CD8+ Anti–Human Immunodeficiency Virus Suppressor Activity (CASA) in Response to Antiretroviral Therapy: Loss of CASA Is Associated with Loss of Viremia

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CD8+ anti–human immunodeficiency virus (HIV) suppressor activity (CASA) defines the noncytolytic suppression of HIV mediated by secreted soluble factors. Changes in CASA in patients receiving combination antiretroviral therapy have not been described. Thirty-two HIV-infected patients receiving mono- or dual therapy for 52 weeks followed by highly active antiretroviral therapy (HAART) for a further 52 weeks were analyzed. T cell number and functional subsets, cutaneous delayed-type hypersensitivity, and plasma HIV RNA were assessed in 17 patients for CASA. Prior to therapy, CASA correlated inversely with HIV RNA (P < .001). Dual therapy yielded greater and more sustained changes in CASA than monotherapy, but HAART decreased CASA to levels observed in HIV-uninfected individuals. The magnitude of HIV RNA suppression correlated significantly with a decrease in activated CD8+ T lymphocytes (CD38+HLA-DR+), increases in naive CD4+ T lymphocytes (CD45RA+62L+), and increases in the delayed-type hypersensitivity score. However, changes in CASA did not correlate with changes in any T lymphocyte subset. CASA increases with improving immune function but appears more dependent on ongoing HIV replication.

Materials and Methods

Subjects. Thirty-two patients were enrolled at St. Vincent’s Hospital, Sydney, in a multicenter, double-blind, randomized placebo-controlled trial of indinavir (800 mg 3 times a day) and stavudine (d4T; 30–40 mg 2 times a day), which were administered alone or in combination. The trial was open to adult HIV-infected patients who had had at least 6 months of zidovudine therapy and had a CD4+ cell count of 50–500 cells/μL. Following 52 weeks of therapy, 29 of the 32 patients rolled over to a triple therapy regimen with d4T-lamivudine-indinavir (HAART) for a further 52 weeks. All patients had sequential CD4+ and CD8+ lymphocyte counts and plasma viral RNA load assessed every 4 weeks for the 104 weeks. Changes in CD4+ and CD8+ lymphocyte subsets were measured in 25 patients by 4-color flow cytometry (the remaining 7 patients were missed at baseline, so subset data were not obtained). In 17 patients, CASA was assessed at weeks 0, 4, 8, 24, 52, 56, 64, 76, and 104 (the CTL responses of the remaining 15 patients were measured in 25 patients by 4-color flow cytometry (the remaining 7 patients were missed at baseline, so subset data were not obtained).
Monoclonal antibodies (MAbs) to CD3-PerCP, CD4-phycoerythrin (PE), CD38-PE, HLA-DR±fluorescein isothiocyanate (FITC) were purchased from Becton Dickinson (San Jose, CA). MAbs to CD4-ECD, CD8-ECD, and CD45RA(2H4)-RD1 were purchased from Coulter Electronics (Hialeah, FL), and MAbs to CD45RO-FITC were from DAKO (Carpinteria, CA). All MAbs were the laboratory strain HIV-1SF-33 and plated out in 24-well tissue culture plates (Corning, Corning, NY) at cells/well. Patient lymphocytes (target cells) or patient CD8+ lymphocytes (effector cells) were immunomagnetically separated by use of MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity of the selected populations was assayed by flow cytometry, and only cell fractions displaying $>$95% purity were used.

Donor CD4+ lymphocytes were infected with 5000 TCID$_{50}$ of the laboratory strain HIV-1$_{SF-33}$ and plated out in 24-well tissue culture plates (Corning, Corning, NY) at $3 \times 10^5$ cells/well. Patient CD8+ lymphocytes were then added in duplicate wells at ratios of 2:1, 1:1, 1:2, 1:5, and 1:10 to a final volume of 1.2 mL. HIV replication was monitored by assaying reverse transcriptase activity [10] in the culture supernatants every 2 days from day 4. Reverse transcriptase measurements of the various ratios of effector to target (E/T) cells were compared with infected control CD4+ cells cultured alone. The effective E/T ratio was defined for each patient time point as the ratio at which viral replication was inhibited by $>$90% of the control cultures [7]. To allow for graphical representation, log transformations of E/T ratios are presented and were used for subsequent statistical analyses. Two patients (1 receiving d4T, 1 receiving indinavir) did not have measurable CASA at the highest E/T ratio tested (2:1) and were excluded from regression analysis; however, they were assigned a ratio of 2:1:1 (log 0.32) for subsequent analyses. CASA was always $\geq$2:1 in the 5 controls over an 18-month period.

T cell-mediated cytotoxicity and spontaneous CD4+ cell death in the suppressor assay were assessed by the Cytotoxic 96 cytotoxicity assay (Promega, Madison, WI) and found to be $<$10% of maximal release at an E/T ratio of 2:1.

Flow cytometric analysis of circulating T lymphocyte subsets. Monoclonal antibodies (MAbs) to CD3-PerCP, CD4-phycocyanin (PE), CD38-PE, HLA-DR–fluorescein isothiocyanate (FITC), CD8–FITC, CD25-PE, CD28-PE, and CD62L(LEU-8)-FITC were purchased from Becton Dickinson (San Jose, CA). MAbs to CD4-ECD, CD8-ECD, and CD45RA(2H4)-RD1 were purchased from Coulter Electronics (Hialeah, FL), and MAbs to CD45RO-FITC were from DAKO (Carpinteria, CA). All MAbs were used at the manufacturers' recommended concentrations.

Whole blood (100 $\mu$L) was incubated with MAbs for 10 min at room temperature, and cells were lysed and fixed [OptiLyse C reagent; Immunotech, Marseille, France]. Samples were analyzed by flow cytometry (EPICS XL; Coulter). Lymphocyte gates were set manually on forward and side scatter, and 10,000 lymphocytes were analyzed. For activation marker analysis, CD3-PerCP and either CD4-ECD or CD8-ECD staining were used to set gates for subsequent analysis of 2-color FITC versus PE histograms. In the case of CD45RA and CD45RO, cells were identified as CD45RA”CD45RO” and CD45RA CD45RO” reciprocal populations.

DTH responses. CD4+ T cell function in vivo was evaluated by use of the CMI MULTITEST kit (Pasteur Mérieux, Lyon, France). Both total DTH score and the number of positive responses were assessed. The total DTH score is the sum of the mean diameters of each positive antigen response (a positive response was defined as induration with a mean diameter $>2$ mm). DTH scores $>10$ mm were classified as normal. Scores of 2–10 mm were classified as hyporeactive, and scores $<2$ mm were classified as anergic [11].

Virus load. Plasma HIV RNA was measured by polymerase chain reaction (PCR) assay (Amplicor HIV-1 Monitor; Roche, Pleasanton, CA). Plasma virus loads $<$400 HIV RNA copies/mL (log 2.6) were considered below the detectable level of the assay, and patients with such levels who were receiving HAART were retested using the Amplicor HIV-1 Monitor Ultra-Sensitive PCR assay (Roche).

Statistical analysis. Computer software (Statview 4.5; Abacus, Berkeley, CA) was used for all statistical calculations. Comparisons between treatment groups with respect to suppressor responses and T cell subsets were carried out by use of the Mann-Whitney U-test, with a cutoff of significance of $P < .05$. Regression analysis was performed to determine correlations between E/T ratio and biologic markers at baseline. The Wilcoxon signed rank test was used to determine therapy-associated changes in CASA from baseline.

Results

Baseline characteristics. Data for T lymphocyte counts and CASA are summarized in table 1 for the original three treatment arms. There was no significant difference between the treatment arms for any of the measured parameters.

Correlation of biologic markers with CASA prior to therapy. Baseline CASA inversely correlated with plasma virus load ($r = .74, P < .001$; figure 1). No correlation was found between baseline CASA and CD4+ or CD8+ lymphocyte numbers, including CD45RA”, CD45RO”, CD45RA”/CD62L”, CD28”, and CD38”/HLA-DR” subsets.

Effect of therapy on CASA. Mean CASA increased significantly (P = .03) from baseline to week 52 (figure 2A), although 4 patients (2 receiving indinavir, 2 receiving d4T-indinavir) showed no change. Therapy-associated changes in CASA were seen in 13 patients; 4 had a decrease in CASA (3 receiving indinavir, 1 receiving d4T), and 9 had an increase during the trial. The greatest increases in CASA were observed in patients receiving d4T-indinavir—the changes were twice those seen with d4T alone. Peak increases in CASA occurred at weeks 4, 52, and 8 for the d4T-indinavir, indinavir, and d4T treatment arms, respectively. CASA returned to baseline by week 52 in the d4T arm, whereas the increase in CASA was sustained to week 52 in patients receiving d4T-indinavir. The mean changes in E/T ratio over the course of therapy were 0.48, 1.15, and
CASA was associated with lower virus loads.

0.74 for the d4T-indinavir, indinavir, and d4T treatment arms, respectively. These changes were significant between the d4T-indinavir and indinavir arms (P < .05). This difference in variation in mean CASA between patients with and those without detectable HIV RNA remained, with the more stringent cutoff (<50 copies/mL) for patients without detectable levels; however, the difference was significant at week 56 only. Three patients with a virus load between 50 and 400 copies/mL demonstrated no change in variation in mean CASA, suggesting a threshold effect.

Patients receiving d4T who switched to HAART demonstrated transient increases in CASA. The increases were associated with initial decreases in HIV RNA and increases in CD4+ and CD8+ lymphocyte counts that then faded to control levels as virus load reached undetectable levels. This arm contained the largest number of patients (71%) with HIV RNA levels that remained undetectable at the end of HAART (week 104); 44% and 38% of the patients in the indinavir and d4T-indinavir treatment arms had levels that remained undetectable.

**T lymphocyte subsets.** Changes in absolute CD4+ T lymphocyte counts are shown in table 1. The characteristic early increase in CD4+CD45RO+ cells was observed in patients receiving d4T-indinavir or indinavir alone. In contrast to the d4T-treated patients, in whom a decrease of 20 CD4+ lymphocytes was seen at week 52, patients receiving d4T-indinavir or indinavir alone showed mean increases of 16 and 18 cells/µL, respectively. Although increases in absolute CD8+ T lymphocyte number from baseline were seen in all three treatment arms, only the d4T arm demonstrated a significant increase at week 52 (P = .02). Up to week 6, there was an initial increase in the mean percentage of CD3+CD8+ lymphocytes staining for both CD38- and HLA-DR- in patients receiving indinavir, which was followed by a decrease in all three treatment arms to levels <log 2.6) lost CASA. Significant differences were seen in the variation in mean CASA between patients with and those without a detectable virus load at weeks 56 and 104 (P < .05; figure 4). This difference in variation in mean CASA between patients with and those without detectable HIV RNA remained, with the more stringent cutoff (<50 copies/mL) for patients without detectable levels; however, the difference was significant at week 56 only. Three patients with a virus load between 50 and 400 copies/mL demonstrated no change in variation in mean CASA, suggesting a threshold effect.

HAART led to a prompt decrease in CASA, as demonstrated by a mean peak increase in the E/T ratio from 0.49 at week 52 to 1.8 at week 64, representing peak mean changes from baseline of 1.31 between weeks 52 and 64 and of 1.01 between weeks 52 and 104. This reduction in CASA decreased to levels observed in the HIV-uninfected controls in 11 of the 14 patients and coincided with a reduction in virus load toward undetectable levels (figure 3). At week 24 of HAART (week 76 of study), CASA correlated with virus load in a manner opposite to that observed at baseline (r = .99, P < .001, n = 6) such that weak CASA was associated with lower virus loads.

Changes in plasma HIV RNA are shown in figure 2B. With therapy, 23% of the patients had virus loads that remained below the detectable level at week 52: 40% of the patients receiving d4T-indinavir had undetectable levels at week 52, compared with 10% receiving indinavir and 17% receiving d4T.

HAART resulted in a mean peak reduction in HIV RNA of −1.2 log copies/mL to mean log 2.8, which was maintained to week 104, with 50% of these patients having virus loads below the level of detection. When patients were grouped by virus load in response to HAART, CASA remained in those patients who demonstrated a virus burden; those with undetectable levels.
Figure 2. Effect of mono-, dual, and triple therapy (highly active antiretroviral therapy [HAART]) on CD8⁺ anti–human immunodeficiency virus suppressor activity (CASA) (A) and plasma virus load (B). Dotted horizontal line = level for HIV-uninfected persons. Plots show mean ± SE for original randomization and switch to HAART. No. of patients for each time point is noted below (absent values are the same as week 0). BDL = below level of detection; E/T = effector-to-target ratio, IDV = indinavir, d4T = stavudine.
Figure 3. Association between plasma virus load and CD8\(^+\) anti-human immunodeficiency virus suppressor activity with partially suppressive regimen (mono- or dual therapy) and fully suppressive regimen (highly active antiretroviral therapy [HAART]). Plot shows mean values ± SE, with no. of patients for each time point noted below. E/T = effector-to-target ratio.

Figure 4. Change in CD8\(^+\) anti-human immunodeficiency virus (HIV) suppressor activity from baseline (week 52) for patients with levels of HIV RNA ≥400 copies/mL or below level of detection (BDL; <400 copies/mL) with highly active antiretroviral therapy. Plot shows mean values ± SE, with no. of patients at each time point noted below. Comparisons between 2 groups were carried out by use of Mann-Whitney U test, with cutoff of significance of \(P < .05\). E/T = effector-to-target ratio.
ment arms. At week 52, the decrease in the percentage of CD8+ CD38+ DR+ lymphocytes in the d4T group (5%) was less than that in the d4T-indinavir (16%; P = .04) or indinavir (10%) group.

Additional increases in CD4+ and CD8+ T lymphocytes and a further decrease in CD8+ T lymphocyte activation, as measured by CD8+ CD38+ HLA-DR+ expression, were seen with HAART. The best outcomes at week 104 were observed in the d4T-indinavir arm, with continued reductions in HIV RNA and increasing numbers of CD4+ and CD4+CD45RA+62L+ T lymphocytes. The indinavir treatment arm had the least favorable response to HAART, with a rising virus load from week 64 to log 3.3 at week 104.

There was no correlation between CASA and T lymphocyte number or between changes in number for CD4+ or CD8+ T lymphocytes or their measured subsets.

DTH responses. The pretherapy mean total DTH score for the 28 patients was 1.8 ± 4.4 mm: 1 (4%) patient had a normal response, 4 (14%) had hypogic responses, and 23 (82%) had anergic responses. The mean number of positive responses was 0.36 ± 0.9. These responses were an order of magnitude lower than those expected for healthy men in this age group [11]. Increases in DTH response with treatment peaked at week 24 and remained above baseline at 52 weeks. The number of responders (patients demonstrating an increase in DTH score from baseline) at week 52 was greater in the d4T-indinavir (3/11 patients) and indinavir (3/10) groups than in the d4T group (1/7). Changes in CASA could not be correlated with improved DTH responses.

Discussion

CASA increases with improving immune function but is most dependent on HIV replication: CASA is abolished if HIV RNA is undetectable. In this study, we observed an increase in immune function with mono- and dual therapies, with CASA also increasing with this improvement. With HAART, once the virus load is below detectable levels and replication is low, a further increase in immune function does not translate to an increase in CASA. In fact, a loss in viremia is associated with a loss in CASA to levels observed in HIV-uninfected individuals. Pretherapy baseline CASA inversely correlated with virus load such that patients with lower levels of HIV RNA demonstrated stronger CASA, suggesting a greater ability of CD8+ lymphocytes to suppress HIV replication, which is consistent with findings in infants [12].

Antiretroviral therapy leading to incomplete virus suppression generally resulted in an increase in CASA for periods of >1 year, and changes in CASA correlated with changes in HIV RNA, not with the type of antiretroviral agent used per se. Increases in CASA observed with d4T-indinavir represent a ~3-fold improvement above that seen with d4T monotherapy, the latter being comparable to CASA improvements seen with zidovudine monotherapy [13]. Transient increases in CASA with HAART may contribute to reduction in virus load together with cytotoxic CD8+ lymphocytes, which may in turn be CD4+ dependent [4, 5, 14, 15].

HAART that suppressed plasma HIV RNA to undetectable levels resulted in a loss of CASA. CASA remained in patients with a detectable virus load, and it remained at a reduced level in 3 patients with 50–400 copies/mL, suggesting a threshold value. As a parallel, a similar response has been documented with CTL and plasma virus load. Ogg et al. [16] found a significant inverse correlation between HIV-specific CTL frequency and plasma RNA, a finding that is associated with improving immune function in the absence of viremia.

We, however, measured CASA in the blood only, which may not be representative of tissue CASA. CASA may still be measurable in the lymph nodes, where HIV may continue to replicate and where HIV antigen and whole viral proteins are still being produced [17]; these whole viral proteins may be responsible for secretion of the soluble suppressor factors involved in CASA. It would be of interest to evaluate lymph nodes from patients with undetectable plasma HIV RNA and correlate any persistent HIV replication with CASA within these nodes. The absence of CASA with undetectable HIV RNA suggests that the immune system may be unable to maintain viral suppression without continued antiretroviral therapy. This response alone cannot maintain suppression, nor is it present as a bystander effect should HIV replication resume.

We were unable to find any correlation between CASA and CD8+ or CD4+ T lymphocyte number or subset at baseline or with antiretroviral therapy. In addition, increases in CD8+ lymphocytes within the three treatment arms did not relate to changes in CASA. Treatment with d4T-indinavir caused the lowest rise in CD8+ T lymphocyte counts with the largest increase in CASA, whereas treatment with d4T caused the largest increase in CD8+ T lymphocytes with the lowest increase in CASA. Even when CASA returned to control levels in patients receiving HAART, an increase in CD8+ T lymphocyte count was still observed. In addition, there is still uncertainty as to the identity of the subset of CD8+ effector cells responsible for suppressor activity. Subsets of CD8+ T lymphocytes expressing HLA-DR+ [18], CD57+ [19], CD11b+ [20], S6F1+ (LFA-1), CD25+, CD38+, and CD45RA+ have all been implicated in CASA, although a recent report suggests that CD8+ CD28+ lymphocytes are primarily responsible for CASA [22]. In support of the findings of Kootstra et al. [23], we could not demonstrate a correlation between CASA and the number or phenotype of CD4+ T lymphocytes; however, individually, patients with a high CD4+ lymphocyte count and large increases in the number of CD4+ cells could be associated with stronger CASA (data not shown).

In addition to the examination of CASA and the phenotypic analysis of T lymphocyte subsets, the cellular arm of the immune system, targeted by HIV, can be assessed in vivo by cu-
taneous DTH responses. Increases in DTH response were observed in the d4T-indinavir and indinavir arms up to week 52. This increase in cell-mediated immunity, resulting from the interaction of T memory cells with antigen-presenting cells in the skin, although conferring a positive response regarding reconstitution of the immune system, could not be correlated with changes in CASA.

Indinavir therapy has been shown to significantly increase the circulating levels of interleukin-16, macrophage inflammatory protein (MIP)–1α, MIP-1β, and RANTES [24]. Interleukin-16 and the combination of MIP-1α, MIP-1β, and RANTES have been shown to have a suppressive effect on HIV replication in vitro [25, 26]; however, these are not thought to be the sole agents responsible for CASA [27], and the identity of CD8+ antiviral factor (CAF) remains unknown. A reduction in HIV replication leading to a decrease in activated CD8+ T lymphocytes could be responsible for the loss in CASA, since these lymphokines have been correlated with CD8+ T lymphocyte number [28] and with activated CD8+CD38+ cells but not virus load [29]. Although no significant correlations were observed between CASA and CD8+ T lymphocytes or their subsets, the lymphokines produced by these cells are the likely source of Levy’s CAF.

In conclusion, the baseline correlation between virus load and CASA and the loss of CASA with undetectable viral replication suggest that the presence of HIV is necessary to drive CASA. Immune restoration following potent antiviral therapy is not limited to changes in lymphocyte number or phenotype but includes an improvement in in vitro immune function. Treatment regimens that lower virus load and increase CD4+ lymphocyte numbers also increase an individual’s ability to suppress HIV through a cell-mediated suppressive action. Since CASA is not present while patients are receiving HAART, it may be necessary to continue HAART to control viral replication. It has been speculated that an improved immune function could effectively suppress HIV replication following withdrawal of antiretroviral therapy in those patients with long-term undetectable plasma HIV RNA [30]. Unfortunately, the present data show that CASA is lost as HIV RNA reaches 50–400 copies/mL. CASA, therefore, may not be able to contribute to the above-mentioned immune-mediated suppression of HIV replication.

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References

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