

ANTIRETROVIRAL THERAPY INFLUENCES CELLULAR SUSCEPTIBILITY TO APOPTOSIS *IN VIVO*

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1. ABSTRACT

It has been proposed that antiretroviral therapies (ART) possess both antiviral and immunomodulatory activities when used in HIV infected patients. Few studies have addressed whether these putative immunomodulatory effects are also seen in HIV negative patients, for example, when used for post exposure prophylaxis (PEP). We chose to evaluate immunologic function in HIV negative patients who received Nelfinavir and Combivir (AZT and 3TC) as PEP. Lymphocytes from patients taken immediately before, during, and after PEP were analyzed. No changes were seen in absolute or percent CD4 or CD8 T lymphocyte numbers, nor in markers of activation, memory, or co-stimulatory molecules. Surface expression of apoptosis-related ligands and receptors were unaltered, but apoptosis susceptibility was significantly inhibited by PEP (P<0.05). These data confirm *in vitro* that apoptosis susceptibility is altered by ART, including in HIV-negative patients who take PEP.

2. INTRODUCTION

Although evidence from several laboratory and clinical-based studies suggest that antiretroviral therapies (ART) possess intrinsic immune modulating effects (1), this has yet to be demonstrated in HIV negative patients. In

the few studies conducted using these drugs in HIV negative patients, there have been conflicting results: an increase in CD4 T lymphocyte count following zidovudine (2,3), a reduction in total and percent CD4 and CD8 T lymphocyte count in two subjects receiving indinavir monotherapy (4), and no changes in T cell number or phenotype in a larger study of HIV-seronegative patients receiving post exposure prophylaxis (PEP) (5). Overall, these data do not suggest that ART effects CD4 or CD8 T lymphocyte counts in HIV negative patients.

More recently, other measures of immune function have been reported to be altered by HIV medications. Mice receiving zidovudine show a decreased ability to respond to foreign antigens including gp120 and coalbumin (6), have altered expression of the apoptosis regulatory molecules, Fas (CD95) and Fas ligand (7), suppression of GM-CSF receptor type alpha (8), and premature cellular senescence with consequent enhanced apoptosis (9). Similarly immunologic changes may be seen in individuals who initiate PI based antiretroviral therapy. Lymphoid tissues of treated patients demonstrate a reduction in levels of apoptosis, reduced expression of markers of activation, and improved cytokine production profiles, CD4+ T cell helper function and increases in naive

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CD4+ cell number (10-13). Given the immunologic effects of these therapies on HIV infected patients, as well as HIV uninfected lab animals, we reasoned that some similar changes must occur in HIV uninfected patients, for example, patients who initiate PEP.

### 3. METHODS

Blood specimens from consenting, healthy adult volunteers initiating PEP were collected for immunologic evaluation immediately prior to the initiation of PEP, during PEP, and at least one month following PEP. Blood was collected in heparin and EDTA-containing tubes. This study was approved by The Ottawa Hospital Research Ethics Board.

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation, using Ficoll Hypaque Plus (Pharmacia Biotech, Baie d'Urfé, QC). Following centrifugation, cells were washed and resuspended in RPMI 1640 (GIBCO Laboratories, Burlington, ON) supplemented with 10% FCS serum (GIBCO), penicillin, streptomycin and glutamine (Sigma, Oakville, ON). PBMCs ( $1 \times 10^6$  cells per ml) were incubated at 37°C in a 5% CO<sub>2</sub> humidified environment.

Immunophenotyping was performed on whole blood and on isolated PBMCs following a 10-minute blocking step with BSA. Following incubation, samples were lysed and fixed using a Multi Q-Prep (Beckman Coulter). PBMCs were isolated using Ficoll Hypaque Plus as described above.  $1 \times 10^6$  cells were stained with the following antibodies: FasL PE (NOK1, Pharmingen), TRAIL L-PE (M180, Immunex Corp.), the remaining antibodies to CD3, CD56, CD19, CD4, CD8 CD38, HLA DR, CD45RO, CD45 RA CD62L and CD28 were purchased from Beckton Coulter and contained FITC, PE or PC5 Fluorochrome tags. Samples were analyzed using an Epics ELITE flow cytometer (Beckman Coulter) with 2 or 3 color immunofluorescence and 10,000 events were counted for each sample.

Proliferation assays were performed on isolated PBMCs as previously described (14). Briefly, 100ul of cell suspension was aliquoted into 96-well plates (Falcon) and was stimulated in triplicate wells with HIV p24 antigen (Chiron Biomaterials; supplied by the National Institutes of Health AIDS Research and Reference Reagent Program; final concentration, 1 ug/mL), phytohemagglutinin-M (PHA; Gibco BRL; final dilution, 1: 200), or medium alone. After 6 days of incubation at 37°C with 5% CO<sub>2</sub>, cells were pulsed with 1 uCi of [3H]thymidine. After a further 18-h incubation, cells were harvested (Harvester 96; Tomtec), and [3H]thymidine incorporation was measured as counts per minute (cpm) of radioactivity on a scintillation counter (1450 Microbeta Plus; Wallac Oy). Stimulation index (SI) was defined as the average cpm in the presence of a stimulus divided by average cpm in its absence. We considered an SI >3 to represent a proliferative response. Thus, we term subjects with such responses "responders" at the time point the SI was >3.

Apoptosis of PBMCs was induced with camptothecin (CPT; 10µM, Sigma) or an agonistic anti-Fas antibody, clone CH11 (CH11; 0.5µg/ml, Beckman Coulter, Mississauga, ON). Control cells were cultured in media only. Following incubation for 16 hours, apoptosis was measured by Annexin-V (BD Biosciences, Palo Alto, CA) and propidium iodide (Sigma) staining. Prior to this study, we validated this assay against a terminal deoxyuridine nucleotide end labeling (TUNEL) assay. Samples were analyzed using an Epics ELITE flow cytometer (Beckman Coulter), and 10,000 events were counted for each sample. Statistical analysis was by paired t tests.

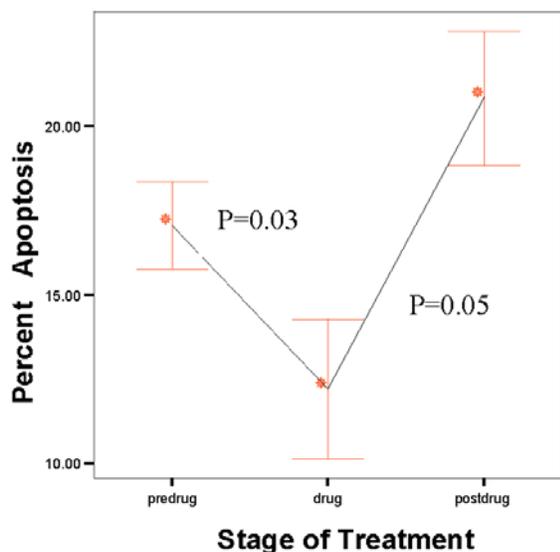
### 4. RESULTS

Eight healthy subjects (5 male) without evidence of immune disease were enrolled. None were on immune modifying medications. The median age of this cohort was 31 years (range: 24 to 61) and median duration of antiretroviral exposure prior to "on therapy" lymphocyte analysis was 9 days (range: 4 to 28). No patients acquired any infectious disease as a result of their exposure. No changes were observed with respect to the following: CD4%, CD4 absolute number, CD8%, CD8 absolute number, CD4/CD38%, CD8/CD38%, CD4/HLA-DR%, CD8/HLA-DR%, CD4/CD38/HLA-DR%, CD8/CD38/HLA-DR%CD4/CD4RO%, CD4/CD45 RA%, CD8/CD45 RO%, CD8/CD45 RA, CD4/CD45 RA/CD62L%, CD8/CD45 RA/CD62L%, CD4/CD28%, CD8/CD28%, CD4/CD95%, CD8/CD95%.

As therapy of HIV infected patients is associated with enhanced lymphoproliferative responses (LPR), we assessed whether these agents affect LPR when used as PEP. LPR, in response to *Candida* antigen or tetanus were unchanged by PEP, as were the antigen non-specific responses to PWM.

Apoptosis is a form of cell death, which is necessary for normal cellular homeostasis, tissue turnover and immune regulation. When this system is dysregulated, enhanced cell death may ensue, for example, in ischemia, immunodeficiency, viral, bacterial, and protozoan pathologies, as well as in diseases of unknown etiology such as inflammatory bowel disease. Alternatively, changes which inhibit apoptosis may result in malignancies or autoimmunity. As the potential impact of disordered apoptosis is serious, the control of apoptosis is complex and occurs at many levels. For example, the prototypic death receptor Fas (CD95) is constitutively expressed at low levels on a wide variety of cell types, including T cells, yet receptor expression alone is insufficient to result in apoptosis even after ligation of the receptor with its cognate ligand. Sensitivity towards Fas receptor induced apoptosis requires cellular activation in addition to Fas receptor expression. Activation causes downregulation of apoptosis inhibitory molecules, including cFLIP and Bcl2 family members, as well as increasing the density of Fas receptor expression. Thereafter, upon exposure to Fas ligand, the activated, Fas expressing, apoptosis sensitive, T cell will undergo apoptotic cell death.

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**Figure 1.** Effect of Antiretroviral therapy on Camptothecin-Induced Apoptosis. Error bars show mean  $\pm 1.0$  standard error.

We analyzed spontaneous apoptosis in four patients before, during and after PEP. Consistent with previous publications, spontaneous apoptosis was minimal (mean 4.6%) and unchanged by therapy. Fas induced apoptosis was also measured, and consistent with the low levels of activation and low levels of Fas receptor expression, minimal Fas induced apoptosis was seen (mean 5.5%) and unchanged by PEP. Camptothecin is a chemotherapeutic agent which acts directly on mitochondria to induce mitochondrial pore opening with consequent release of cytochrome c, caspase 9 activation and then apoptosis. As such, all cells are sensitive to CPT induced apoptosis, irrespective of activation status. CPT induced apoptosis was reduced by PEP (Figure 1). Changes from before treatment to during treatment samples ( $P=0.03$ ) and during treatment to posttreatment ( $P=0.05$ ) samples were significantly inhibited by PEP.

It has been previously reported that PI down regulate Fas ligand (15-16), therefore we evaluated expression of FasL and the related protein TRAIL, on CD3+ T cells, CD56+ NK cells and CD19+ B cells. No consistent changes were observed in FasL nor TRAIL expression on any cell subset.

## 5. DISCUSSION

We demonstrate an inhibitory effect of short-term combination protease inhibitor and nucleoside reverse transcriptase inhibitor therapy on apoptosis signaling in HIV seronegative patients, yet no impact on a variety of cellular receptors and ligand expression, nor on functional T cell responses. These data are consistent with *in vitro* observations that PI inhibit apoptosis in response to a variety of stimuli, and that the mode of inhibition is localized to mitochondrial pore function.

The impact of these changes in HIV negative patients who take such medication for short terms, as a part of PEP, are likely minimal. Conversely, the impact of such immunologic changes in patients who use these medications for prolonged periods is unknown, but may impact such disease processes as neoplasia development, degree of immune reconstitution, and normal cellular turnover. Further investigation of this phenomenon to determine if this observation is antiretroviral class- or drug-specific is warranted.

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## 7. REFERENCES

1. Phenix B. N., C. Cooper, C. Owen & A. D. Badley: Modulation of apoptosis by HIV protease inhibitors. *Apoptosis* 7, 295-312. (2002)
2. Levy J. A., B. Ramachandran, E. Barker, J. Guthrie & T. Elbeik: Plasma viral load, CD4+ cell counts, and HIV-1 production by cells. *Science* 271, 670-671 (1996)
3. Milazzo L., L. M. Vaira & L. Cremonesi: CD4+ lymphocyte count variations in HIV-negative subjects treated with zidovudine. *Aids* 10, 1444-1445 (1996)
4. Barker E., J. Kahn, S. Fujimura & J. A. Levy: Protease inhibitors do not increase the CD4+ cell count in HIV-uninfected individuals. *Aids* 12, 1117-1118 (1998)
5. Puro V. & G. Ippolito: Brief report: effect of antiretroviral agents on T-lymphocyte subset counts in healthy HIV-negative individuals. The Italian Registry on Antiretroviral Postexposure Prophylaxis. *J Acquir Immune Defic Syndr* 24, 440-443 (2000)
6. McKallip R. J., M. Nagarkatti & P. S. Nagarkatti: Immunotoxicity of AZT: inhibitory effect on thymocyte differentiation and peripheral T cell responsiveness to gp120 of human immunodeficiency virus. *Toxicol Appl Pharmacol* 131, 53-62 (1995)
7. Bowden R., S. Soto & S. Specter: Modulation of fas/fasL in a murine retroviral infection by AZT and methionine enkephalin. *Adv Exp Med Biol* 493, 143-152 (2001)
8. Chitnis S., D. Mondal & K. C. Agrawal: Zidovudine (AZT) treatment suppresses granulocyte-macrophage colony stimulating factor receptor type alpha (GM-CSFR alpha) gene expression in murine bone marrow cells. *Life Sci* 71, 967-978 (2002)
9. Tejera A. M., D. F. Alonso, D. E. Gomez & O. A. Olivero: Chronic *in vitro* exposure to 3'-azido-2', 3'-dideoxythymidine induces senescence and apoptosis and reduces tumorigenicity of metastatic mouse mammary tumor cells. *Breast Cancer Res Treat* 65, 93-99 (2001)

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10. Angel J. B., K. G. Parato, A. Kumar, S. Kravcik, A. D. Badley, C. Fex, D. Ashby, E. Sun & D. W. Cameron: Progressive human immunodeficiency virus-specific immune recovery with prolonged viral suppression. *J Infect Dis* 183, 546-554. (2001)
11. Lederman M. M., E. Connick, A. Landay, D. R. Kuritzkes, J. Spritzler, M. St Clair, B. L. Kotzin, L. Fox, M. H. Chiozzi, J. M. Leonard, F. Rousseau, M. Wade, J. D. Roe, A. Martinez & H. Kessler: Immunologic responses associated with 12 weeks of combination antiretroviral therapy consisting of zidovudine, lamivudine, and ritonavir: results of AIDS Clinical Trials Group Protocol 315. *J Infect Dis* 178, 70-79. (1998)
12. Connors M., J. A. Kovacs, S. Krevat, J. C. Gea-Banacloche, M. C. Sneller, M. Flanigan, J. A. Metcalf, R. E. Walker, J. Falloon, M. Baseler, I. Feuerstein, H. Masur & H. C. Lane: HIV infection induces changes in CD4+ T-cell phenotype and depletions within the CD4+ T-cell repertoire that are not immediately restored by antiviral or immune-based therapies. *Nat Med* 3, 533-540. (1997)
13. Autran B., G. Carcelain, T. S. Li, C. Blanc, D. Mathez, R. Tubiana, C. Katlama, P. Debre & J. Leibowitch: Positive effects of combined antiretroviral therapy on CD4+ T cell homeostasis and function in advanced HIV disease. *Science* 277, 112-116. (1997)
14. Clerici M., N. I. Stocks, R. A. Zajac, R. N. Boswell, D. R. Lucey, C. S. Via & G. M. Shearer: Detection of three distinct patterns of T helper cell dysfunction in asymptomatic, human immunodeficiency virus-seropositive patients. Independence of CD4+ cell numbers and clinical staging. *J Clin Invest* 84, 1892-1899 (1989)
15. Sloand E. M., N. S. Young, T. Sato, S. Kim & J. P. Maciejewski: Inhibition of interleukin-1beta-converting enzyme in human hematopoietic progenitor cells results in blockade of cytokine-mediated apoptosis and expansion of their proliferative potential. *Exp Hematol* 26, 1093-1099 (1998)
16. Weichold F.F., J.L. Bryant, S. Pati, O. Barabitskaya, R.C. Gallo & M.S. Retitz Jr.: HIV-1 protease inhibitor ritonavir modulates susceptibility to apoptosis of uninfected T cells. *J Hum Virol* 2, 261-269 (1999)

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