Alterations in the Immune Response of Human Immunodeficiency Virus (HIV)–Infected Subjects Treated with an HIV-Specific Protease Inhibitor, Ritonavir

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Effects of a human immunodeficiency virus (HIV) type 1 protease inhibitor, ritonavir, were evaluated in 21 patients enrolled in a phase I/II study. The magnitude and rates of CD4 and CD8 lymphocyte increase, changes in subsets of CD4 and CD8 lymphocytes, and proliferative responses to mitogen and antigens were analyzed. Significant increases were noted in CD4 and CD8 lymphocyte counts; numbers of CD4CD45RO lymphocytes increased significantly by week 1 of therapy. Increases in the CD4CD45RA subset were observed at week 4. Reductions in the percentage of CD4 and CD8 lymphocytes expressing CD38 were noted. Increases in proliferative responses to phytohemagglutinin were noted in 6 of 7 patients and correlated with duration of virus load suppression. Increased responses to recall antigens and to HIV-specific proteins were observed. Treatment with ritonavir produced alterations in the immune system that included changes in T cell subset distribution and increases in CD4 and CD8 lymphocyte numbers and of lymphocyte function.

Assessment of immune response after antiretroviral therapy of human immunodeficiency virus (HIV) disease is usually restricted to measurement of CD4 lymphocytes, because it is often assumed that any improvement in immune function will be reflected by changes in CD4 lymphocyte counts. Nevertheless, repeated observations have shown that CD4 lymphocyte function, as assessed by in vivo and in vitro techniques, is reduced to a much greater extent than can be explained on the basis of reduced CD4 lymphocyte numbers [1–4]. In patients with advanced HIV disease, therapy with zidovudine or didanosine results in a transient improvement in lymphocyte function [5–12]. The magnitude and duration of these changes have not correlated well with CD4 lymphocyte increases or in reductions in virus load [5–7].

Changes in CD4 and CD8 lymphocyte subsets have been studied as additional markers of disease progression. Reduction in the number of CD4 lymphocytes with a memory phenotype (as defined by surface expression of CD29 or CD45RO proteins) has been noted by some researchers [13–16], but not all

[17–20]. Reductions in the resting or naive subset (as defined by expression of CD45RA) become significant late in disease [13]. CD4CD45RO lymphocytes are preferentially infected by HIV, and reduced function of this subset may lead to the early loss of responses to recall and HIV-specific antigens [21, 22]. The relative preservation of the CD4CD45RA subset may explain why responses to mitogens and alloantigens are maintained until later in the disease process [15, 21, 22].

The expression of activation markers CD38 and HLA-DR is increased on CD4 and CD8 lymphocytes of HIV-infected subjects [13, 20, 23–26]. High-level CD38 expression on CD8 lymphocytes correlates with disease progression [27, 28]. A significant proportion of in vitro cytotoxic T lymphocyte (CTL) activity is derived from the CD8CD38 subset [29]. There is also a marked increase in the number of CD8 lymphocytes with a cytotoxic phenotype, as defined by expression of an epitope of the lymphocyte function–associated antigen-1 bound by the monoclonal antibody (MAb) S6F1 [30–32].

Relatively little is known about the changes in these subsets during antiretroviral therapy. Studies of zidovudine have shown decreased levels of T cell activation after initiation of therapy, with decreased expression of CD38 on CD8 lymphocytes [23, 33]. However, this effect may be lost with prolonged therapy [20].

Ritonavir is an HIV-1 protease inhibitor that has been shown in two phase I/II trials to significantly increase CD4 lymphocyte counts and reduce plasma viral RNA by more than one order of magnitude [34, 35]. The drug appears to be well tolerated, and there is a dose-response effect: Higher doses (500 or 600 mg twice a day) have a more prolonged effect on both CD4 cell count and virus load than do lower doses [34].

Two groups have used the effects of ritonavir on both virus load and CD4 lymphocyte count to estimate rates of virus doubling time and CD4 lymphocyte destruction [36, 37]. Using various assumptions, they concluded that the half-life of a CD4

lymphocyte in an HIV-infected patient was ~2 days. These publications generated considerable discussion [38–43].

The aim of the present study was to explore the effect of treatment with ritonavir on the HIV-infected immune system. The effects of ritonavir therapy on both CD4 and CD8 lymphocytes were studied in terms of the rate, magnitude, and duration of change in these populations, changes in lymphocyte subsets as determined by cell surface protein phenotype, and alteration in lymphocyte function by T cell proliferative responses.

Methods

Subjects.   Patients were enrolled as part of a multicenter, double-blind, dose-ranging study of ritonavir that was placebo controlled for the first 4 weeks. The trial was done in two phases. In the first, 39 patients were randomized to either ritonavir (300 or 400 mg twice a day) or placebo; the remaining 45 patients were given 500 or 600 mg of drug twice a day or placebo. After 4 weeks, those receiving the placebo were rerandomized to one of the active treatment arms. The principal inclusion criteria were a CD4 lymphocyte count of >50 cells/ μL, HIV-1 p24 antigenemia assessed by an immune complex–dissociated p24 antigen assay, and no active AIDS-related illness (except cutaneous Kaposi's sarcoma). All patients ceased other antiretroviral agents for $\geq$2 weeks before beginning the blinded therapy. The subgroup that we report is the 21 patients randomized at St. Vincent's Hospital, Sydney. All 21 had sequential estimations of CD4 and CD8 lymphocyte counts and plasma viral RNA copy number weekly for the first 8 weeks, every other week for the next 8 weeks, and every 4 weeks thereafter. The last 11 patients randomized had CD4 and CD8 lymphocyte subset analyses done; the last 7 had proliferative assays.

Flow cytometry.   Blood samples from all subjects were collected in acid citrate dextrose (ACD). Simultaneous collections for total lymphocyte count were made in tubes containing potassium-EDTA and allowed determination of absolute counts.

Estimations of CD4 and CD8 lymphocyte counts and subsets were assessed by two-color immunofluorescence flow cytometry using a panel of MAbs to the following cell surface proteins: fluorescein isothiocyanate (FITC)– or phycoerythrin (PE)-conjugated MAb to CD4 and CD8 (Coulter Electronics, Hialeah, FL); FITC-conjugated CD45RO (UCHL1; Dako, Glostrup, Denmark), HLA-DR, T cell receptor (TCR)-$\alpha\beta$ and TCR-$\gamma\delta$ (both Becton Dickinson, San Jose, CA); PE-conjugated CD45RA(2H4) and CD11a/CD18 (S6F1) (both Coulter), and CD25, CD28, CD62L (Leu-8), CD38 (Leu-17), and HLA-DR (all Becton Dickinson).

All antibodies (at the manufacturers' recommended concentrations) were incubated with 100 μL of whole blood for 5 min at 25°C before red cell lysis and fixation using Immunoprep reagents and Q-prep equipment (Coulter), according to the manufacturer's directions. Cells were then stored at 4°C for up to 24 h before analysis by flow cytometer (EPICS XL; Coulter). A total of 5000 cells were analyzed in a manually set lympho-

cyte gate. Positive cutoffs for fluorescence were set to include <2% of negative control mouse IgG conjugates (Coulter). Anti–CD38-PE–positive cells were identified using a cutoff with 2-fold greater intensity of staining than other markers. Compensation levels were determined using CD4 and CD8 paired monoclonal conjugates and checked daily.

Lymphocyte proliferation assay.   Whole blood samples were collected in ACD from the last 7 patients randomized to the high-dose group, 8 HIV-seronegative controls, and 2 patients who, after failure of their initial randomized therapy (adverse event), received rechallenge doses of ritonavir (300 mg twice a day). These 2 patients initially received the drug for <2 weeks. Both tolerated ritonavir on rechallenge.

Blood was collected at 0, 2, 4, 6, 8, 12, 16, 20, and 24 weeks. Peripheral blood mononuclear cells (PBMC) were isolated within 4 h of collection using the standard Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) density separation method and washed twice with Dulbecco's calcium- and magnesium-free PBS. Cells were resuspended at 2×10^6/mL in RPMI 1640 medium supplemented with complete medium (2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin [all GIBCO BRL, Grand Island, NY], and 7.5% heat-inactivated AB serum [ICN Pharmaceuticals, Costa Mesa, CA]). Cells (10^5) for phytohemagglutinin (PHA; Murex Diagnostics, Paris) stimulation or for antigen-driven stimulation (2×10^5) were cultured in quadruplicate in 96-well round-bottom tissue culture plates (Corning, Corning, NY). Cell suspensions were incubated with 0.5 μg/mL PHA for 72 h and with tetanus toxoid (TT; 2 Lf/mL; CSL, Melbourne), streptokinase/streptodornase (SK/SD; 125 U/mL; Lederle, Sydney [both TT and SK/SD were dialyzed before use to remove thiomersol]), and two recombinant HIV-related proteins, p24 and p17 (1 μg/mL each; gift of British Biotechnology, Oxford, UK) for 7 days. Unstimulated cultures consisted of PBMC incubated in complete medium without antigen or mitogen. Tritiated thymidine (0.5 μCi; Amersham Laboratories, Amersham, UK) was added to each well for the last 4 h of the PHA cultures and for the last 18 h of the antigen-stimulated cultures. Cells were harvested onto filter paper using a 12-well semiautomatic harvester (Skatron, Lier, Norway). Filter papers were dried, immersed in scintillant (Ready Safe; Beckman Instruments, Palo Alto, CA), and assessed for 2 min on a liquid scintillation spectrometer (Tricarb 1500; Packard, Groningen, Netherlands).

Thymidine incorporation induced by antigen or mitogen stimulation was determined using either Δ counts per minute (cpm) or the stimulation index. In the former, we determined the mean cpm of stimulated wells – the mean cpm of unstimulated wells. In the latter, we divided the mean cpm of stimulated wells by the mean cpm of unstimulated wells. An SI >2 was considered a positive response.

Virus load.   Estimations of virus load were made by RNA copy number per milliliter of plasma using a branched-chain (b) DNA assay (Chiron, Emeryville, CA) according to the manufacturer's instructions.

Statistics. We used Statview 4.02 software (Abacus, Berkeley, CA) for all statistical calculations. Comparisons of responses between dosage groups were done using the Kruskal-Wallis test. For flow cytometry subset data, arithmetic means of change from baseline were calculated and comparisons to baseline counts were determined using the nonparametric Wilcoxon signed rank test. Because not all subjects had detailed lymphocyte phenotyping done at the same visit after the first 4 weeks of therapy, data from weeks 6–8 and 10–16 of active treatment were pooled. Spearman's ranked sum test was used to determine correlation coefficients. The rate of initial CD4 lymphocyte increase (the increase during the first 21–28 days of therapy) and the rate of increase to peak CD4 lymphocyte count were derived from the slope of linear regression plots of each subject's CD4 lymphocyte count and the log transformed CD4 count against time. Rates of CD8 lymphocyte increase to peak were calculated in a similar fashion.

Results

Changes in CD4 lymphocyte count. Baseline CD4 and CD8 lymphocyte counts and plasma viral RNA levels for each subgroup are shown in table 1. After treatment, increases in CD4 cells were observed in all 21 patients. The mean increase to peak CD4 lymphocyte count was 245 cells/μL. The time to peak CD4 lymphocyte count varied widely, ranging from 2 to 14 weeks (mean, 6). Of the CD4 lymphocytes, <1% expressed TCR-$\gamma\delta$ at all time points; conversely, >90% expressed TCR-$\alpha\beta$ (data not shown).

Rates of increase of CD4 lymphocytes. Each subject's CD4 lymphocyte and log transformed CD4 lymphocyte counts were plotted against time. The rate of increase was derived from the line of best fit using linear regression analysis. Both the initial

Table 1. CD4 and CD8 lymphocyte counts and virus load data for patients treated with ritonavir.

	All patients (n = 21)	Phenotypic subset analysis (n = 11)	Proliferative response analysis (n = 7)
CD4 cells/μL			
Baseline	153 (116)	146 (115)	179 (135)
Peak	404 (183)	357 (191)	385 (233)
Increase to peak	245 (131)	273 (184)	194 (125)
CD8 cells/μL			
Baseline	813 (302)	901 (252)	935 (261)
Peak	1781 (594)	1896 (580)	1674 (568)
Increase to peak	964 (532)	994 (524)	739 (413)
Plasma viral RNA			
Baseline copies			
10^3/mL	368 (423)	251 (197)	192 (163)
Reduction*	1.16 (0.52)	1.04 (0.47)	0.92 (0.47)

NOTE. Data are mean ($\pm$SD)
* Log_{10} RNA copies/mL.

Regression Plot
95% Confidence Bands

A

CD4 count (cells/μL)

Y = 204.93 + 3.37 * X; R^2 = .47

Regression Plot
95% Confidence Bands

B

log CD4 count (cells/μL)
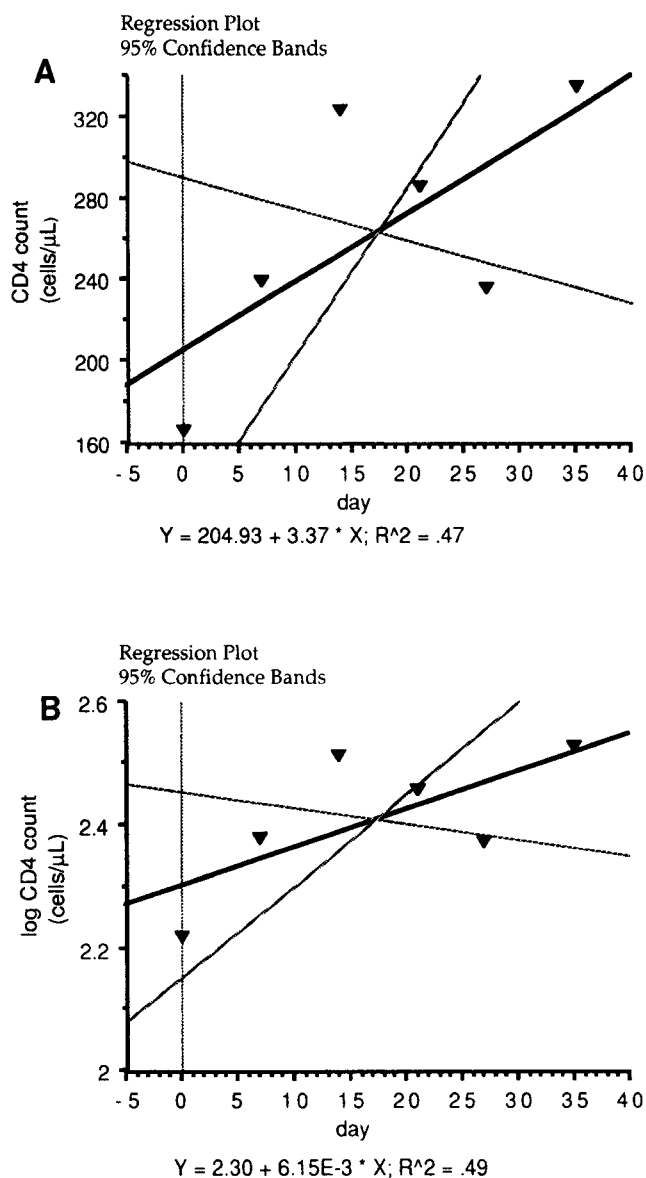
Y = 2.30 + 6.15E-3 * X; R^2 = .49

Figure 1. Regression analyses of slope to peak CD4 cell count of representative subject (no. 122) using raw (**A**) and log transformed (**B**) CD4 cell counts. 95% confidence intervals for slopes and formula for regression line are shown. For **A**, slope: 3.37 cells/day; SE, 1.78, R^2 = .47. For **B**, slope: 0.62 log cells/day; SE, 0.31, R^2 = .49.

rate and the rate of increase to peak CD4 count were determined. No significant relationship between dose regimen and rate of CD4 cell increase was found. From these data, it was not clear whether the increase was truly exponential or linear (figure 1). The initial rates of increase ranged from -0.19 to 24.68 CD4 cells/μL/day (mean, 7.53). However, once the R^2 and SE data were considered, in the majority of subjects, linear regression analyses of raw or log transformed CD4 lymphocyte counts over time did not provide reliable estimates of rates of CD4 lymphocyte increase. In fact, the slopes of these regression lines were only statistically significant in 5 of 21 cases (data not shown).

In an attempt to improve the reliability of the estimation, rates of increase to peak CD4 cell count were analyzed in the same manner. These analyses were based on more data points than the initial slope method in all but 5 subjects. The range of slope to peak CD4 cell count was 1.18–24.68 CD4 cells/μL day (mean, 7.53); however, even with this method, the slope of the regression lines was statistically significant in only 11 of the 21 patients. This was true whether raw or log transformed CD4 cell counts were used.

Despite these findings, the mean and range of rates of CD4 cell increase were similar to those described previously [36, 37]. A negative correlation was noted between initial CD4 lymphocyte count and rate of log transformed CD4 lymphocyte increase when either initial rate ($\rho = -0.66$, $P = .003$) or rate to peak ($\rho = -0.59$, $P = .009$) is considered. These correlations need to be interpreted with caution in view of the uncertainty of the slope data. No such correlation was observed with the linearly derived data ($\rho = -0.26$, $P = .25$).

Changes in CD4 lymphocyte subsets. Figure 2 shows changes in CD4 lymphocyte subsets and total CD4 lymphocyte counts in the 11 patients analyzed by two-color immunofluorescence. All patients received one of the high-dose regimens (500 or 600 mg twice a day). In those receiving placebo during the first 4 weeks of the trial, no significant changes in CD4 or CD8 lymphocyte subsets were noted (data not shown). CD4CD45RO lymphocytes made up most of the initial rise in total CD4 lymphocytes, with significant increases in absolute numbers of CD4CD45RO cells after 1 week of therapy ($P = .008$). No changes in numbers of CD4CD45RA cells occurred during the first 3 weeks of therapy. An increase was first noted
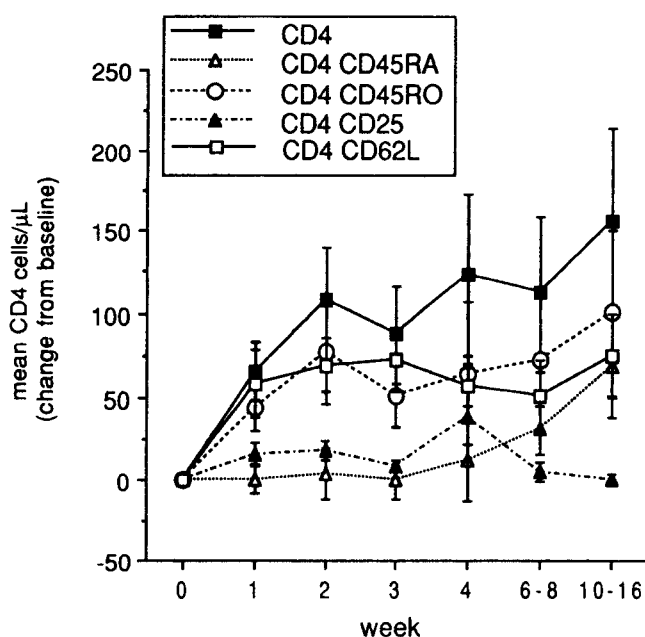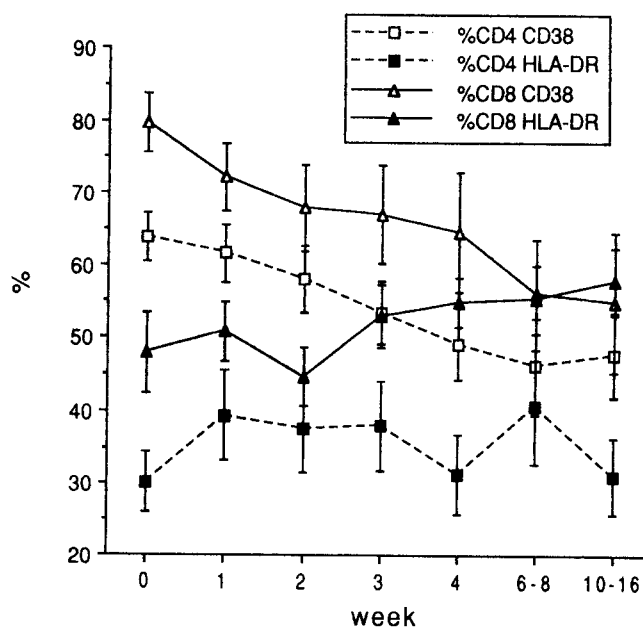

Figure 3. Changes in expression (%) of activation markers CD38 and HLA-DR on CD4 and CD8 cells.

at 4 weeks and became significant at the 6- to 8-week time point ($P = .02$).

The percentage of CD4CD38 lymphocytes decreased significantly by week 2 ($P = .02$). The percentage of CD4 HLA-DR cells increased slightly by week 1 and then remained constant throughout the observation period (figure 3). Given that the absolute number of CD4 lymphocytes increased, the number of CD4 cells not expressing CD38 or HLA-DR must have increased with therapy.

CD4CD28 lymphocytes were counted at selected times for 9 subjects. Significant increases in absolute numbers of this subset were noted within 1 week ($P = .008$) and were maintained for ≥ 4 weeks (data not shown).

Initial increases in absolute numbers of CD4 lymphocyte were associated with increases in CD4CD62L cells ($P = .007$ at 2 weeks), which then plateaued. There was a small, transient increase in the number of CD4CD25 lymphocytes ($P = .007$ at 1 week, figure 2). In fact, as a percentage of total CD4 lymphocytes, this subset decreased by weeks 6–8 ($P = .03$, data not shown).

CD8 lymphocytes. Accompanying the increase in CD4 lymphocytes was a significant increase in CD8 lymphocytes (table 1). The average peak CD8 lymphocyte increase was 964 cells/μL. No significant difference was seen between dosage regimes in terms of magnitude or rate of increase. The kinetics of the CD8 cell increase was modeled poorly by linear regression analysis. From the available data, it was not possible to determine whether the increase was linear or exponential (data not shown). There was no correlation between initial CD8 lymphocyte count and magnitude or rate of CD8 cell increase. The magnitude of the CD8 lymphocyte increase correlated with the magnitude of CD4 lymphocyte increase ($\rho = 0.522$, $P =$

Figure 2. Changes from baseline CD4 cell count and in CD4 CD45RA and CD4 CD45RO, CD4 CD62L, and CD4 CD25 subsets from day 1 of ritonavir therapy (± 1 SE of mean).

.02). The time to peak CD4 lymphocyte count correlated with the time to peak CD8 lymphocyte count ($\rho = 0.524$, $P = .02$).

CD8 lymphocyte subsets. Changes in the expression of CD45RO and CD45RA on CD8 lymphocytes were less clear than with CD4 lymphocytes (figure 4). However, as with CD4 cells, there was a late increase in the number of cells expressing CD45RA. Expression of HLA-DR on CD8 lymphocytes increased in absolute counts ($P = .008$ at week 1) and in percentage terms ($P = .03$ at 6–8 weeks) over the treatment period (figure 3). In percentage terms, the expression of CD38 on CD8 lymphocytes decreased over the observation period; the decrease was significant at 1 week ($P = .004$; figure 3). S6F1 binding to CD8 lymphocytes was found on >90% of the CD8 lymphocytes before the onset of therapy, and this percentage was maintained throughout the observation period (data not shown).

Proliferative responses to PHA. Marked increases in PBMC proliferative responses to PHA were seen in 6 of 7 patients within 4 weeks of the start of active therapy (figure 5). In contrast, over the initial 4 weeks of the study, there was no change in the proliferative response in PBMC isolated from patients receiving placebo. Given that the number of PBMC per well was kept constant, there was a 6-fold increase in proliferative capacity on a per lymphocyte basis by 4 weeks. There was no correlation between the magnitude of the CD4 lymphocyte increase and improvement in PHA responses. Mean and median duration of improved proliferative responses to PHA above baseline was 16 weeks.

Proliferative responses to antigens. Patients receiving placebo had no changes in proliferative responses to any antigen during the first 4 weeks of the study (data not shown). Of 7 subjects given ritonavir, 5 had improved proliferative responses

to SK/SD, 2 to TT, and 2 to HIV-specific p24 and p17 (figure 6).

When all data shown in figure 5 are considered together, 1 subject had improvements to all three sets of antigens tested. Two other subjects had improvements in proliferative responses to two of the three sets of antigens tested. Two subjects had improved responses to one antigen. Two subjects did not demonstrate any improvement in responses to any antigen; 1 of them also did not have improved PHA responses. Both patients had CD4 counts of <50 cells/μL on day 1 of active therapy.

Proliferative responses in rechallenge patients. Two patients were studied after rechallenge with low-dose ritonavir. Both had increases in proliferative responses to PHA and negative responses to all antigens at day 1 of rechallenge. One developed a positive response to SK/SD that was maintained for 12 weeks. No other improvements were noted (data not shown).

Changes in virus load. Before treatment, the median plasma virus load in the 21 subjects was 264,600 copies/mL (range, 10,000–1,524,000). Except for 1 subject who had undetectable viral RNA at baseline, all other patients had rapid declines in plasma viral RNA levels. In 17 subjects, plasma viral RNA reached levels below the assay's level of detection (10,000 copies/mL) within the first 2 weeks of therapy. Because of the relatively high limits of detection of the bDNA assay, the reduction in plasma viral RNA was probably underestimated. The rate of reduction was not estimated because of the limited number of data points available before viral RNA become undetectable.

There was a weak negative correlation between initial CD4 lymphocyte count and baseline virus load ($\rho = -0.46$, $P = .04$). No correlation was noted between log change in plasma viral RNA copy and magnitude or rate of CD4 or CD8 lymphocyte increase. A correlation between the duration of >50% reduction in plasma viral RNA and time to peak CD4 ($\rho = 0.58$, $P = .01$) and CD8 ($\rho = 0.66$, $P = .004$) lymphocyte count indicates that the longer the reduction in virus load persists, the later the peak in CD4 and CD8 lymphocyte counts. However, there was no correlation between the duration of reduction in virus load and magnitude of increase in CD4 or CD8 lymphocyte count.

No correlation between log reduction in plasma viral RNA and improvements in PHA responses was observed. There was, however, good correlation between duration of improvement in PHA response and duration of reduction in virus load to >50% of baseline ($\rho = 0.84$, $P = .05$).

Discussion

In the subset of 21 subjects enrolled in a phase I/II trial of ritonavir therapy, all had increases in CD4 lymphocyte counts, and all 20 patients with detectable plasma viral RNA had reductions in virus load. Significant increases in CD8 lymphocyte counts were noted, a phenomenon that has not been reported
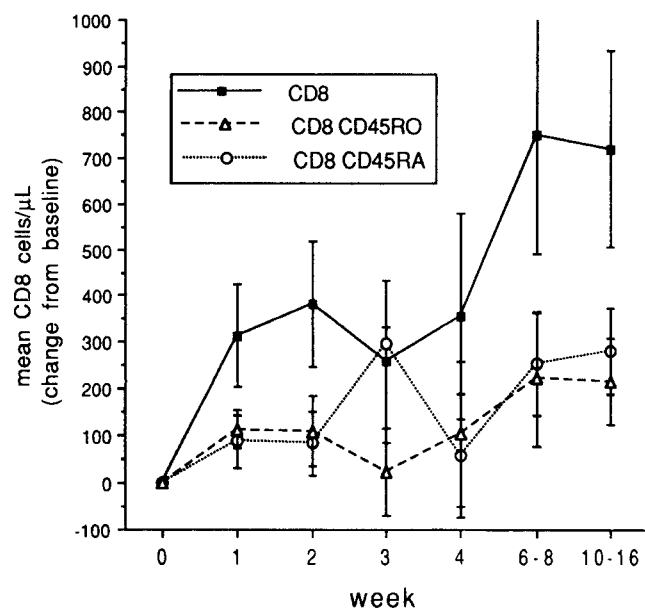

Figure 4. Changes from baseline CD8 cell count and in CD8 cell subsets, CD8CD45RA and CD8CD45RO, from day 1 of ritonavir therapy ($\pm$1 SE of mean).
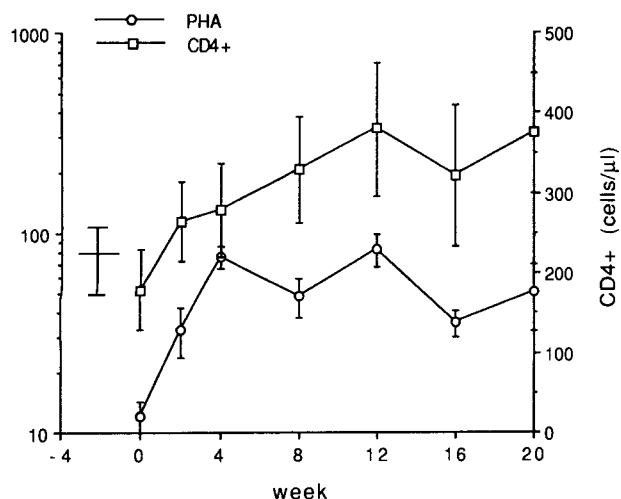
Figure 5. Proliferative response by peripheral blood mononuclear cells to phytohemagglutinin (PHA) in 7 patients treated with ritonavir (500 or 600 mg twice a day). Plot shows mean Δ counts per minute (cpm) $\times$ 10^{-3} (mean cpm in stimulated wells – mean cpm in unstimulated wells) on left axis and mean CD4 cells on right axis (error bars = 1 SE). Single error bar represents mean $\pm$1 SD for PHA reponse in 8 seronegative controls in samples run simultaneously with trial samples. No change in PHA response was found in placebo phase (data not shown).

with any other HIV antiviral therapy. This increase may have great significance if the putative role of CD8 lymphocytes in controlling HIV infection, either by CTL or by soluble inhibitory factors, can be confirmed [44, 45].

The increases in CD4 and CD8 lymphocyte counts were studied in more detail to determine the nature of these cells. By using a model proposed in previous publications [36, 37], we plotted both raw and log transformed CD4 and CD8 counts against time and did linear regression analyses to derive rates of increase. These data permit a number of observations. First, there is wide intersubject variability in both the linear and exponential (ranges) rates of increase; these did not relate to dose regimen. Second, the estimates of rates of increase of CD4 and CD8 cells were associated with significant uncertainty, as indicated by both the size of the SE and P values. Any correlations or predictions based on these data would be difficult to interpret. Finally, the increase in both CD4 and CD8 cells did not appear to have either exponential or linear kinetics, which prevented any firm conclusion as to whether the source of these cells is peripheral (exponential) or central (linear).

This type of approach has been used to predict sources of the returning CD4 cells, their half-lives, and total body CD4 cell turnover [36, 37]. There are two problems with this approach. First, there is an inability to ascribe with certainty either linear or exponential kinetics to the increase. This is true in our hands and in published data. This may result from either the inherent variability of CD4 cell counts [46] or it may indicate that the CD4 cell increase is the result of a more complex process in which the kinetics are neither linear nor exponential. This goes to the second problem: The proposed model [36, 37]

does not take into account any trafficking phenomenon and assumes that any increase in peripheral blood CD4 cell count can be extrapolated to reflect an increase of the same magnitude in total body CD4 cell count. In our hands, this model cannot be used to predict with any certainty either the source of the observed lymphocyte increase or the rate of lymphocyte turnover.

The phenotypic data presented here, however, may give further insight into the dynamics of the lymphocyte increase following therapy with ritonavir. Considerable differences were observed in the time course of change of various CD4 and CD8 lymphocyte subsets. During the first 4 weeks of therapy, there was a rapid increase in the CD4CD45RO subset that paralleled the CD4 lymphocyte increase; however, this increase made up less of the overall increase. If this subset is preferentially infected by HIV [21, 22], this increase may have resulted either from decreased cell death or proliferation in the periphery. By contrast, the absolute CD4CD45RA subset number did not increase until week 4 of therapy and rose steadily thereafter. It is generally accepted that CD45RA marks naive cells that are recent thymic emigrants [47, 48]. This delayed increase in CD45RA cells may indicate a contribution from thymic processing, although these data alone cannot exclude a second source derived from CD4CD45RO cells cycling back to CD4CD45RA cells upon reduction in antigenic stimulation [43, 49]. The time courses of the CD45 subsets of CD8 cells are more difficult to interpret, perhaps indicating a more complex process.

CD28 is an important molecule for the transmission of costimulatory signals in T cell activation, and its expression is decreased on CD4 lymphocytes of subjects infected with HIV [50, 51]. Recent functional studies have suggested that defects in proliferative responses observed in the PBMC of persons infected with HIV arise from the CD4 lymphocyte subset not expressing CD28 [52]. The rapid rise in numbers of CD4CD28 cells observed here reverses the depletion normally seen and may partly explain the improved proliferative responses observed.

The expression of the adhesion molecule CD62L (L-selectin) is markedly reduced in subjects with HIV infection [23, 53]. After therapy, there was an immediate rapid rise in the CD4CD62L lymphocyte count, which plateaued after only 1 week. Thereafter, despite continuing rises in CD4 cell counts, there was no significant increase in numbers of CD4CD62L cells. If trafficking into the periphery is the major cause of the CD4 cell increase, as has been suggested [38, 39], then loss of cells expressing CD62L from the peripheral circulation might result in a fall in CD4 cell count. This was not observed.

The relative reduction in the expression of activation markers CD38 and CD25 on CD4 cells and of CD38 on CD8 cells may be an indication that the populations of CD4 and CD8 lymphocytes are becoming less activated, possibly reflecting decreased virus load. It can be inferred that except in the initial weeks of therapy that the cells contributing to the increases in CD4 and CD8 cell counts did not express CD38. By contrast,
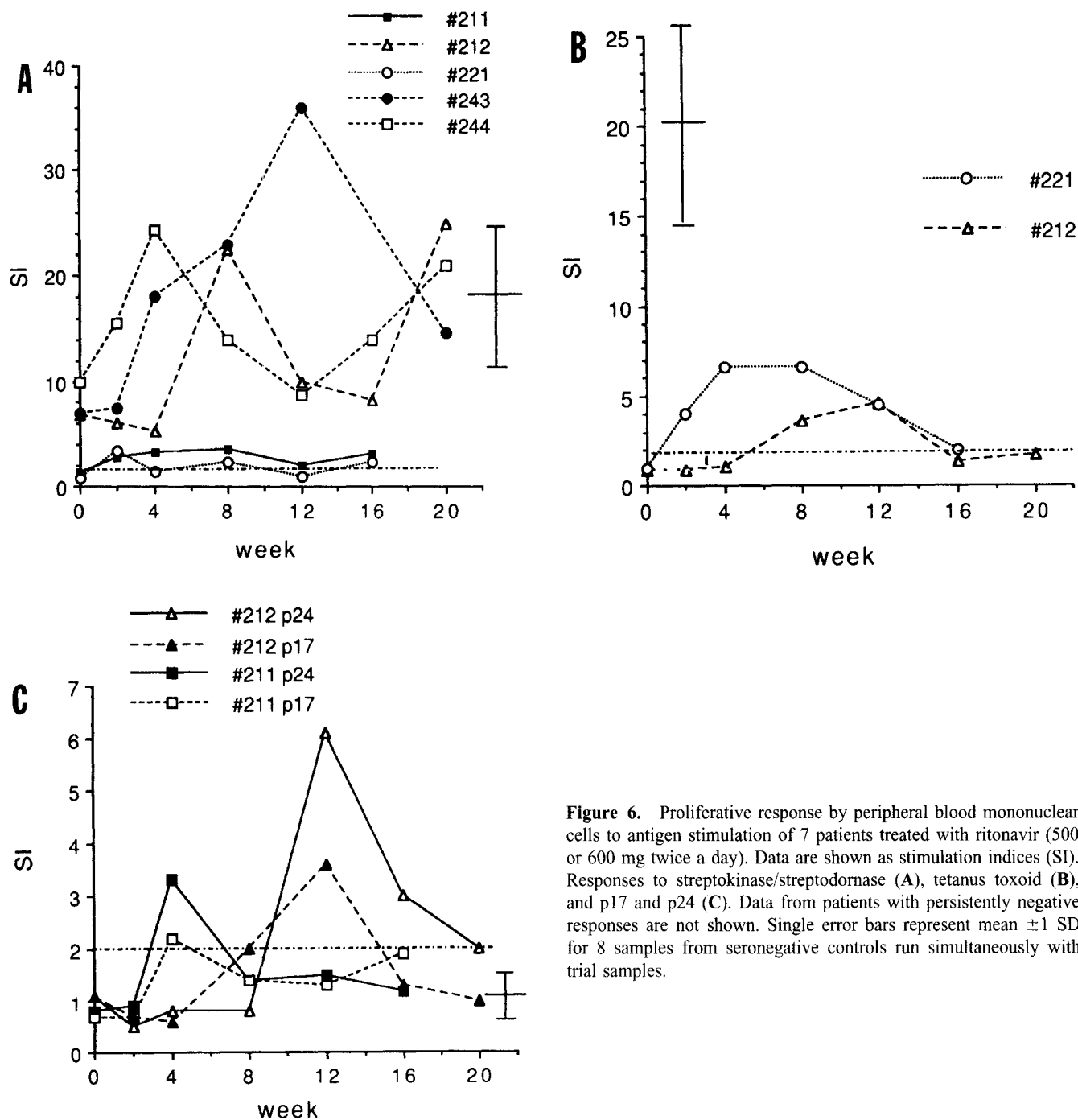
Figure 6. Proliferative response by peripheral blood mononuclear cells to antigen stimulation of 7 patients treated with ritonavir (500 or 600 mg twice a day). Data are shown as stimulation indices (SI). Responses to streptokinase/streptodornase (A), tetanus toxoid (B), and p17 and p24 (C). Data from patients with persistently negative responses are not shown. Single error bars represent mean ±1 SD for 8 samples from seronegative controls run simultaneously with trial samples.

in percentage terms, the expression of HLA-DR remained relatively constant on CD4 cells and increased on CD8 cells. Differential expression of CD38 and HLA-DR on CD8 lymphocytes may identify cell subpopulations with distinct developmental and functional characteristics [28–30]. Since the overwhelming majority of CD8 cells appear to be of cytotoxic phenotype, as identified by the binding of MAb S6F1, the observed changes may reflect a change from a population of cytotoxic effectors to cytotoxic precursors [28–30, 32].

The duration of improvement in T cell function in terms of PHA proliferative responses correlated with duration of virus

load reduction. The length of this improvement compares favorably with those induced by zidovudine [6]. The proliferative responses to antigens may be restored in the reverse order to which they are lost [1–3]. The 2 patients who had <50 lymphocytes/μL at the start of active therapy did not show any improvement in response to recall or HIV-specific antigens. The lack of improvement in TT responses in 5 of 7 patients, to SK/SD in 2 of 7, and to p17 or p24 in 5 of 7 may indicate deletion of clones due to progressive T lymphocyte depletion by HIV.

Overall, in terms of absolute numbers of CD4 and CD8 lymphocytes, virus load, lymphocyte proliferative responses,

and the distribution of phenotypic subsets within both CD4 and CD8 lymphocytes, treatment with ritonavir tended to give patients with late-stage disease a numerical and functional T lymphocyte profile more akin to subjects in the asymptomatic phase of HIV infection [53].

Changes in CD4 and CD8 lymphocyte subsets, as determined by surface protein phenotype, indicate that the dynamics of lymphocyte recovery following therapy with potent antiretrovirals, such as ritonavir, may be more complex than previously realized. Although recirculation phenomena has been invoked as the major cause of the observed CD4 cell increase [38, 39], the late increase in CD4 CD45RA cells, the relative reduction in the contribution of CD62L cells to CD4 cell increase, the increase in CD28 expression, and the restoration of lymphocyte function argue that this is not the entire explanation. Furthermore, several of the predictions as to the phenotype of the lymphocytes contributing to the observed increases are not supported by our phenotypic data [39, 41, 43].

On the basis of our findings, we propose the following model. Treatment with an effective antiretroviral causes a rapid reduction in virus load. This results in a significant perturbation of the HIV-infected immune system, which is reflected in increased numbers of CD4 lymphocytes. A proportion of the initial increase may result from recirculation of lymphocytes. However, the sustained increases result from decreased cell death and restoration of lymphocyte production, initially in the periphery and subsequently from central locations. The concomitant improvement in CD4 lymphocyte function allows normalization of cytokine secretion and T cell help, resulting in improved CD8 lymphocyte number. The reduction in virus load results in less activation of CD4 and CD8 cells. These changes are dependent upon reduction in virus load.

More detailed analysis of the phenotypic changes in both the CD4 and CD8 lymphocyte subsets may be achieved by the use of three- and four-color flow cytometry. These techniques may give insight into the possibility of thymic reconstitution, as may molecular analysis of CD45 isoforms. Further definition of changes in CD4 and CD8 lymphocyte function may be revealed by changes in profiles of cytokine secretion and how these cell types perform in various in vitro functional assays.

Acknowledgments

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