Improvement in Cell-Mediated Immune Function during Potent Anti-Human Immunodeficiency Virus Therapy with Ritonavir plus Saquinavir

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Inhibiting human immunodeficiency virus (HIV) replication with potent antiretroviral therapy may result in improved immune function, and this may lead to favorable outcomes, independent of changes in CD4⁺ lymphocyte count. The effect of combination protease inhibitor therapy (ritonavir plus saquinavir) on functional measures of cell-mediated immunity in 41 HIV-infected patients from one center of a multicenter trial was investigated. After 24 weeks, median plasma virus load decreased from 4.74 log₁₀ copies/mL to below the detection limit of the assay (2.30 log₁₀), and mean CD4⁺ lymphocyte count increased from 284 cells/μL to 413 cells/μL. Proliferative responses to phytohemagglutinin developed in 21 of 34 patients in whom responses were absent at baseline. Increases were observed in interleukin-2, -12, and -10 production and in the expression of CD28 on CD8⁺ lymphocytes. Initiation of potent anti-HIV therapy results in a degree of immune restoration, suggesting that HIV-induced immune suppression is a dynamic and potentially reversible process.

Progressive loss of cell-mediated immunity (CMI) is the hallmark of human immunodeficiency virus (HIV) disease and is responsible for the ultimate development of serious opportunistic infections and malignancies. Although the CD4⁺ T cell count is useful in the prognosis and therapy of HIV-infected persons, it is an imperfect measure of cellular immune function. Cell-mediated immune dysfunction, as determined by impaired peripheral blood mononuclear cell (PBMC) proliferation or interleukin (IL)-2 production, can be detected early in the course of HIV infection [1–4] and provides prognostic information independent of CD4⁺ T cell number [5, 6]. Restoration of cell-mediated immune responses may, therefore, be of considerable benefit to HIV-infected patients.

In addition to PBMC proliferation and IL-2 production, CMI can be evaluated by a number of other measures. Generation of cell-mediated immune responses is associated with the production of IL-12, whereas IL-10 down-regulates such responses [7–9]. Progression of HIV disease appears to be associated with decreased production of IL-12 [10–12] and an overexpression of IL-10 [13], and the impaired proliferative capacity of PBMC from HIV-infected persons can be restored in vitro by the addition of IL-12 or anti–IL-10 antibodies [12–16]. This suggests that a reversible loss of CMI exists in HIV-infected persons and is, at least in part, due to dysregulation of IL-12 and IL-10 production.

Optimal T cell function requires the interaction of the T cell receptor with the major histocompatibility complex–peptide complex on the antigen-presenting cell (APC) in association with a costimulatory signal. This costimulatory signal involves the interaction of the B7 molecule on the APC with the CD28 molecule, which is expressed on the surface of ~95% of CD4⁺ T cells and 50% of CD8⁺ T cells in peripheral blood of HIV-seronegative persons [17–20]. Decreased CD28 expression on CD8⁺ T cells, and to a lesser degree on CD4⁺ T cells, has been shown to be associated with progression of HIV disease [21–24] and is felt to be another indicator of impaired CMI.

Along with their ability to decrease virus load, improve CD4⁺ T cell counts, and improve clinical outcomes, antiretroviral agents have been shown in a small number of studies to restore CMI, as demonstrated by an increase in PBMC proliferation or IL-2 production [25–30]. One report, which correlated changes in immune function with clinical outcomes, showed that improvement in T helper cell function, but not changes in CD4⁺ lymphocyte counts, correlated with a decreased incidence of infections [28]. These studies are limited by the fact that all patients were receiving relatively weak anti-HIV therapy (monotherapy) and they involved small numbers of patients, many with advanced disease. Likely as a result, improvements in immune responses were often transient.

We are currently involved in a multicenter study of the use of combination antiretroviral therapy with two protease...
inhibitors, ritonavir and saquinavir, in HIV-infected persons. These two agents have a synergistic pharmacokinetic interaction that increases saquinavir bioavailability and have largely nonoverlapping viral resistance patterns [31, 32]. This has given us an opportunity to study the effect of this potent antiretroviral therapy on several aspects of immune function in a relatively large number of patients.

Materials and Methods

Patients. From April 1996 until July 1996, 42 patients were enrolled into a clinical trial entitled “Safety and Efficacy of Ritonavir in Combination with Saquinavir in HIV-Infected Patients” (Abbott Laboratories Protocol M96-462) at the Ottawa General Hospital. Inclusion criteria included a CD4+ lymphocyte count of 100–500 cells/μL, discontinuation of antiretroviral therapy for at least 2 weeks, and no prior use of protease inhibitors. Patients were randomized to one of four combinations of ritonavir plus saquinavir, and all patients received at least 400 mg of ritonavir twice daily and 400 mg of saquinavir twice daily.

HIV RNA level and CD4+ lymphocyte counts. At frequent intervals, including baseline, week 4, and week 24, blood was collected in an acid citrate dextrose-containing tube, and plasma was immediately separated and transported to a central laboratory (SciCor, Indianapolis) for quantitation of plasma HIV RNA by reverse transcriptase–polymerase chain reaction (Roche Laboratories; detection limit, 200 [2.30 log10] RNA copies/mL). At the same time points, blood was collected in an EDTA-containing tube and transported to a central laboratory (SciCor) for quantitation of CD4+ lymphocyte number.

PBMC isolation and proliferation assays. At baseline and at weeks 4 and 24, blood was drawn into sodium citrate–containing tubes and PBMC were isolated by centrifugation over Ficoll-Paque (Pharmacia, Baie d’Urfe, Canada), and resuspended at 3 x 10^6 cells/mL in endotoxin-free RPMI 1640 with 5% pooled HIV-seronegative AB serum from a single lot (Advanced Biotechnologies, Columbia, MD). Proliferation assays were performed as previously described [1]. Cell suspension was aliquoted (100 μL) into 96-well plates (Falcon, Lincoln Park, NJ) and stimulated in triplicate with HIV p24 antigen (Chiron Biomaterials, Emeryville, CA; final concentration, 1 μg/mL) or phytohemagglutinin-M (PHA; Gibco BRL, Grand Island, NY; final dilution, 1:200). After 6 days of incubation at 37°C and 5% CO2, cells were pulsed with 1 μCi of [3H]thymidine. After a further 24 h of incubation, cells were harvested (Harvester 96; Tomtec, Orange, CT), and [3H]thymidine incorporation was read as counts per minute (cpm) of β-radioactivity by a scintillation counter (1450 Microbeta PLUS; Wallac, Turku, Finland). Stimulation index (SI) was defined as cpm in the presence of a stimulus divided by cpm in its absence, thereby providing an internal control for CD4+ lymphocyte number. An SI of >3 was considered to represent a proliferative response. When there was a proliferative response to a stimulus at baseline, an increase in the SI of >2.5-fold (250%) was considered to indicate an improvement in the proliferative response.

Measurement of cytokine production. PBMC suspension (1 mL) was aliquoted into 12 x 75 mm polypolyethylene tubes (Falcon) and supplemented with 500 μL of either PHA (final dilution, 1:100), lipopolysaccharide (Salmonella enteritidis LPS; Sigma, St. Louis; catalog L-2012; final concentration, 1 μg/mL) or Staphylococcus aureus Cowan strain 1 (SAC; Calbiochem, La Jolla, CA; final dilution, 1:10,000) and incubated at 37°C and 5% CO2. After 24 h, cells were pelleted, and supernatants were collected and frozen at −70°C. After week 24, samples were processed, and supernatants from all time points were thawed and assayed for IL-2 (BioSource International, Camarillo, CA), IL-12 p40 (BioSource), and IL-12 p70 (R&D Systems, Minneapolis) by a commercially available ELISA. IL-10 was measured by an established sandwich ELISA as previously described [33]. Briefly, 96-well plates were coated overnight at 4°C with the anti–human IL-10 monoclonal antibody (MAb) JES3-9D7 (PharMingen, San Diego). Plates were washed and blocked with PBS–10% fetal calf serum, and IL-10 was detected with a second biotinylated anti–IL-10 MAb, 18562D (PharMingen). The limits of detection of the assays were 8.7 pg/mL IL-2, 1 pg/mL IL-12 p40, 5 pg/mL IL-12 p70, and 16 pg/mL IL-10.

Flow cytometric analysis of CD28 expression. At baseline and at weeks 4 and 24, blood was collected in acid citrate dextrose–containing tubes, and CD28 expression on CD4+ and CD8+ lymphocytes was analyzed by flow cytometry. Whole blood (100 μL) was distributed into 12 x 75 polypolyethylene tubes (Sarstedt, Newton, NC) containing saturating amounts (5 μL) of phycoerythrin–conjugated mouse anti–CD4 antibody (Sigma) or phycoerythrin–conjugated mouse anti–CD8 antibody (Becton Dickinson, Lincoln Park, NJ) and fluorescein isothiocyanate–conjugated mouse anti–CD28 antibody (PharMingen). Red blood cells were lysed, and remaining cells were fixed (Q-Prep; Coulter Electronics, Hialeah, FL). Blood samples processed in the absence of antibodies (autofluorescence) and isotype-matched MAbs were included as negative controls. Isotype-matched MAbs for CD4, CD8, and CD28 were IgG1 clone MOPC-21 (PharMingen), IgG1 clone X40 (Becton Dickinson), and IgG1 clone MOPC-21 (PharMingen), respectively. The data were acquired on an Excel flow cytometer (Coulter Electronics) and saved as listmode files. The percentages of CD4+ and CD8+ cells that expressed CD28 were determined.

Data analysis. Data are presented as mean ± SD if normally distributed; otherwise, they are presented as median and interquartile range (IQR). Cytokine production at the three time points was compared using a Kruskal-Wallis test with pairwise comparisons of on-treatment versus baseline levels (Dunnnett test). Development of or improvement in proliferative responses and changes in cytokine production were correlated with changes in virus load and CD4+ lymphocyte count using Spearman’s correlation. The percentages of CD4+ and CD8+ lymphocytes that were CD28+ were compared using a one-way analysis of variance and a Dunnett test for pairwise comparisons.

Results

Plasma HIV RNA levels and CD4+ T cell counts. Of the 42 patients enrolled, 1 withdrew from the study within the first week. All but 1 of the evaluable 41 patients had detectable plasma HIV RNA at baseline. This patient was subsequently found to have HIV-1 subtype D and was excluded from evaluations of virus load. The median baseline plasma HIV RNA concentration was 4.74 log10 (IQR, 4.36–5.15 log10) copies/mL. This decreased to
2.82 log_{10} \text{(IQR,} <2.30 – 3.20 \text{ log}_{10}) \text{ copies/mL after 4 weeks and}
<2.30 \text{ log}_{10} \text{(IQR,} <2.30 – 2.30 \text{ log}_{10}) \text{ copies/mL after 24 weeks.}
The mean CD4^{+} \text{ lymphocyte count at baseline was } 284 \pm 121
\text{ cells/μL and increased to } 374 \pm 118 \text{ cells/μL after 4 weeks and}
413 \pm 121 \text{ cells/μL after 24 weeks of therapy.}

\text{Proliferative responses. To evaluate T cell function,}
\text{PBMC proliferative responses to p24 antigen and PHA were}
\text{measured at predetermined intervals. PBMC of 41 patients}
\text{were analyzed for proliferative responses to p24 antigen}
\text{and PHA at baseline and after 4 and 24 weeks of therapy.}
\text{At baseline, PBMC of 2 (4.9\%; 95\% confidence interval [CI],}
0–11.5\%) \text{ subjects proliferated (SI} >3\text{) in response to p24}
\text{antigen. This increased to 9 (22.0\%; 95\% CI, 9.3\%–34.6\%)
after 4 weeks and 10 (24.4\%; 95\% CI, 11.2\%–37.5\%)
after 24 weeks (figure 1). Median cpm in unstimulated PBMC was}
191 (IQR, 93–598). In patients who exhibited proliferative
\text{responses to p24 antigen, the median cpm were 937 (IQR,}
782–1091; \text{n} = 2), 917 (IQR, 290–1257; \text{n} = 9),
\text{and 2360 (IQR, 1748–2800; \text{n} = 10) at weeks 0, 4, and 24, respectively.}
In patients who did not exhibit a proliferative response to PHA,
\text{median cpm were 104 (IQR, 75–161; \text{n} = 39), 232 (IQR,}
91–978; \text{n} = 32), and 795 (IQR, 329–990; \text{n} = 31). Of the
39 patients who were initially unresponsive to p24 antigen,
\text{17 developed a proliferative response at 4 or 24 weeks. Of}
\text{patients with proliferative responses to p24 antigen at baseline,}
\text{both became unresponsive by 24 weeks of therapy. Seven pa-
\text{tients initially unresponsive to p24 antigen, who developed a}
\text{proliferative response at 4 weeks, subsequently became unre-
\text{sponsive at 24 weeks.}

\text{In response to PHA, PBMC of 7 (17.1\%; 95\% CI, 5.6\%–}
28.6\%) \text{ subjects proliferated at baseline. The number of pa-
\text{tients whose PBMC proliferated in response to PHA increased}
\text{to 26 (63.4\%; 95\% CI, 48.7\%–78.2\%)} \text{ after 4 weeks and 25}
(61.0\%; 95\% CI, 46.0\%–75.9\%)} \text{ after 24 weeks (figure 1).}
\text{In patients who exhibited proliferative responses to PHA, the}
\text{median cpm were 319 (IQR, 240–804; \text{n} = 7), 3483 (IQR,}
626–4674; \text{n} = 26), and 1974 (IQR, 1557–4520; \text{n} = 25) at}
\text{ weeks 0, 4, and 24, respectively. In patients who did not exhibit}
\text{a proliferative response to p24 antigen, the median cpm were}
158 (IQR, 72–279; \text{n} = 34), 1778 (IQR, 330–3134; \text{n} = 15),
\text{and 1140 (IQR, 591–1776; \text{n} = 16). The relatively low cpm}
\text{measured at predetermined intervals. PBMC of 41 patients}
were analyzed for proliferative responses to p24 antigen and observed in response to PHA are a reflection of the culture
\text{conditions used (i.e., stimulation with PHA, final dilution,}
1:200, for 6 days). In addition, the median cpm of the “re-
\text{sponders” at baseline were considerably lower than values at}
\text{4 and 24 weeks, suggesting that although proliferative re-
\text{sponses may have been present prior to therapy, such responses}
\text{were much more pronounced while patients were receiving}
ritonavir plus saquinavir. Thirty of the 34 patients who were
initially unresponsive to PHA at baseline developed prolifera-
tive responses, while 3 of the 7 patients who were initially
responsive at baseline exhibited an increase (>2.5-fold in-
crease in SI) in their proliferative response at either 4 or 24
weeks. Of patients with proliferative responses to PHA at base-
line, 4 became unresponsive at 24 weeks of therapy. Eight pa-
tients initially unresponsive to PHA, who developed a prolif-
erative response at 4 weeks, subsequently became unresponsive
at 24 weeks. Changes in proliferative responses did not corre-
late with baseline values or quantitative changes in either virus
load or CD4^{+} \text{ lymphocyte count.}

\text{Cytokine production. To further assess T cell function, IL-2}
\text{production in response to stimulation with PHA was measured.}
\text{PHA-stimulated PBMC produced a median of 268 (IQR,}
105–803) \text{ pg/mL IL-2 at baseline. This increased to 716 (IQR,}
302–1247) \text{ pg/mL at week 24 (} P < .05 \text{ for week 24 vs. week}
0; \text{n} = 41; \text{table 1).}

![Figure 1. Proportion of patients at each time point whose peripheral blood mononuclear cells had proliferative response (stimulation index >3) when cultured in presence of HIV p24 antigen or phytohemagglutinin (PHA).]
was 1193 (IQR, 488±2611) pg/mL at baseline and increased
P
pg/mL at week 24 (
(IQR, 238±1194) pg/mL at week 4 and 1510 (IQR, 715±2975) his proliferative response to PHA was also lost (®gure 2).

studied. PHA-stimulated PBMC produced a median of 258 and his proliferative response to p24 antigen was lost. Four
of PHA, a T cell mitogen, and LPS, a potent inducer of mono-
tinuing his medication because of gastrointestinal intolerance,

Produced a median of 543 (IQR, 276±1006) pg/mL IL-12 p40 Of interest is the anecdotal observation of 1 patient who, after

41; table 1). There was, however, no change observed in median
SAC-induced IL-12 p40 production. Both SAC- and LPS-in-
duced IL-12 p70, the biologically active heterodimer, were
undetectable at all time points in virtually all patients assayed.

IL-10* PHA 258 78±407 635 ² 238±1194 1510 ² 715±2975

NOTE. IQR, interquartile range (n = 41); IL, interleukin; PHA, phytohemagglutinin; SAC, Staphylococcus aureus
Cowan strain 1; LPS, lipopolysaccharide.
* Significant difference between time points by Kruskal-Wallis test (P < .001 for IL-2, P < .05 for LPS-induced
IL-12 p40, P < .001 for PHA-induced IL-10, P < .001 for LPS-induced IL-10).

P < .05, Dunnett’s test for pairwise comparison vs. week 0.

Impact of discontinuation of therapy on immune function. Of interest is the anecdotal observation of 1 patient who, after being compliant for 24 weeks, discontinued therapy at week 26. This case was unique in that this was the only patient to entirely discontinue antiretroviral therapy. His PBMC were initially unresponsive to both p24 antigen and PHA. At 4 weeks of therapy, his plasma HIV RNA level was below the detection limit of the assay, and he developed a proliferative response to PHA. At 24 weeks, his plasma HIV RNA level remained below the detection limit of the assay, and he developed a proliferative response to p24 antigen. Two weeks after discontinuing his medication because of gastrointestinal intolerance, his plasma HIV RNA level rebounded to 98,600 copies/mL and his proliferative response to p24 antigen was lost. Four weeks later (week 32), prior to restarting antiretroviral therapy, his plasma HIV RNA level rebounded to 98,600 copies/mL and his proliferative response to p24 antigen was lost. Four weeks later (week 32), prior to restarting antiretroviral therapy, his proliferative response to PHA was also lost (figure 2).

Discussion

We demonstrate that, in the population studied, initiation of therapy with potent antiretroviral activity is paralleled by a rapid and sustained improvement in a number of functional measures of CMI. Treatment-induced changes were observed

Table 2. Percentage of CD4+ and CD8+ lymphocytes expressing CD28 (mean ± SD, n = 13).

<table>
<thead>
<tr>
<th>Week 0</th>
<th>Week 4</th>
<th>Week 24</th>
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<tbody>
<tr>
<td>CD4</td>
<td>89.17 ± 8.33</td>
<td>88.61 ± 10.12</td>
</tr>
<tr>
<td>CD8</td>
<td>32.34 ± 9.83</td>
<td>39.39 ± 14.71</td>
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* Significant difference between time points by one-way analysis of variance (P < .02) and P < .05 by Dunnett’s test for pairwise comparison vs. week 0.
in responses indicative of improvement in both T cell and APC function.

Since T helper cell dysfunction is an indicator of poor prognosis [5, 6], the improvements observed here (development or improvement in proliferative responses to p24 antigen or PHA and increased IL-2 production) may ultimately contribute to improved clinical outcomes. The majority of patients developed proliferative responses to PHA at 4 weeks and sustained this at 24 weeks (14 patients), first developed a proliferative response to PHA at 24 weeks (8 patients), or exhibited, at 24 weeks, an increase from their initial proliferative response to PHA (2 patients). Although the number of patients with proliferative responses to p24 antigen was greater at both 4 and 24 weeks than at baseline, this was less impressive than the response to PHA, and many responses appeared to be transient. Since proliferative responses to recall antigen are lost prior to those in response to mitogen [1], it is logical that PHA-induced proliferation would be restored prior to p24 antigen-induced proliferation. The reason that some patients exhibited transient improvements in proliferative responses, despite optimal virologic responses, is unclear.

After 24 weeks of potent antiretroviral therapy, the percentage of CD8 T lymphocytes expressing CD28 increased from 32% to 46%, similar to the level of expression in HIV-seronegative persons [21, 23]. Since IL-2 production and proliferation are impaired in CD28−, as compared to CD28+CD8+ lymphocytes [21, 34], an increase in the percentage of CD8+ lymphocytes expressing CD28 at 4 weeks and a further increase at 24 weeks may be another indication of a continuous improvement in T cell function. Whether an increase in the proportion of CD8+ T cells expressing CD28 leads to improved CTL activity or increased secretion of soluble inhibitors of HIV replication (i.e., β chemokines, IL-16) remains to be established.

Since in vitro infection with HIV inhibits IL-12 production [10, 11] and PBMC from HIV-infected persons produce less IL-12 than PBMC from HIV-seronegative persons [10–12], it was anticipated that decreasing plasma viremia would lead to increased IL-12 production. In fact, LPS-induced IL-12 p40 production increased after 4 weeks and further increased after 24 weeks of therapy. The reason that there was an increase in LPS- but not SAC-induced IL-12 p40 production is not clear. The mechanisms by which these compounds induce cytokine production differ, and it is possible that decreasing HIV replication by protease inhibition over a period of 6 months reverses HIV-induced abnormalities of LPS signaling but not of SAC signaling.

The observation that both SAC- and LPS-induced IL-12 p70 were undetectable in virtually all patients is consistent with published observations [11] and may not exclude an increase in IL-12 bioactivity. Since an increase in IL-12 p40 is felt to reflect an increase in vivo IL-12 bioactivity [35], the increase in LPS-induced IL-12 p40 observed here may represent improvement in monocyte/macrophage function, further contributing to improved cellular immune responses. Since the addition of exogenous IL-12 to PBMC from HIV-seropositive persons restores their proliferative capacity in response to antigen or mitogen [14–16], the improvement in PBMC proliferative responses after 4 and 24 weeks of therapy is consistent with an increase in IL-12 bioactivity.

IL-10 plays a complex and likely vital role in HIV immunopathogenesis. Although IL-10 may inhibit cellular immune responses, some investigators have observed decreased produc-
tion of inducible IL-10 in HIV-infected persons [33, 36], and in most in vitro systems IL-10 inhibits HIV replication [37–40]. It has also been reported that in vivo infusion of IL-10 results in both a decrease in plasma viremia and a decreased susceptibility to in vitro infection with HIV [41]. The increase in IL-10 production observed in this study may represent a potentially beneficial effect of antiretroviral therapy.

The observation that PHA-induced IL-10 was significantly enhanced by 4 weeks but no change was observed in LPS-induced IL-10 production until 24 weeks may be due to the fact that PHA is a T cell mitogen, whereas LPS primarily stimulates monocytes/macrophages. Since HIV turnover is much less rapid in monocyte/macrophages than in lymphocytes, prolonged virus suppression may be required to result in such functional changes in the monocyte/macrophage population.

Why some patients do not have improvements in some measures of CMI despite apparent optimal antiretroviral activity and increase in CD4+ lymphocyte count is not clear. It is possible that suppression of viral replication for periods of >24 weeks is required for the restoration of such immune responses in some individuals. If the degree of virus suppression correlates quantitatively with improvement in CMI, it may be difficult to decipher this from the present study since the majority (75%) of patients had a decrease in their HIV RNA to below the detection limit of the assay, effectively resulting in a homogeneous population. The case described above of the individual who discontinued therapy (figure 2), if representative, may reflect the association between drug therapy, virus suppression, and immune reconstitution. It is also possible that factors other than quantitative changes in virus load and CD4+ T cell count play a role in the improvement of CMI.

We demonstrate that potent combination protease inhibitor therapy with ritonavir and saquinavir is associated with a rapid and sustained improvement in a number of aspects of immune function, including PBMC proliferation, IL-2 production, IL-12 production, and CD28 expression on CD8+ lymphocytes, suggesting that HIV-induced immune suppression is both dynamic and potentially reversible. This may explain, at a cellular level, how antiretroviral therapy leads to a decrease in the development of infectious and malignant complications of HIV infection. Whether this is associated with improved clinical outcomes and whether this is dependent on or independent of the degree of viral suppression or other host and virus characteristics can only be addressed with longer follow-up and further studies.

References
on CD4\(^+\) and CD8\(^+\) lymphocytes during HIV-1 infection. J Acquir Immune Defic Syndr 1994; 7:245–53.


