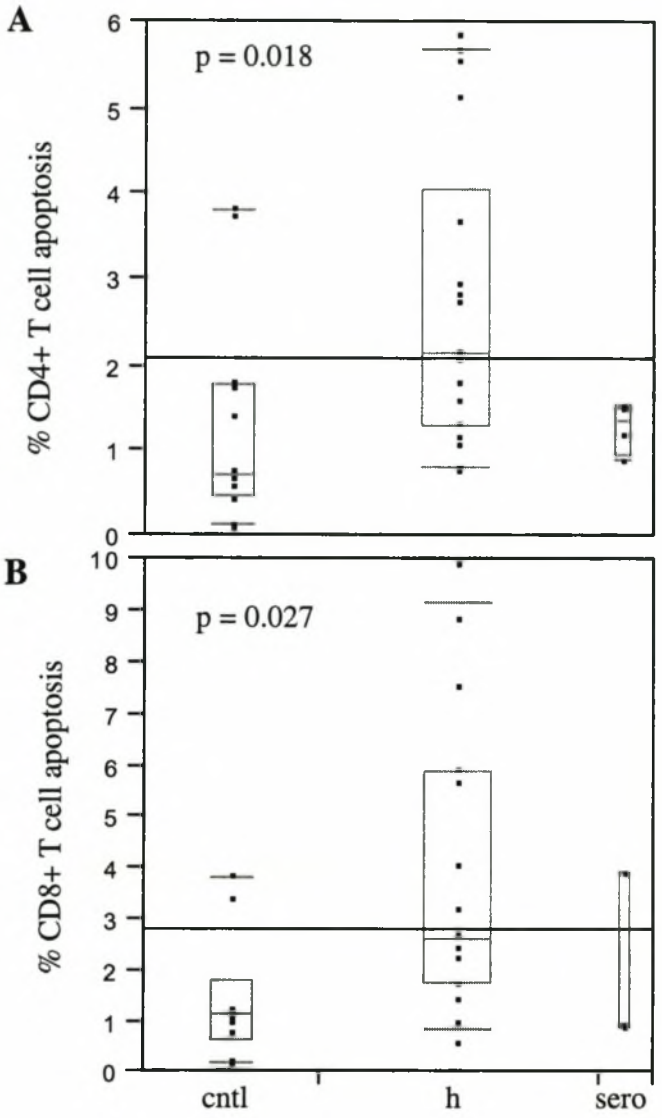


Figure 13. Percentage CD4+ (A) and CD8+ (B) T cell apoptosis in symptomatic South African HIV-infected children is higher than in seronegative controls and seroreverters. cntl - control; h - HIV-1-infected study subject; sero - seroreverter.

Analysis was by Kruskal-Wallis test. Graphical data are presented as quantile boxes. The lowest line represents 10th and uppermost the 90th centile. The box represents the 25th to 75th centiles and the horizontal line in each box represents the median for that sample.

The dotted line represents the median value of the total sample. CD4+: Control n = 13; HIV+ n = 18; Seroreverter n = 4. CD8+: Control n = 13; HIV+ n = 16; Seroreverter n = 3.

6.4.3. Effect of age and weight for age Z scores on percentage apoptosis in seronegative controls

Even though the seronegative controls were significantly older than study subjects, there was no correlation between age and either CD4+ or CD8+ T cell apoptosis (CD4+: $R^2 = 0.054$; $p = 0.41$; $n = 15$) (CD8+: $R^2 = 0.02$; $p = 0.64$; $n = 13$). In a separate analysis, CD4+ and CD8+ T cell apoptosis was compared in adult and paediatric seronegative controls and found not to be significantly different. [8]

Seronegative controls also had significantly higher weight for age Z scores than HIV-1-infected patients. However, linear regression analysis showed no correlation for

---

[8] Comparison of CD4+ and CD8+ T cell apoptosis in seronegative paediatric and adult controls

	Paediatric	Adult	p value
number	10	5	
age (years)	6.4 (2.1 - 11.2)	40.8 (14.5 - 41.3)	
CD4+ T cell apoptosis	1.6 (0.6 - 3.8)	0.4 (0.1 - 1.8)	0.09
CD8+ T cell apoptosis	1.1 (0.6 - 3.4)	0.7 (0.2 - 3.9)	0.54

weight for age Z scores and percentage CD4+ and CD8+ T cell apoptosis in either study subjects or seronegative controls. [9]

Seronegative controls were therefore compared to HIV-infected children for comparison of apoptosis data despite differences in age and weight for age Z score as these parameters were considered not to have a significant impact on the seronegative study subjects.

6.4.4. Correlations between CD4+ and CD8+ T cell apoptosis and disease progression in symptomatic HIV-1-infected children

Comparisons between CD4+ and CD8+ T cell apoptosis and CD4 percentage in HIV-1-infected patients are shown in Figure 14. There was a significant negative correlation between the percentage CD4+ apoptosis and CD4+ T cell percentage (R^2 = 0.27; p = 0.011; n = 23). There was a trend towards a similar correlation for CD8+ T cell apoptosis (R^2 = 0.13; p = 0.11; n = 21) but this is not statistically significant. Similarly, there was a significant negative correlation between CD4/8 ratios and CD4+ T cell apoptosis (R^2 = 0.32; p = 0.0046; n = 23) and a trend for CD8+ T cell apoptosis (R^2 = 0.16; p = 0.075; n = 21) (data not shown graphically).

---

[9] Regression analyses comparing weight for age Z scores and CD4+ and CD8+ T cell apoptosis in seronegative controls:

CD4+: R^2= 0.2; p = 0.26; n = 8

CD8+: R^2 = 0.09; p = 0.48; n = 8

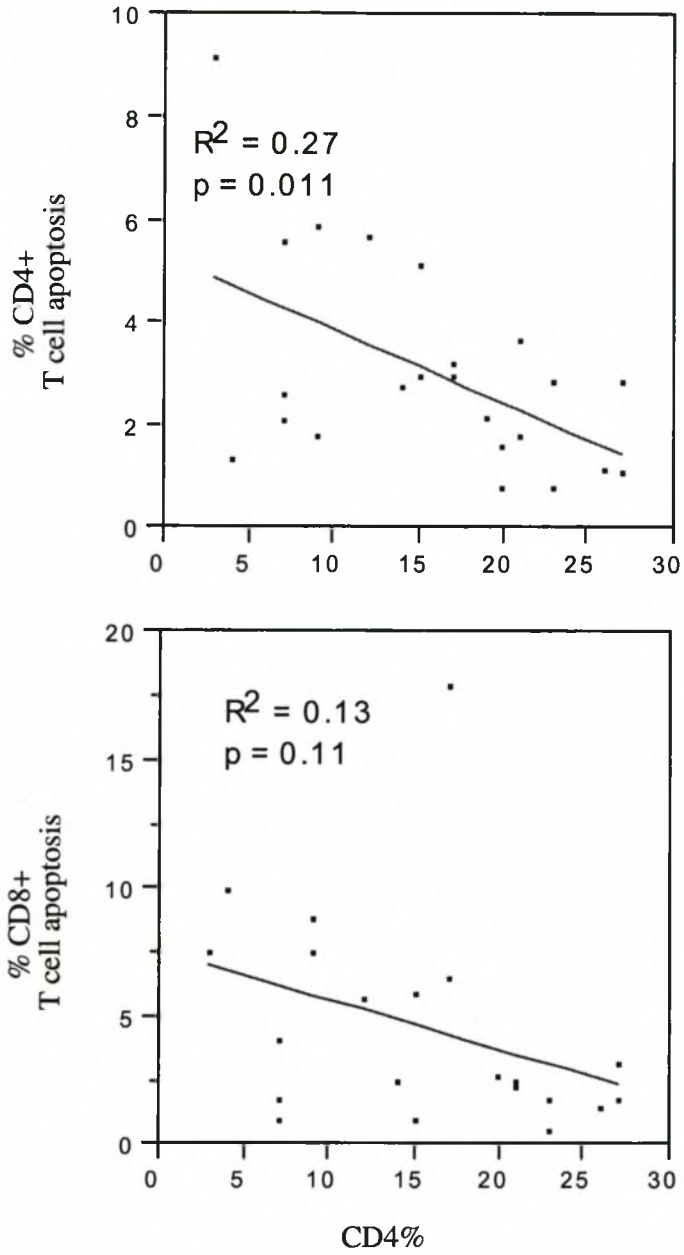


Figure 14. CD4+ T cell apoptosis correlates with CD4+ T cell depletion in symptomatic South African HIV-infected children. CD8+ T cell apoptosis shows trend towards correlation. Linear regression analysis. CD4+: n = 23; CD8+: n = 21. Apoptosis measured by scatter assay.

6.4.5. Effect of clinical status of HIV-1-infected child on percentages CD4+ and CD8+ T cell apoptosis

Note was taken of the clinical status of the study subjects at the time of the apoptosis assay. Hospitalized patients were either symptomatic due to or recovering from intercurrent disease. Patients attending the outpatient clinic were relatively asymptomatic. The reason for this stratification was that children hospitalized for intercurrent infection might have differences in immunological activity when acutely sick, recovering or if asymptomatic, possibly because of differences in T cell activation in response to pathogens. Eight patients were initially assayed as outpatients, eight while recovering from intercurrent infection and two while acutely ill.

CD4+ T cell apoptosis was significantly higher in the recovery phase. For CD8+ T cell apoptosis, there was a non-significant trend towards increased apoptosis in the recovery phase, as illustrated in Figure 15.

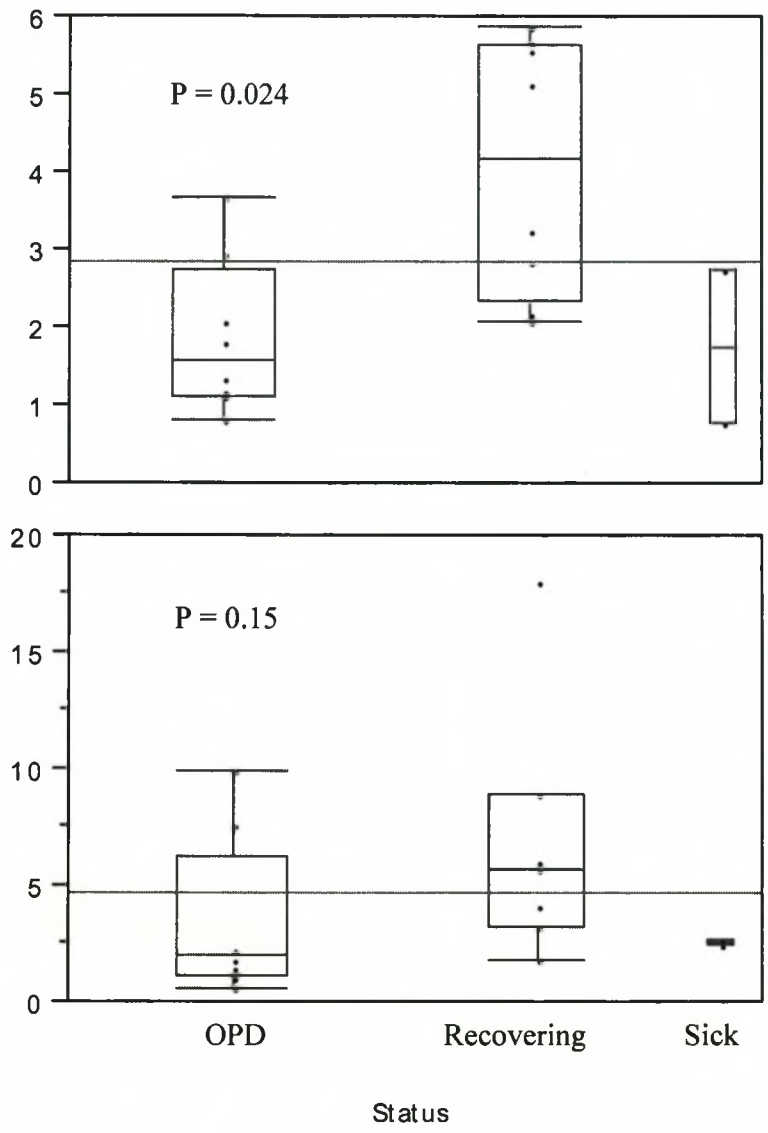


Figure 15. CD4+ T cell apoptosis is significantly elevated in symptomatic South African HIV-infected children recovering from intercurrent disease than when either acutely ill or relatively asymptomatic as an outpatient. A) CD4+ and B) CD8+ T cell apoptosis by hospitalization status. opd - outpatient; rec - recovering from intercurrent illness; sick - clinically ill due to intercurrent disease. Statistical analysis was by Kruskal-Wallis (rank sums) test. Graphical data are presented as quantile boxes. The lowest line represents 10th and uppermost the 90th centile. The box represents the 25th to 75th centiles and the

horizontal line in each box represents the median for that sample. The dotted line represents the mean value of the total sample. Numbers: opd - 8; recovering - 8 (7 for CD8+); sick - 2.

CD4+ T cell apoptosis: Rec vs sick: $p = 0.11$; rec vs opd $p = 0.01$; opd vs sick $p = 0.6$ (Wilcoxon)

6.4.6. Comparisons between HIV-1-infected children with moderate (B) or severe (C) infection

Anthropometric, clinical, immunological and apoptosis data were similar in HIV-infected children with stages "B" and "C" disease (Table XXIII). However, patients in "B" tended to be older, reflecting their increased survival. Patients in "C" tended to have lower weight for age Z scores with a tendency to be hospitalized more often for acute intercurrent illness. Plasma HIV RNA levels were similar in the small subset where it was measured. Differences did not reach statistical significance at the 0.05 level. CD4+ T cell percentages and CD4/8 ratios were similar in the two groups.

Percentages of both CD4+ and CD8+ T cell apoptosis in the two groups were similar, although there was a non-significant trend towards more apoptosis in Group "C"

Table XXIII. Clinical, anthropometric and apoptosis data in symptomatic HIV-1 infected children with stage B and C infection.

	B	C	p value
Number	6	12	
Weight for age (Z-score)	-2.1 (-5.7 - 0.6)	-4.1 (-6.6 - -0.8)	0.17
Status: acutely ill convalescence. outpatient visit	 0 2 4	 3 7 2	0.15*
Age (years)	3.3 (0.5 - 10.4)	0.9 (0.2 - 4.8)	0.07
Male: female	1	0.7	0.8
Plasma HIV-1 RNA	149 368 (460 - 220 000) (N = 3)	412 500 (21 000 - >750 000) (N = 4)	0.59
CD4/8 ratio	0.4 (0.06 - 0.8)	0.4 (0.1 - 0.5) (N = 11)	0.76
CD4 %	19 (4 - 27)	15 (7 - 27) (N = 11)	0.72
% CD4+ T cell	1.2 (0.8 - 5.6)	2.5 (0.8 - 5.9)	0.19

apoptosis		N = 19	
% CD8+ T cell apoptosis	1.6 (0.6 - 9.9) N = 6	3 (1.8 - 8.9) N = 10	0.14

Median (range). Analysis by Wilcoxon rank sums test. * Chi Squares analysis. Only initial values included for study subjects in whom multiple samples available. CD8+ T cell apoptosis not measured in 2 patients in class "C" as insufficient PBMCs were obtained.

6.5. **Discussion**

The present study represents some of the first data on HIV-related apoptosis in a developing country. The same apoptotic trends observed in North American patients were confirmed. For example, the South African patients also showed significantly higher levels of CD4+ and CD8+ T cell apoptosis than seronegative controls and a significant negative correlation with CD4+ T cell percentage. [10]

South African children, classified as "B" or "C", like their North American counterparts, showed similar percentages of apoptosis although there was a tendency to increased apoptosis in those with the most severe disease (C). No South African patient was either asymptomatic (N) or had mild disease (A) most likely due to the absence of screening of pregnant women for HIV infection. Thus children are only diagnosed when presenting with severe intercurrent disease. In contrast, the majority of patients studied in Denver were either asymptomatic or had mild disease. An additional observation of note is that both CD4+ and CD8+ T cell apoptosis in seroreverters were similar to that of seronegative controls.

There are few data on apoptosis in HIV infection from Africa. Michel *et al* found more CD4+, CD8+ and B cell apoptosis in Senegalese adult patients with HIV-1 than HIV-2 infection [134]. They also found high correlations between B2 microglobulin, a marker of immune activation, and CD4+ T cell apoptosis and concluded that chronic

---

[10] The direct labelling method for CD4 and CD8 was used throughout in the demonstration of the direct correlation between CD4+ and CD8+ T cell apoptosis and CDC4+ T cell depletion. In the previous chapter, the significant correlation was only shown in indirectly labelled and not directly labelled samples. One explanation is the difference in sample size: 11 (Denver USA) versus 21 in RSA.

immune activation contributed to continued cell loss and disease progression. Likewise, they found an inverse correlation between CD4+ T cell count and increased percentages of CD4+ T cell apoptosis in those with the most severe disease [134].

A novel finding in my study was that of significantly increased CD4+ T cell apoptosis in a group of hospitalized HIV-1-infected children recovering from intercurrent infection in comparison with ill patients and a group of outpatients. This finding supports the hypothesis that CD4+ T cell numbers decline as a result of intercurrent infection through apoptosis, but that this is only evident during recovery.

I had previously described the demographics and clinical outcome of HIV-1-infected children attending Tygerberg Children's Hospital. The majority of HIV-infected children are impoverished with 38% coming from squatter communities and 9% from farms. Thirty-six (40%) of 91 patients hospitalized between 1991 and 1996 had died with the median age at death being 8.4 months [23]. Few patients receive ART. In my cohort, one patient was on ZDV monotherapy.

The effects of malnutrition, tuberculosis and intercurrent illness on apoptosis in seronegative patients are unknown. Additional studies on seronegative children recovering from malnutrition or intercurrent illness are needed for comparison with HIV-infected children in order to evaluate their impact on apoptosis. In our patients, as none had a positive tuberculin skin test or culture of *Mycobacterium tuberculosis*, it is probable that none had TB at the time of study. However, PTB in children is difficult to confirm by culture. Our experience, however, confirms the importance of a positive (>15 mm) Mantoux skin test in HIV-infected children in a region of high endemicity for TB [128].

6.6. Conclusions

My data confirms that CD4+ and CD8+ T cell apoptosis are elevated in symptomatic HIV-1-infected South African children and also that CD4+ T cell apoptosis correlates inversely with CD4+ T cell percentage. My data supports the hypothesis that ongoing cell loss by apoptosis contributes to the pathogenesis of HIV infection and that this process may occur during the recovery phase of intercurrent infection. Patients recovering from intercurrent disease that necessitated hospitalization had higher levels of CD4+ T cell apoptosis than those acutely ill or well enough to attend the outpatient clinic.

CHAPTER 7

COMPARISON OF CD4+ AND CD8+ T CELL APOPTOSIS IN HIV-1-INFECTED CHILDREN FROM NORTH AMERICA AND SOUTH AFRICA: FURTHER EVIDENCE OF APOPTOSIS AS A CORRELATE OF PROTECTION

7.1. Introduction

There have been no studies, to my knowledge, comparing apoptosis in HIV-1-infected children from developed and developing countries. Even in the absence of ART, children from developed countries tend to survive for longer periods [24, 126]. There are many factors that influence the prognosis of HIV-1 infection. These include socio-economic circumstances, as even before ART became available, outcome varied geographically. For example, prior to the use of PCP prophylaxis and ART, Scott and colleagues showed a median survival of 38 months from time of diagnosis in a cohort of perinatally infected children in Florida, USA [135]. Eighty percent presented under the age of two years. In contrast, Bobat *et al* showed in Durban, RSA that 83% of deaths occurred in infants below 10 months of age, also in the absence of ART [136].

Hussey *et al* documented the survival of HIV-1-infected children in Cape Town where ART is not available. Median survival of untreated infants presenting with symptomatic disease below 1 year of age was between 32 and 36 months. Seventy-eight percent of those presenting later survived for at least 48 months [24]. In my own study at Tygerberg Children's Hospital, the median age of presentation of symptomatic children requiring hospitalization was seven months and the median age of death was eight months. Over the 5-year study period 39.6% (36 of 91) had died [23].

The predominant South African HIV sequence subtype is clade C as opposed to clade B in North America. It is therefore possible that there are biological differences between the two clades, with differing effects on CD4+ or CD8+ T cells [2, 137]

7.2. Aim

My aim was to compare CD4+ and CD8+ T cell apoptosis in HIV-1-infected children from RSA and North America with either moderate or severe HIV-related disease. I hypothesized that if CD4+ and CD8+ apoptosis were correlates of disease progression, the highly symptomatic South African cohort could exhibit more apoptosis. Should it, however, be a correlate of protection, the North American children should have higher levels. Alternatively, apoptosis might be a reflection of severe disease irrespective of geographical and socio-economic co-factors.

7.3. Methods

7.3.1. Patients

HIV-1-infected children from Denver, Colorado or Tygerberg, RSA were eligible for inclusion. Individual patients from both regions had more than one apoptosis assay

performed, and in the North American patients, both direct and indirect cell labelling procedures had been used. Both factors might affect comparisons. Therefore, only the initial apoptosis values after direct labelling were compared. Patients were staged according to criteria of the CDC, Atlanta, GA [26]. Asymptomatic patients and those with mild disease (stages N and A) were excluded as all South African patients had either moderate or severe disease (stages B and C).

7.3.2. Laboratory methods

Methods were the same as outlined in previous chapters. PBMCs were assayed for apoptosis after overnight incubation using the scatter-based assay [69]. A FACScan (BD) was used for flow cytometry in both centers. All PBMCs were labelled with PE-conjugated MAbs for CD4 and CD8 (direct labelling method).

7.3.3.Statistics

Data are presented as median and range. The Shapiro-Wilk test was applied to continuous variables to test for normality. A p-value of less than 0.05 implies that the data does not have a normal distribution. Because of non-normal distribution of data, a non-parametric test was used. Analysis was by the Wilcoxon rank-sum for continuous variables and Chi-squares for discrete numerical data. Percentage apoptosis above control values were compared. The reason for correction was to control for different region sizes used in flow cytometric analysis in the North American patients. Region size can influence the percentage of apoptosis cells. For the Tygerberg patients, the same regions were used throughout, with minimal adjustment.

7.4. Results

7.4.1. Patient demographics

Twenty-one South African and 7 North American children were compared. All were vertically infected. Demographic, anthropometrical and laboratory characteristics are shown in Table XXIV. South African patients were significantly younger than their North American counterparts and less likely to be taking ARVs. All North American patients were seen as outpatients whereas the majority of South African children were hospitalized for intercurrent disease. CD4/8 ratio, degree of immunosuppression and severity of HIV disease, were, however, equivalent. The chronological ages of seronegative controls were not significantly different in the two groups.

South African patients had significantly lower weight for age Z scores indicative of more compromised nutritional status. There was a tendency for South African patients to have lower serum albumin and cholesterol levels although measurements were not available for all patients. Also, serum gamma globulin levels were significantly higher in South African patients, suggesting more immune dysregulation. Again, these assays were not available for all study patients.

Table XXIV. Demographic and laboratory characteristics of North American and South African HIV-1-infected children with either stage B or C HIV disease and seronegative controls

	Tygerberg, RSA (N = 21)	Denver, USA (N = 7)	p value
HIV-1-infected study subjects			
Male: female	1.3	0.4	0.38=
Serum chemistry			
Albumin (g/l)	36 (14 - 44)	45 (33 - 47) (n = 5)	0.06
Gamma globulin (g/l)	51 (42 - 76) (n = 15)	32 (21 - 42) (n = 5)	0.012
Cholesterol (mg/100ml)	102.2 (44.5 - 174.5)	149 (78 - 176) (n = 4)	0.075
Weight for age Z score	-3.9 (-6.6 - 0.6)	-1.2 (-2.2 - 0.3)	0.007
Status:			0.01=
Outpatient	9	7	
Hospitalized	12	0	
Age (y)	1.3 (0.2 - 10.4)	9.9 (0.53 - 13.1)	0.005
Number on ARVs*	1	6	0.0000
CD4/8 ratio	0.3 (0.04 - 0.79)	0.14 (0.03 - 0.82)	0.32

	(N = 20)		
CD4%	15 (3 - 27) (N = 20)	9 (0 - 32)	0.36
Disease classification B C	 7 14	 2 5	1.0=
Seronegative controls			
Number	20	7	
Age (y)	8 (2.3 - 41.3)	11.1 (0.25 - 40.01)	0.37

Y – year; N - number.

*USA: 4 on monotherapy (ZDV - 1; ddI - 3) and 2 on dual NRTI therapy (I - ZDV/ddC; 1 - ZDV/ddI). Median duration of therapy 20.5 was (1.1 - 58.8) months 1 patient from RSA on monotherapy (ZDV) for 6 weeks.

Data presented as median (range). Comparisons by Wicoxon rank sums. = Fisher's exact two-tail test.

7.4.2. CD4+ and CD8+ T cell apoptosis in South African and North American HIV-1-infected children and seronegative controls

7.4.2.1. No effect of age on CD4+ and CD8+ T cell apoptosis in seronegative controls

Because I elected to compare "corrected" apoptosis values in study subjects, I analyzed data from seronegative controls in order to look for an age effect. In Table XXIV, I had not shown an age difference between North American and South African seronegative controls, although for both groups, age was significantly higher than in the concurrently studied HIV-infected patients. The comparison of CD4+ and CD8+ T cell apoptosis in seronegative controls from either Denver or Tygerberg is shown in Table XXV. No differences in either CD4+ or CD8+ T cell apoptosis were noted.

Table XXV. No difference in either CD4+ or CD8+ T cell apoptosis in seronegative control patients from RSA and USA when children are compared to adults and adolescents

	Adult / adolescent *(n = 6)*	*Paediatric* *(n = 13)*	*p value*
% CD4+ T cell apoptosis	0.8 (0.1 - 4.3)	1.4 (0.6 - 3.8)	0.3
% CD8+ T cell apoptosis	1.4 (0.2 - 3.9)	1.1 (0.4 - 3.4)	0.9

Data presented as median (range). Analysis by Wilcoxon rank sums test. Children <13 years of age. One seronegative control used for three HIV-infected children and four compared to two HIV-infected children simultaneously.

Separate regions were drawn for each North American sample and its concurrently studied seronegative control. In the South African patients, the same region sizes were used throughout with minimal adjustment.

RSA two adults (> 18 years), one adolescent (13 - 18 years) and nine children (below 13 years. North America: two adults, one adolescent and four children.

7.4.2.2. Comparison of percentages CD4+ and CD8+ T cell apoptosis above control in North American and South African HIV-1-infected study subjects

Comparison of "corrected" percentages of apoptosis is shown in Table XXVI. There was no difference in CD4+ T cell apoptosis but the North American children had significantly more CD8+ T cell apoptosis. Data are also presented graphically in Figure 16.

Table XXVI. Percentage CD4+ and CD8+ T cell apoptosis above control in North American and South African HIV-1-infected children

	Tygerberg, RSA (n = 20)*	Denver, USA (n = 7)	p value
CD4+ T cell apoptosis (% above control)	1.35 (-2.42 - 8.25)	1.9 (-2.2 - 18.3)	0.87
CD8+ T cell apoptosis (% above control)	1.5 (-1.66 - 17.42)	4.4 (3 - 9.3)	0.04

Data presented as median (range). Comparisons by Wicoxon rank sums test. Percentage T cell apoptosis of seronegative controls subtracted from that of HIV-1-infected study subjects. Apoptosis measured by scatter assay.

* No seronegative control for one South African study subject.

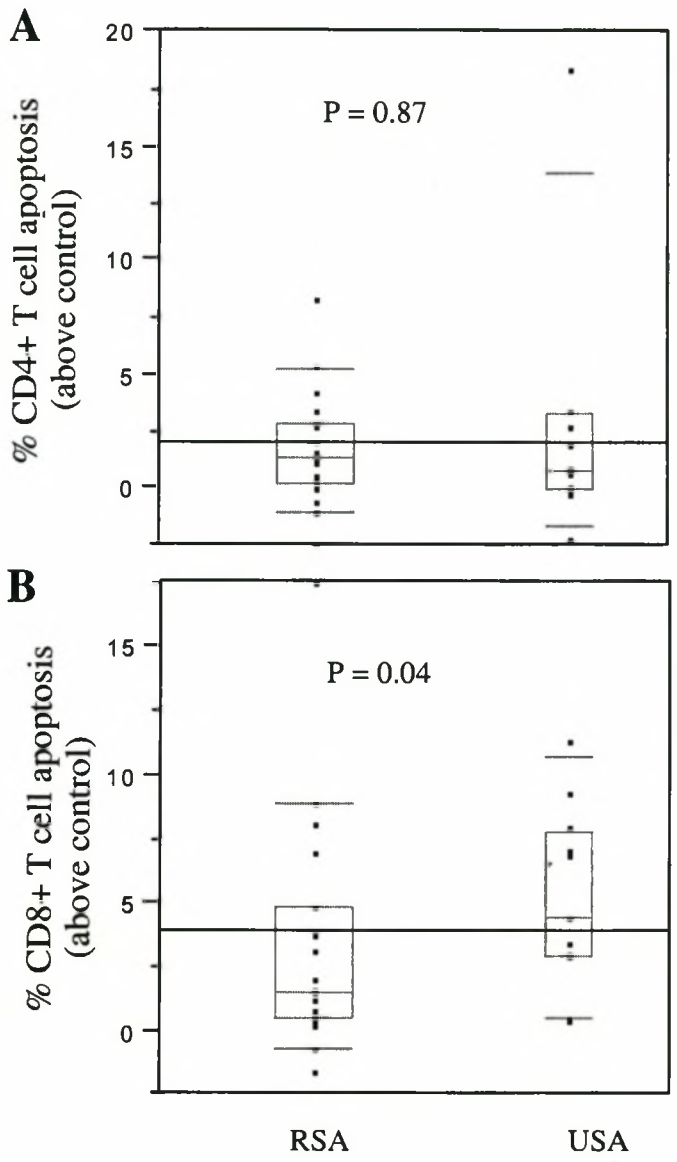


Figure 16. South African children with moderate or severe HIV infection have similar percentages of CD4+ T cell apoptosis but lower levels of CD8+ T cell apoptosis than North American children (A. CD4+ and B. CD8+ T cell apoptosis). Comparisons by Wilcoxon rank sums test. (RSA: n = 20; USA: n = 7). Percentage T cell apoptosis of seronegative controls subtracted from that of HIV-1-infected study subjects. Apoptosis measured by scatter assay. Data presented as quantile boxes. Lowest line represents 10th

and uppermost line the 90th centile. Box represents 25th to 75th centiles and horizontal line in each box represents the median of each sample.

7.5. Discussion

Because of the wide use of triple therapy in USA since 1995, it is no longer possible to compare disease-matched study subjects in USA and RSA as almost all HIV-infected children in USA receive highly active triple ART. Therefore, the comparisons in the thesis represent a unique opportunity for this comparison.

I compared apoptosis in HIV-1-infected children from two geographically distinct populations, one from Denver, Colorado and the other from the Western Cape province of RSA and found significantly elevated CD8+ T cell apoptosis in North American children. In contrast, CD4+ T cell apoptosis was similar. The North American children were significantly older, better nourished, more likely to be receiving ART (mainly monotherapy) and less likely to be hospitalized than their South African counterparts.

My findings suggest that CD8+ T cell apoptosis reflects ongoing immunological activity in children with advanced HIV-1 disease and that this is more evident in North American children. Possibly this increased immunological activity reflects the better outcome in North American children. All North American children were studied as outpatients and had not had recent intercurrent disease. In contrast, 57% of South African patients were sampled during hospitalization for intercurrent infection, and should therefore have manifested more CD8+ T cell apoptosis, possibly due to Fas expression and T cell activation in response to intercurrent infection. In the previous chapter, I had presented evidence of significantly increased CD4+ and a trend towards increased CD8+

T cell apoptosis in South African HIV-1-infected patients *recovering* from intercurrent infection, suggesting a possible delay in immunological activity in response to infection. Even in the recovery phase, this may be of a lower magnitude than in North American patients.

The Fas (CD95)-Fas-L interaction is important in the regulation of T cell responses. CD95 is expressed in activated lymphocytes and marks them for apoptosis [50]. CD95 antigen expression is upregulated on lymphocytes from HIV-infected patients and correlates with CD4+ T cell depletion [93]. Katsikis *et al* showed a positive correlation between anti-CD95 Ab-induced CD4+ T cell apoptosis and CD4+ T depletion in HIV-infected adults. [62]. I did not measure CD95 expression in our patients but speculate that Fas expression may be increased in North American patients because of increased immunological activity against HIV or conversely, that Fas expression in symptomatic South African HIV-1-infected children might be diminished.

Progressive lymph node destruction has been well demonstrated in HIV infection [75, 76]. I have previously hypothesized that with progressive disruption of lymph node architecture, more apoptotic lymphocytes may escape into the peripheral bloodstream for destruction elsewhere in the reticuloendothelial system [138]. Our previously reported lymph node study provides supportive evidence, as in the child with the most severe CD4+ T cell depletion, apoptotic cells were rarely found [97]. Lymph node architecture was not studied in either of my study populations, but could be more disrupted in North American patients because of longer duration of disease.

That no difference in CD4+ T cell apoptosis was detected supports the hypothesis that it reflects ongoing CD4+ T cell loss indicative of advanced HIV disease, irrespective

of age and geographical location. By extrapolation, CD4+ T cell apoptosis is not influenced by differences between clades B and C viruses.

Although the two studies were conducted on different continents and at different times, the same flow cytometer, reagents, cell labelling and PBMC separation were used. The same investigator performed all flow cytometric analyses. That the South African children were younger and hospitalized may, however, have influenced the findings.

7.6. Conclusion

I have demonstrated that symptomatic North American HIV-1-infected children have significantly higher percentages of CD8+ T cell apoptosis than South African children with similar severity of HIV disease. North American children were significantly older, were not hospitalized and better nourished. The most likely explanation is that apoptosis reflects better immunological function in the North American children.

7.7. Recommendations

My data represents a retrospective analysis of data from RSA and North America and should be repeated as a prospectively designed study. Apoptosis data needs to be collected using identical flow cytometric regions and differences in CD95 expression as well as plasma HIV RNA levels should be explored.

CHAPTER 8

GENERAL CONCLUSIONS

HIV infection has the potential to be as devastating to under-resourced communities as the Bubonic plague was to Europe in the 14th and 15th centuries. In wealthy countries HIV infection also causes much morbidity and mortality. For these reasons, knowledge of the pathogenesis of HIV infection and the resultant immunodeficiency is important for the design of therapeutic strategies.

The central role of apoptosis in normal cellular homeostasis and in many pathological processes has become apparent since the early nineties. Studies of the relevance of apoptosis in HIV infection have been outlined in this thesis. The major emphasis of this thesis has been on children.

I describe the development of a simple scatter-based assay for determining apoptosis in PBMCs. The assay is especially useful in resource-constrained countries where flow cytometric facilities have already been established as neither additional reagents nor laboratory time are needed. As shown in later chapters, the assay may have contributed towards new insights in the pathogenesis of HIV infection.

The manner in which PBMCs are labelled with MAb may be a factor in inducing apoptosis. I compared samples labelled either directly with PE-conjugated MAb or indirectly, first with biotinylated MAb and thereafter with streptavidin-FITC and provided evidence supporting enhanced apoptosis in the indirectly labelled specimens. A

speculation, requiring further study, is the possibility that the indirect method may be more sensitive for detecting apoptosis.

CD4+ and CD8+ T cell apoptosis were studied immediately *ex vivo* in HIV-1-infected children, adolescents and adults. Earlier studies had documented elevated apoptosis after overnight culture either spontaneously or after stimulation. My finding of enhanced apoptosis immediately *ex vivo* is therefore relevant to the pathogenesis of disease. Both CD4+ and CD8+ T cell apoptosis *ex vivo* correlated with spontaneous apoptosis after overnight incubation and support the validity of measuring apoptosis immediately *ex vivo*. Although others had correlated the degree of CD4+ T cell apoptosis with CD4+ T cell depletion, my studies provide some of the first data in children. I also demonstrated a correlation between CD8+ T cell apoptosis and CD4+ T cell depletion. My study was the first to use disease-matched controls, seronegative patients with cystic fibrosis, to demonstrate that low-grade sinusitis and bronchiectasis alone do not contribute to the elevated apoptosis seen in children with advanced HIV disease. My data provided preliminary evidence of a tendency towards diminished CD4+ T cell apoptosis in patients who had responded the best to ART by an elevation in CD4+ T cell percentage.

I then studied apoptosis in symptomatic South African HIV-1-infected children and provided some of the first data from developing communities. Once again both CD4+ and CD8+ T cell apoptosis were significantly elevated in HIV infection and correlated with CD4+ T cell depletion. When children were stratified according to hospitalization status, those recovering from intercurrent infection appeared to have more CD4+ T cell apoptosis than those who were either acutely ill or relatively asymptomatic. This finding

supports a role for loss of CD4+ T cells through apoptosis as a result of intercurrent infection and illustrates how intercurrent disease might contribute to HIV progression through CD4+ depletion. The dynamics of CD4+ T cell apoptosis in response to intercurrent infection needs to be studied in more patients and also in HIV-uninfected disease-matched controls.

Lastly, I compared CD4+ and CD8+ T cell apoptosis in North American and South African HIV-infected children and showed elevated CD8+ T cell apoptosis in North American children. South African children were also significantly younger, more malnourished and had higher levels of serum gamma globulin than their North American counterparts. My results support the hypothesis that CD8+ T cell apoptosis might be defective in the South African patients possibly due to diminished Fas expression. Diminished Fas expression, if present, is most likely due to nutritional factors although differences in clade B and C virus might also be present. As my data are derived from retrospective analyses, it is important that they are confirmed in prospective studies and that Fas expression should also be investigated.

Future studies

As a result of studies reported in the present thesis, a number of new avenues need to be explored. These include:

1. The effect of direct and indirect labelling of CD4+ and CD8+ T cells on apoptosis in HIV-infected patients
2. A prospective study of CD4+ and CD8+ T cell apoptosis in HIV-infected children in order to determine whether it distinguishes slow from rapid progressors, its

correlation with plasma HIV RNA and other immunological parameters and whether it predicts ART success or failure.

3. A comparative study of children from resource-rich and poor communities in order to confirm findings in the present thesis, including an evaluation of Fas expression.
4. The influence of nutritional parameters and intercurrent disease such as TB on CD4+ and CD8+ T cell apoptosis.

REFERENCES

1. Centers For Disease Control and Prevention. *Pneumocystis carinii* pneumonia in Los Angeles. MMWR 1981; 30:250-252.
2. Levy JA. HIV and the pathogenesis of AIDS. Vol. 57. Washington DC: ASM Press, 1998.
3. Clavel F, Guetard G, Brun-Vezinet F, et al. Isolation of a new human retrovirus from West African patients with AIDS. Science 1986; 233:343-346.
4. Coffin JM. Retroviridae and their replication. In: Fields BN, ed. Virology. New York: Raven Press, 1990:1437-1500.
5. Greene WC. The molecular biology of human immunodeficiency virus type 1 infection. N Engl J Med 1991; 324:308-317.
6. Maldonado YA. Human immunodeficiency virus and the acquired immunodeficiency syndrome. In: Long SS, Pickering LK, G.Prober C, Eds. Pediatric Infectious Diseases. New York: Churchill Livingstone, 1997:738-748.
7. Connor EM, Sperling RS, Gelber R, Kiselev P, Scott G, al. e. Reduction of maternal-infant transmission of HIV-1 with zidovudine treatment. N Engl J Med 1994; 331:1173-1180.
8. Bertolli J, St. Louis ME, Simonds RJ, et al. Estimating timing of mother-to-child transmission of HIV in a breast-feeding population in Kinshasa, Zaire. J Infect Dis 1996; 174:722-726.

9. Centers For Disease Control and Prevention. HIV and AIDS - United States, 1981 - 2000. MMWR 2001; 50:430-434.

10. Report on the global HIV/AIDS epidemic. Geneva: UNAIDS, 2000.

11. Department of Health. Seventh national HIV survey of women attending antenatal clinics of the public health services in the Republic of South Africa, October/November 1996. Epidemiol Comments 1997; 23:4-16.

12. 2000 Antenatal survey: Department of Health of South Africa, 2001.

13. Centers For Disease Control and Prevention. Guidelines for national human immunodeficiency case surveillance including monitoring for human immunodeficiency virus infection and acquired immunodeficiency syndrome. MMWR 1999; 48:RR-13.

14. Zuchel U, Kauffman SHE. Role of heat shock proteins in protection from and pathogenesis of infectious diseases. Clin Microbiol Rev 1999; 12:19-39.

15. Gottlieb MS, Schroff R, Schanker H, M., et al. Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence for a new acquired cellular immunodeficiency. N Engl J Med 1981; 305:1425-1431.

16. Klatzmann D, Barre-Sinoussi F, Nugeyre MT, et al. Selective tropism of lymphadenopathy associated virus (LAV) for helper-inducer T lymphocytes. Science 1984; 225:59-64.

17. Fauci AS. The human immunodeficiency virus: infectivity and mechanisms of pathogenesis. Science 1988; 239:617-622.

18. Moore JP. Co-receptors: implications for HIV pathogenesis and therapy. Science 1997; 276:51-52.

19. Nowak MA, McMichael AJ. How HIV defeats the immune system. Sci Am 1995; 273:58-65.

20. Ho DD, Neumann AU, Perelson AS, Chen W, Leonard JM, Markowitz M. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature 1995; 373:123-126.

21. Wei X, Gosh SK, Taylor ME, et al. Viral dynamics in human immunodeficiency virus type 1 infection. Nature 1995; 373:117-122.

22. Edelson PJ. Immunology of HIV infection in children and adolescents. In: Long SS, Pickering LK, Prober CG, Eds. Principles and practice of pediatric infectious diseases. New York: Churchill Livingstone, 1997:748-750.

23. Cotton MF, Schaaf HS, Willemsen E, Van Veenendal M, Janse v Rensburg A, Janse v Rensburg E. The burden of mother to child transmission of HIV-1 disease in a "low" prevalence region, a five year study of hospitalized children. S Afr J Epidemiol Infect 1998; 13:46-49.

24. Hussey GD, Reijnhart RM, Sebens AM, Burgess J, Schaaf S, Potgieter S. Survival of children in Cape Town known to be vertically infected with HIV-1. S Afr Med J 1998; 88:554-558.

25. Barnhart HX, Caldwell MB, Thomas P, et al. Natural history of HIV disease in perinatally infected children: an analysis from the pediatric spectrum of diseases project. Pediatrics 1996; 97:710-716.

26. Centers For Disease Control and Prevention. 1994 Revised classification system for human immunodeficiency virus infection in children less than 13 years of age. MMWR 1994; 43:rr1-12.

27. Virchow R. Passive processes. Fatty degenerations. Cellular pathology as based upon physiological and pathological histology. London: John Churchill, 1858.

28. Virchow R. Normal and pathological new formation. Cellular pathology as based upon physiological and pathological histology. London: John Churchill, 1858:418-419.

29. Majno G, Jorris I. Apoptosis, oncosis, and necrosis. An overview of cell death. Am J Pathol 1995; 146:3-15.

30. Kerr JFR, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972; 26:239-255.

31. Wyllie AH, Kerr JFR, Currie AR. Cell Death: the significance of apoptosis. Int Rev Cytol 1980; 68:251-304.

32. Wyllie AH. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 1980; 284:555-556.

33. Glucksman A. Cell deaths in normal vertebrate ontogeny. Biol Rev Camb Philos Soc 1951; 26:59-86.

34. Hetts SW. To die or not to die. An overview of apoptosis and its role in disease. JAMA 1998; 279:300-307.

35. Savill J, Fadok V, Henson P, Haslett C. Phagocyte recognition of cells undergoing apoptosis. Immunol Today 1993; 14:131-136.

36. Martin DJ, Sim JG, Sole GJ, et al. CD4+ lymphocyte count in African patients co-infected with HIV and tuberculosis. J Acquir Immune Defic Syndr Hum Retrovirol 1995; 8:386-391.

37. Zychlinski A, Zheng LM, Liu C-C, Young JD-E. Cytolytic lymphocytes induce both apoptosis and necrosis in target cells. J Immunol 1991; 146:393-400.

38. Russell JH, Masakowski V, Rusinski T, Phillips G. Mechanisms of immune lysis. III. Characterization of the nature and kinetics of the cytotoxic T lymphocyte-induced nuclear lesion in the target. J Immunol 1982; 128:2087-2094.

39. Stacey NH, Bishop CJ, Halliday JW, et al. Apoptosis as the mode of antigen-dependent lymphocytotoxicity. J Cell Sci 1985; 74:169-179.

40. Kisielow P, Von Boehmer H. Negative and positive selection of immature thymocytes: timing and the role of the ligand for alpha/beta T cell receptor. Semin Immunol 1990; 2:35-44.

41. Swat W, Ignatowicz L, Kisielow P. Detection of apoptosis of immature $CD4^{+}8^{+}$ thymocytes by flow cytometry. J Immunol Methods 1991; 137:779-87.

42. Razvi EN, Welsh RM. Programmed cell death of T lymphocytes during acute viral infection: a mechanism for virus-induced immune deficiency. J Virol 1993; 67:5754-5765.

43. Ito M, Watanabe M, Ihara T, Kamiya H, Sakurain N. Fas antigen and bcl-2 expression on lymphocytes cultured with cytomegalovirus and varicella-zoster virus antigen. Cell Immunol 1995; 160:173-177.

44. Uehara T, Miyawaki T, Ohte K, et al. Apoptotic cell death of primed $CD45RO^{+}$ T lymphocytes in Epstein Barr virus-induced infectious mononucleosis. Blood 1992; 80:452-458.

45. Pignata C, Fiore M, De Filippo S, Cavalcanti M, Gaetaniello L, Scotese I. Apoptosis as a mechanism of peripheral blood mononuclear cell death after

measles and varicella-zoster virus infections in children. Pediatr Res 1998; 43:77-83.

46. Hedgecock EM, Sulston JE, Thomson JN. Mutations affecting programmed cell deaths in the nematode *Caenorhabditis elegans*. Science 1983; 220:1277-1279.

47. Golstein P. Controlling cell death. Science 1997; 275:1081-1082.

48. Berke G. Killing mechanisms of cytotoxic lymphocytes. Curr Opin Hematol 1997; 4:32-40.

49. Leithauser F, Dhein J, Mechtersheimer G, et al. Constitutive and induced expression of APO-1, a new member of the nerve growth factor/tumor necrosis factor receptor superfamily, in normal and neoplastic cells. Lab Invest 1993; 69:415-429.

50. Lynch DH, Ramsdell F, Alderson MR. Fas and FasL in the homeostatic regulation of immune responses. Immunol Today 1995; 16:569-574.

51. Strand S, Hofmann WJ, Hug H, et al. Lymphocyte apoptosis induced by CD95 (APO-1/Fas) ligand-expressing tumor cells-a mechanism of immune evasion? Nat Med 1998; 2:1361-1366.

52. Gura T. How TRAIL kills cancer cells, but not normal cells. Science 1997; 277:768.

53. Nicholson DW, Thornberry NA. Caspases: Killer proteases. Trends Biochem Sci 1997; 22:299-306.

54. Stennicke HR, Salvesen GS. Properties of the caspases. Biochim Biophys Acta 1998; 1387:17-31.

55. Darzynkiewicz Z, Juan G, Li X, Gorczyka W, Murakami T, Traganos F. Cytometry in cell necrobiology: analysis of apoptosis and accidental cell death (necrosis). Cytometry 1997; 27:1-20.

56. Cree IA, Nurbhai S, Milne G, Swanson Beck J. Cell death in granulomata: the role of apoptosis. J Clin Path 1987; 40:1314-1319.

57. Duke RC, Cohen JJ. Morphological and biochemical assays of apoptosis. Curr Prot Immunol 1992; Supplement 3:3.17.1-3.17.16.

58. Salzman GC, Singham SB, Johnston RG, Bohren CF. Light scattering and cytometry. In: Melamed MR, Lindmo T, Mendelsohn ML, Eds. Flow cytometry and sorting. New York: Wiley-Liss, 1990:81-107.

59. Darzynkiewicz Z, Bruno S, Del Bino G, et al. Features of apoptotic cells measured by flow cytometry. Cytometry 1992; 13:795-808.

60. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Meth 1991; 139:271-279.

61. Dive C, Gregory CD, Phipps DJ, Evans DL, Miller AE, Wyllie AH. Analysis and discrimination of necrosis and apoptosis (programmed cell death) by multiparameter flow cytometry. Biochim Biophys Acta 1992; 1133:275-285.

62. Katsikis PD, Wunderlich ES, Smith CA, Herzenberg LA, Herzenberg LA. Fas antigen stimulation induces marked apoptosis of T lymphocytes in human immunodeficiency virus-infected individuals. J Exp Med 1995; 181:2029-2036.

63. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labelling of nuclear DNA fragmentation. J Cell Biol 1992; 119:493-501.

64. Gorczyca W, Bruno S, Darzynkiewicz RJ, Gong J, Darzynkiewicz Z. DNA strand breaks occurring during apoptosis: Their early in situ detection by the terminal deoxynucleotidyl transferase and nick translation assays and prevention by serine protease inhibitors. Int J Oncol 1992; 1:639-648.

65. Van Engeland M, Nieland LJW, Ramaekers FCS, Schutte B, Reutelingsperger CPM. Annexin-V affinity assay: a review on an apoptosis detection system based on phospatidylserine exposure. Cytometry 1998; 31:1-9.

66. Potten CS. What is an apoptotic index measuring? A commentary. British Journal of Cancer 1996; 74:1743-1748.

67. Cohen JJ, Duke RC. Apoptosis and programmed cell death in immunity. Annu Rev Immunol 1992; 10:267-293.

68. Cai Q, Huang X-L, Rappocciolo G, Rinaldo CR. Natural killer cell responses in homosexual males with early HIV infection. J Acq Immunodefic Syndr 1990; 3:669--676.

69. Cotton MF, Ikle DN, Rapaport EL, et al. Apoptosis of CD4+ and CD8+ T cells isolated immediately *ex vivo* correlates with disease severity in human immunodeficiency virus type 1 infection. Pediatric Res 1997; 42:656-644.

70. Carbonari M, Marina Cibati M, Cherchi M, et al. Detection and characterization of apoptotic peripheral blood lymphocytes in human immunodeficiency virus

infection and cancer chemotherapy by a novel flow immunocytometric method. Blood 1994; 83:1268-1277.

71. Schnittman SM, Psallidopopoulis MC, Lane HC, et al. The reservoir for HIV-1 in human peripheral blood is a T cell that maintains expression of CD4. Science 1989; 245:305-308.

72. Clerici M, Stocks NI, Zajac RA, et al. Detection of three distinct patterns of T helper cell dysfunction in asymptomatic human immunodeficiency virus-seropositive patients. Independence of T cell numbers and clinical staging. J Clin Invest 1989; 84:1892-1899.

73. Miedema F, Petit AJ, Terpstra FG, et al. Immunological abnormalities in human immunodeficiency virus (HIV)-infected asymptomatic homosexual men. HIV affects the immune system before CD4+ T helper depletion occurs. J Clin Invest 1989; 82:1908-1904.

74. Ameisen JC, Capron A. Cell dysfunction and depletion in AIDS: the programmed cell death hypothesis. Immunol Today 1991; 12:102-105.

75. Embretson J, Zupanic M, Ribas JL, et al. Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS. Nature 1993; 362:292-293.

76. Pantaleo G, Graziozi C, Demarest JF, et al. HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. Nature 1993; 364:291-292.

77. Laurent-Crawford AG, Krust B, Muller S, et al. The cytopathic effect of HIV is associated with apoptosis. Virology 1991; 185:829-839.

78. Terai C, Kornbluth RS, Pauza CD, Richman DD, Carson DA. Apoptosis as a mechanism of cell death in cultured T lymphoblasts acutely infected with HIV-1. J Clin Invest 1991; 87:1710-1715.

79. Groux H, Torpier G, Monte D, Mouton Y, Capron A, Ameisen JC. Activation-induced death by apoptosis in $CD4^+$ T cells from human immunodeficiency virus-infected asymptomatic individuals. J Exp Med 1992; 175:331-340.

80. Meyaard L, Otto SA, Jonker RR, Mijnster MJ, Keet RPM, Miedema F. Programmed death of T cells in HIV-1 infection. Science 1992; 257:217-219.

81. Meyaard L, Otto SA, Keet IPM, Roos MTL, Miedema F. Programmed death of T cells in human immunodeficiency virus infection. No correlation with progression to disease. J Clin Invest 1994; 93:982-988.

82. Pandolfi F, Pierdominici M, Oliva A, et al. Apoptosis-related mortality in vitro of mononuclear cells from patients with HIV infection correlates with disease severity and progression. J Acq Immune Defic Syndr Hum Retrov 1995; 9:450-458.

83. Oyaizu N, McCloskey TW, Coronesi M, Chirmule N, Kalyanaraman VS, Pahwa S. Accelerated apoptosis in peripheral blood mononuclear cells (PBMCs) from human immunodeficiency virus type-1 infected patients and in CD4 cross-linked PBMCs from normal individuals. Blood 1993; 82:33992-3400.

84. Walker BD, Chakrabarti S, Moss B, et al. HIV-specific cytotoxic T lymphocytes in seropositive individuals. Nature 1987; 328:345-348.

85. Sattentau QJ, Dalgleish AG, Weiss RA, Beverley PC. Epitopes of the CD4 antigen and HIV infection. Science 1986; 234:1120-1123.

86. Banda NK, Bernier J, Kurahara DK, et al. Cross-linking CD4 by human immunodeficiency virus gp120 primes T cells for activation-induced apoptosis. J Exp Med 1992; 176:1099-1106.

87. Newell MK, Haughn LJ, Maroun CR, Julius MH. Death of mature T cells by separate ligation of CD4 and the T-cell receptor for antigen. Nature 1990; 347:286-289.

88. Oh S-Y, Cruikshank WW, Raina J, et al. Identification of HIV-1 envelope glycoprotein (gp120) bound to CD4 determinants on normal lymphocytes and expressed by infected cells serves as target for immune attack. J Acq Immunodefic Syndr 1992; 5:251-256.

89. Westendorp MO, Frank R, Ochsenbauer C, et al. Sensitization of T cells to CD95-mediated apoptosis by HIV-1 tat and gp120. Nature 1995; 375:497-500.

90. Aceituno E, Castanon S, Jimenez C, et al. Circulating immune complexes from HIV-1+ patients induces apoptosis on normal lymphocytes. Immunology 1997; 92:317-320.

91. Su L, Kaneshima H, Bonyadi M, et al. HIV-1-induced thymocyte depletion is associated with indirect cytopathicity and infection of progenitor cells in vivo. Immunity 1995; 2:25-36.

92. Cavert W, Notermans DW, Staskus K. Kinetics of response in lymphoid tissues to antiretroviral therapy of HIV-1 infection. Science 1997; 276:960-964.

93. McCloskey TW, Oyaizu N, Kaplan M, Pahwa S. Expression of the Fas antigen in patients infected with the human immunodeficiency virus. Cytometry 1995; 22:111--114.

94. Bohler T, Nedel S, Debatin K-M. CD95-induced apoptosis contributes to loss of primed/memory but not resting naive T cells in children infected with human immunodeficiency virus type 1. Pediatr Res 1997; 41:878-885.

95. Baumler CB, Bohler T, Herr I, A. B, Krammer PH, Debatin K-M. Activation of the CD95 (APO-1/Fas) system in T cells from human immunodeficiency virus infected type 1-infected children. Blood 1996; 88:1268-1277.

96. Herbein G, Mahlknecht U, Batiwalla F, et al. Apoptosis of CD8+ T cells is mediated through macrophages through interaction of HIV gp120 with chemokine receptor CXCR4. Nature 1998; 395:189-194.

97. Finkel TH, Tudor-Williams G, Banda NK, et al. Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV- and SIV-infected lymph nodes. Nat Med 1995; 1:129-134.

98. Goebel WS, Hommel-Berey G, Brahmi Z. Lymphokine-activated killer cells do not induce degradation of viral or cellular RNA in targets in which DNA fragmentation occurs. FASEB J 1992; 6:A1335.

99. McCloskey TW, Ott M, Tribble E, et al. Dual role of HIV tat in regulation of apoptosis in T cells. J Immunol 1997; 158:1014-1019.

100. Sunila I, Vaccarezza M, Pantaleo G, Fauci A, Orenstein JM. gp120 is present on the plasma membrane of apoptotic CD4 cells prepared from lymph node of HIV-1-infected individuals: an immunoelectron microscopic study. AIDS 1997; 11:27-32.

101. Muro-Cacho CA, Pantaleo G, Fauci AS. Analysis of apoptosis in lymph nodes of HIV-infected persons. Intensity of apoptosis correlates with the general state of

activation of the lymphoid tissue and not with stage of disease or viral burden. J Immunol 1995; 154:5555-5566.

102. Huang L, Soldervilla G, Leeker M, Flavell R, Crispe N. The liver eliminates T cells undergoing antigen-triggered apoptosis in vivo. Immunity 1996; 1:741.

103. Mittler R, Del Pozo O, Meisel L, Lam E. Pathogen-induced programmed cell death in plants, a possible defense mechanism. Dev Genet 1997; 21:279-289.

104. Antoni BA, Sabbatini P, Rabson AB, White E. Inhibition of apoptosis in human immunodeficiency virus-infected cells enhances virus production and facilitates persistent infection. J Virol 1995; 69:2384-2392.

105. Chinnaiyan AM, Woffendin C, Dixit VM, Nabel GJ. The inhibition of pro-apoptotic ICE-like proteases enhances HIV replication. Nat Med 1997; 3:333-337.

106. Rosner B. Fundamentals of Biostatistics. Boston: PWS-Kent, 1990:251-252.

107. Cotter TG, Lennon SV, Glynn JM, Green DR. Microfilament-disrupting agents prevent the formation of apoptotic bodies in tumor cells undergoing apoptosis. Cancer Res 1992; 52:997-1005.

108. Wesselborg S, Kabelitz D. Activation-driven death of human T cell clones: time course kinetics of the induction of cell shrinkage, DNA fragmentation, and cell death. Cell Immunol 1993; 148:234-241.

109. JMP User's guide. Version 2 of JMP. In: Inc SI, ed. Cary, North Carolina: SAS Institute Inc, 1989.

110. Schmid I, Uitenbogaart CH, Keld B, Giorgi JV. A rapid method for measuring apoptosis and dual-color immunofluorescence by single laser flow cytometry. J Immunol Methods 1994; 170:145-157.

111. Schmid I, Uittenbogaart CH, Giorgi JV. A sensitive method for measuring apoptosis and cell surface phenotype in human thymocytes by flow cytometry. Cytometry 1994; 15:12.

112. Mittler RS, Rao PE, Talle MA, Look R, Goldstein G. Cell membrane pertubation of resting T cells ad thymocytes causes display of activation antigens. J Exp Med 1983; 158:99-111.

113. Debatin K, Fahrig-Faissner A, Enenkel-Stoodt S, Kreuz W, Brenner A, Krammer PH. High expression of APO-1 (CD95) on T lymphocytes from human immunodeficiency virus-1 infected children. Journal of Infectious Diseases 1994; 83:3101-3103.

114. Bohler T, Baumler C, Herr I, Groll A, Kurz M, Debatin KM. Activation of the CD95 system increases with disease progression in human immunodeficiency virus type 1-infected children and adolescents. Pediatr Infect Dis J 1997; 16:754-759.

115. Centers For Disease Control and Prevention. Revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults. MMWR 1993; 41:RR-17.

116. Gougeon M-L, Garcia S, Heeney J, et al. Programmed cell death in AIDS-related HIV and SIV infections. AIDS Res Hum Retroviruses 1993; 9:553-563.

117. Casella RC, Finkel TH. Mechanisms of lymphocyte killing by HIV. Curr opin Hematol 1997; 4:24-31.

118. Chirmule N, Lesser M, Gupta A, Ravipati M, Kohn N, Pahwa S. Immunological characteristics of HIV-infected children: relationship to age, CD4 counts, disease progression and survival. AIDS Res Hum Retroviruses 1995; 10:1209-1219.

119. Phillips AN, Eron JJ, Bartlett JA, et al. HIV-1 RNA levels and the development of clinical disease. AIDS 1996; 10:859-865.

120. Forte M, Maartens G, Rahelu M, et al. Cytolytic T-cell activity against mycobacterial antigens in HIV. AIDS 1992; 6:407-411.

121. Carmichael A, Jin X, Borysiewicz L. Quantitative analysis of human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic T lymphocyte (CTL) response at different stages of HIV-1 infection: differential CTL responses to HIV-1 and Epstein-Barr virus in late disease. J Exp Med 1993; 177:249-256.

122. Prati E, Gorla R, Malacarne F, et al. Study of spontaneous apoptosis in HIV+ patients: correlation with clinical progression and T cell loss. AIDS Res Hum Retroviruses 1997; 13:1501-1508.

123. Andrieu J, Lu W, Levy R. Sustained Increases in CD4 cell counts in asymptomatic human immunodeficiency virus type 1-seropositive patients treated with prednisolone for 1 year. J Infect Dis 1995; 171:523-530.

124. Bohler T, Walcher J, Holz-Wenig G, et al. Early effects of antiretroviral combination therapy on apoptosis and regeneration of T cells in HIV-1-infected children and adolescents. AIDS 1999; 13:779-789.

125. Pitrak DL, Bolanos J, Hershow R, Novak RM. Discordant CD4 T lymphocyte responses to antiretroviral therapy for HIV infection are associated with ex-vivo rates of apoptosis. AIDS 2001; 15:1317-1319.

126. The European Collaborative Study. Natural history of vertically acquired human immunodeficiency virus-1 infection. Pediatrics 1994; 94:815-819.

127. Jean SS, Reed GW, Verdier R-I, Pape JW, Johnson WD, Wright PF. Clinical manifestations of human immunodeficiency virus infection in Haitian children. Pediatr Infect Dis J 1997; 16:600-606.

128. Schaaf HS, Geldenhuys A, Gie RP, Cotton MF. Culture-positive tuberculosis in human immunodeficiency virus type 1-infected children. Pediatr Infect Dis J 1998; 17:599-604.

129. Martin DJ, Blackburn NK, O'Connell KF, Brant ET, Goetsch EA. Evaluation of the World Health Organization antibody-testing strategy for the individual diagnosis of HIV infection. S Afr Med J 1995; 85:877-880.

130. Engelbrecht S, Janse v Rensburg E. Detection of South African human immunodeficiency virus type 1 by polymerase chain reaction: evaluation of different primer sets and conditions. J Virol Methods 1995; 55:391-400.

131. Sanders-Buell E, Salminen M, McCutchan FE. Sequencing primers for HIV-1. in: Myers G, Korber B, Hahn BH et al, Eds. Human Retroviruses and AIDS. Vol. part III. Los Alamos, New Mexico, U.S.A.: Los Alamos Laboratory, 1995.

132. Coste J, Montes B, Reynes J, et al. Comparative evaluation of three assays for the quantitation of human immunodeficiency virus type 1 RNA in plasma. J Med Virol 1996; 50:293-302.

133. Feinstein AR. Clinical Biostatistics. St Louis: CV Mosby, 1977.

134. Michel P, Balde AT, Roussilhon C, Aribot G, Sarthou J-L, Gougeon M-L. Reduced immune activation and T cell apoptosis in human immunodeficiency

virus type 2 compared with type 1: correlation with T cell apoptosis with B2 microglobulin concentration and disease evolution. J Infect Dis 2000; 181:64-75.

135. Scott GB, Hutto C, Makuch RW, et al. Survival in children with perinatally acquired human immunodeficiency virus type 1 infection. N Engl J Med 1989; 321:1791-1796.

136. Bobat R, Coovadia H, Coutsoudis A, Moodley D. Determinants of mother-to-child transmission of HIV-1 infection in a cohort from Durban, South Africa. Pediatr Infect Dis J 1996; 15:604-610.

137. Engelbrecht S, Smith TL, Kasper P, et al. HIV type 1 V3 domain serotyping and genotyping in Gauteng, Mpumalanga, KwaZulu-Natal and the Western Cape Provinces of South Africa. AIDS Res Hum Retroviruses 1999; 15:325-328.

138. Cotton MF, Casella C, Rapaport EL, Tseng P-O, Marschner S, Finkel TH. Apoptosis in HIV-1 infection. Behring Inst Mitt 1996; 97:220-231.