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High Levels of Anti-Human Immunodeficiency Virus Type 1 (HIV-1) Memory Cytotoxic T-Lymphocyte Activity and Low Viral Load Are Associated with Lack of Disease in HIV-1-Infected Long-Term Nonprogressors

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Lack of disease in long-term nonprogressors with human immunodeficiency virus type 1 (HIV-1) infection was strongly associated with very low copy numbers of HIV-1 DNA and RNA in peripheral blood mononuclear cells and plasma and the presence of high levels of anti-HIV-1 $CD8^+$ memory cytotoxic T lymphocytes specific for Gag, Pol, and Env, compared with levels present in intermediate and advanced progressors. $CD8^+$ memory cytotoxic T lymphocytes may have an important role in controlling HIV-1 replication and preventing disease in long-term nonprogressors.

Human immunodeficiency virus type 1 (HIV-1) infection results in severe immune dysfunction with a progressive and inexorable loss of $CD4^+$ T lymphocytes and development of AIDS in most adults within a median of 10 years (9). Approximately 5% of all HIV-1-infected persons, however, remain asymptomatic with stable numbers of $CD4^+$ T cells for many years after seroconversion to the virus (20, 26). These long-term nonprogressors are of special interest because they may represent individuals who are not susceptible to the pathogenic effects of HIV-1 infection. Knowledge of the immunologic and virologic mechanisms by which HIV-1 infection is impeded in these individuals may have implications for the development of effective HIV-1 vaccines and therapies.

Koup et al. (18) and Borrow et al. (1) recently reported that high levels of precursor frequency of major histocompatibility complex class I-restricted, $CD8^+$ anti-HIV-1 cytotoxic T lymphocytes (CTL) generated by in vitro stimulation of peripheral blood mononuclear cells (PBMC) with anti-CD3 monoclonal antibody (MAb) and interleukin 2 are temporally associated with rapid reduction of plasma viremia in subjects with primary HIV-1 infection. Moreover, these memory CTL (CTLm) specific for HIV-1 are decreased in number in late-stage HIV-1-infected subjects with low $CD4^+$ T-cell numbers (3). These findings support the concept that maintenance of a precursor pool of HIV-1-specific CTLm is a major factor in host control of HIV-1 infection.

We have therefore investigated CTLm activity against a panel of HIV-1 structural and regulatory proteins in homosexual men with long-term, stable HIV-1 infection. This immunologic measure was compared with levels of HIV-1 DNA and RNA in the blood of these nonprogressors and in individuals with moderate or severe loss of circulating $CD4^+$ T cells (intermediate and advanced progressors).

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The participants were enrolled in the Pittsburgh portion of the Multicenter AIDS Cohort Study (MACS) (16), a longitudinal investigation of the natural history of HIV-1 infection in homosexual men. These men have been monitored for clinical, sexual, and laboratory parameters every 6 months since 1984 to 1985. The present CTL and viral load study was a cross-sectional analysis conducted in 1993 to 1994.

Seven subjects were defined as long-term nonprogressors by established criteria (26, 28) of a serologically documented duration of HIV-1 infection of approximately 9.5 years, a stable or increasing slope of $CD4^+$ T cells over this time, and maintenance of absolute $CD4^+$ T-cell counts within the normal range (Table 1). Nine men were intermediate progressors, and 11 were advanced progressors; these men had similar numbers of $CD4^+$ T cells during the first year in the MACS but had negative regression slopes of $CD4^+$ T lymphocytes and decreases in absolute numbers of $CD4^+$ T cells during the years of follow-up in the study.

The nonprogressors and intermediate progressors were asymptomatic, with only one intermediate progressor receiving anti-HIV-1 therapy at the time of the study (Table 1). One of the advanced progressors had AIDS (CDC group IV.C-1), while the others were either asymptomatic (CDC group II) or had minor symptoms (CDC groups IV.A and/or IV.C-2) (4, 5); 7 of these 11 subjects were being treated for HIV-1 infection.

Because high levels of HIV-1 replication are significantly related to progression to AIDS (6, 14, 19, 22, 23, 34), we first investigated the relation of viral load to protection in nonprogressors compared with those in intermediate and advanced progressors. Levels of cellular and plasma HIV-1 RNA and cellular HIV-1 DNA in the blood were quantitated by an internally controlled PCR (ICPCR) assay (13). The ICPCR used coamplification of the target DNA sequence or co-reverse transcription and coamplification of the target RNA sequence with a control plasmid HIV-1 DNA or RNA containing a deletion in the *gag* region. The ICPCR assay is reproducible and can accurately quantify 10^0 to 10^3 copies of HIV-1 DNA

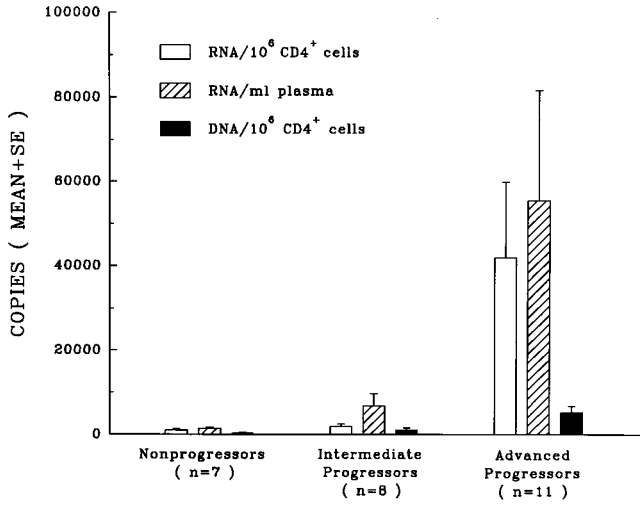

FIG. 1. Increasing levels of HIV-1 RNA in plasma, and RNA and DNA in PBMC, are associated with disease progression. SE, standard error.

and 10^1 to 10^4 copies of HIV-1 RNA within the linear range of amplification.

Quantitative ICPCR analysis demonstrated that the nonprogressors had uniformly low levels of HIV-1 RNA in their plasma and PBMC and similarly low levels of HIV-1 DNA in their PBMC (Fig. 1). All seven nonprogressors had <600 copies of HIV-1 DNA per 10^6 $CD4^+$ cells and <2,300 copies of HIV-1 RNA per ml of plasma and per 10^6 $CD4^+$ cells. In contrast, the HIV-1 DNA and RNA load in the blood components of the intermediate progressors was variable and that in the blood components of the advanced progressors was significantly greater ($P < 0.05$ versus nonprogressors or intermediate progressors; Kruskal-Wallis test, chi-square approximation). Advanced progressors had 40-fold-higher levels of HIV-1 RNA in plasma and $CD4^+$ cells and 15-fold-higher HIV-1 DNA levels in $CD4^+$ cells than did the nonprogressors (Fig. 1). Higher levels of HIV-1 DNA and RNA in the blood were also associated with lower $CD4^+$ T-cell slopes in the total cohort ($r = -0.8$ and $P = 0.001$ and $r = -0.5$ and $P = 0.008$, respectively; Pearson correlation coefficient test). Thus, resistance to the pathogenic effects of HIV-1 infection was significantly related to a low level of HIV-1 in the blood of long-term nonprogressors. This finding is in agreement with those of a recent study of long-term nonprogressors by Cao et al. (2). Furthermore, the low level of HIV-1 in the blood is accompanied by similarly low levels of virus replication in lymph nodes of long-term nonprogressors (28).

To establish whether CTLm specific for HIV-1 were involved in control of HIV-1 disease progression, precursor frequencies of anti-HIV-1-specific CTLm were determined by limiting dilution assay of freshly isolated PBMC after stimulation for 2 weeks with anti-CD3 MAb and interleukin 2 by the method of Koup et al. (17, 18). Target cells were autologous B-lymphoblastoid cell lines (B-LCL) immortalized by infection with Epstein-Barr virus (38). The B-LCL were negative for mycoplasmas as determined with a nucleic acid probe (Genprobe, San Diego, Calif.). The B-LCL were infected with recombinant vaccinia virus (VV) at an input virus-to-cell multiplicity of 4 to 1. VVs used in these studies were as follows: vAbT 141, which contains the *gag* coding sequence for p55 of the BH10 strain of HIV-1; vAbT 204, which contains the complete *pol* coding sequence, including the reverse transcriptase, protease, and integrase of the HxB2 strain of HIV-1; vAbT 271, which contains the *env* coding sequence for gp160 of HIV-1 BH10; vAbT 408, which contains the combined *gag/pol* and *env* coding sequences of the BH10 and HxB2 strains of HIV-1; and vT23, which contains the *nef* coding region of the NL43 strain of HIV-1 (Therion Biologics, Cambridge, Mass.). An additional VV expressed the *tat* exon 1 region of HIV-1 strain BH10 (courtesy of B. Moss, National Institutes of Health), and the VV strain NYCBH (VAC) was used as the control virus. High levels of expression of the VV vectors in the B-LCL were determined as previously described (38). All VV pools were negative for mycoplasmas.

TABLE 1. Clinical characteristics of HIV-1-infected nonprogressors and progressors

Group	Age (yr)[a]	No. of yr HIV-1 positive[a,b]	AIDS diagnosis (no. of subjects positive/total)[c]	Anti-HIV-1 treatment (no. of subjects)[d]	$CD4^+$ T-cell no. (mean ± SE)[e]		$CD4^+$ T-cell slope[a,f]
					First yr of follow-up in MACS	Last yr of follow-up in MACS	
Nonprogressors[g]	38 (35–45)	>9.5 (7.1 to ≥10.0)	0/7	None	765 ± 82	740 ± 69	+15 (−2 to +29)
Intermediate progressors[h]	37 (31–47)	8.8 (5.6 to ≥9.5)	0/9	ZDV, DDI (1) None (8)	758 ± 61	482 ± 44	−34 (−53 to −16)
Advanced progressors[i]	37 (30–65)	8.4 (3.5 to ≥9.5)	1/11	ZDV (1) ZDV, DDI (2) ZDV, DDC (3) ZDV, DDI, DDC (1) None (4)	792 ± 54	174 ± 38	−89 (−218 to −71)

[a] Median (range).
[b] Data represent subjects who were HIV-1 seropositive at entry into the MACS in 1984 to 1985 (seroprevalent subjects [≥9.5 and ≥10 years of infection]) and subjects who seroconverted during the follow-up period (seroconverters).
[c] Centers for Disease Control definition of 1986 and 1987 (4, 5).
[d] Treatment at the time of this study. ZDV, zidovudine; DDI, dideoxyinosine; DDC, dideoxycytosine.
[e] Data were compiled from two to five determinations done on each subject during the first year after entry into the MACS for seroprevalent subjects or during the first year after documented seroconversion during the MACS for seroconverters (first year of follow-up) and in the last year of the MACS during the time of this study (last year of follow-up).
[f] Change in number of $CD4^+$ T cells per semester during the total time of follow-up in the MACS for seroprevalent subjects or after seroconversion for the seroconverters.
[g] $n = 7$.
[h] $n = 9$.
[i] $n = 11$.

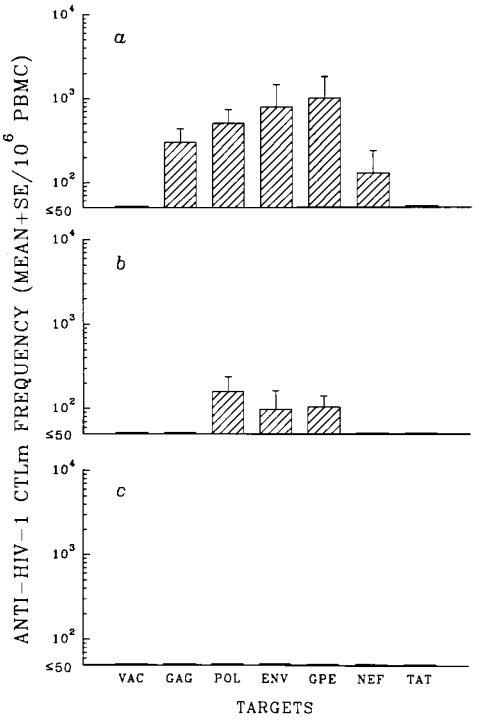

FIG. 2. Anti-HIV-1 CTLm precursor frequency relates to protection against HIV-1 disease progression in 7 nonprogressors (a), 8 intermediate progressors (b), and 11 advanced progressors (c). VAC, VV control; GPE, VV Gag/Pol/Env triplex. The anti-HIV-1 CTLm frequency for PBMC of HIV-1 seronegative controls was <50 per 10^6 cells against all HIV-1–VV-infected targets (data not shown). SE, standard error.

Cytotoxicity was assessed by split-well analysis of the stimulated, cultured PBMC and a standard chromium release assay (17, 18). The precursor cell frequency was estimated by the maximum likelihood method (10) with a statistical program kindly provided by S. Kalams (Boston, Mass.); CTLm activity (precursor frequency) and 95% confidence limits were expressed per 10^6 PBMC. A positive anti-HIV-1-specific CTLm response was defined as a precursor frequency of ≥50 per 10^6 PBMC. This was derived from the mean plus 2 standard deviations of the CTLm responses against the VAC control targets. Validity testing of duplicate blood samples from HIV-1-seropositive subjects showed that there was <10% variation in levels of CTLm activity against the HIV-1 proteins.

High levels of anti-HIV-1 CTLm frequencies above the positive threshold of >50 per 10^6 PBMC were detected in the seven nonprogressors (Fig. 2). The mean level of CTLm responses specific for Gag and Pol was greater for the nonprogressors than for the advanced progressors ($P < 0.05$; one-way analysis of variance). There was no evidence of a selective CTLm response to a particular HIV-1 gene product in the nonprogressors. That is, positive CTLm responses against one or more HIV-1 gene products were noted in all of the nonprogressors, with five of seven subjects having activity against either Gag or Env, four of seven subjects having activity against Pol, and four of five subjects having activity against the Gag/Pol/Env triplex. Positive CTLm responses against Nef and Tat were infrequent (two of six and zero of six subjects, respectively). Positive CTLm responses were much less frequent in the 11 advanced progressors, with activity against only single structural proteins being demonstrable for three subjects and activity against both Nef and Tat being demonstrable for another subject. The intermediate progressors had CTLm reactivity between that of the nonprogressors and that of the advanced progressors, with positive anti-HIV CTLm responses specific for various HIV-1 proteins occurring in six of the eight subjects.

The differential anti-HIV-1 CTLm activity among the three cohorts was not due to differences in either T-cell growth, viability, or phenotype in the CTLm precursor assay. Study subjects with either high or low levels of anti-HIV-1 CTLm activity had similar increases in cell numbers of approximately 250- to 750-fold after the 14 days of culture. Moreover, the cultured cells from the three cohorts had comparable viability levels of 73 to 99%, as determined by trypan blue dye exclusion, with the lower viability levels being observed in wells seeded with the low concentrations of PBMC. The cultured cells were >85% $CD8^+$, and the $CD8^+$ cells expressed similar levels of the HLA-DR activation marker (50 to 60%) and the S6F1 cytotoxicity marker (65%) (24), as determined by flow cytometry (11).

$CD8^+$ and $CD8^-$ cells were compared with regard to CTLm activity. Freshly isolated PBMC were treated with anti-CD16 MAb (Becton Dickinson) (10 μl/3 × 10^6 PBMC) and incubated for 15 min at 4°C. The cells were washed and treated with biotinylated goat anti-mouse immunoglobulin (Antigenix, Franklin Square, N.Y.); washed and mixed with streptavidin-conjugated microbeads and microbeads directly conjugated to anti-CD4, -CD14, and -CD19 MAbs (Miltenyi Biotec, Sunnyvale, Calif.) at 10 μl per 10^7 cells at 4°C; and then loaded onto a magnetic separation column (Miltenyi). The nonadherent cells eluted through the column were stained with fluorescent MAbs specific for CD4, CD8, CD14, CD16, and CD20 and analyzed in a flow cytometer. A purity of ≥90% $CD8^+$ cells with <1% $CD4^+$, $CD14^+$, $CD16^+$, and $CD20^+$ cells was achieved. $CD8^-$ cells were enriched from PBMC by removal of ≥98% of the $CD8^+$ cells in anti-$CD8^+$ MAb-coated flasks (Applied Immune Sciences, Menlo Park, Calif.) (38).

$CD8^+$ T-cell-enriched populations had greater precursor frequencies than did $CD8^-$ cell populations against Gag, Pol, and the Gag/Pol/Env triplex (Table 2), indicating that the CTLm activity against Gag and Pol was mediated by $CD8^+$ cells. CTLm activity against Env was not detected in this experiment. In other experiments, however, CTLm responses to Env were noted for both the $CD8^+$ and $CD8^-$ T-cell-enriched

TABLE 2. $CD8^+$ cells mediate the anti-HIV-1 CTLm response[a]

Effector cells	CTLm precursor frequency (confidence interval) per 10^6 PBMC with:				
	VAC	Gag	Pol	Env	Gag/Pol/Env
PBMC	17 (10–28)	37 (26–54)	1,033 (763–1,399)	26 (17–39)	862 (639–1,163)
$CD8^+$	18 (6–21)	66 (49–89)	2,101 (1,543–2,857)	13 (7–23)	1,905 (1,401–2,591)
$CD8^-$	3 (<1–9)	6 (2–14)	26 (17–40)	6 (2–14)	15 (9–26)

[a] Representative data obtained with a nonprogressor with a $CD4^+$ T-cell slope of +7 for >10 years of infection are shown.

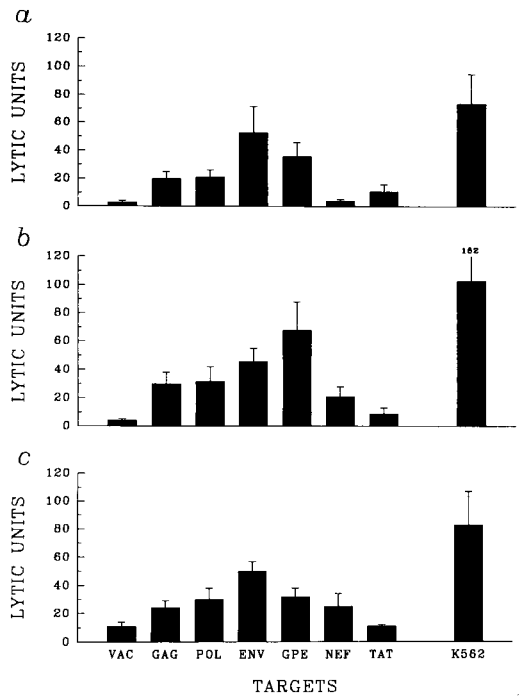

FIG. 3. CTLe activity is not associated with disease progression as shown for seven nonprogressors (a), eight intermediate progressors (b), and nine advanced progressors (c). VAC, VV control; GPE, VV Gag/Pol/Env triplex. Anti-HIV-1 CTLe activity was not detected in PBMC of HIV-1-seronegative, healthy subjects (data not shown). Data are means plus standard errors. NK cell reactivity against K562 cells is shown for comparison.

populations (data not shown). This suggests that cytotoxic reactivity against Env may be mediated in part by $CD8^-$ natural killer (NK) effectors armed with gp160-specific antibodies (21, 33, 41).

The results show that long-term nonprogressors with HIV-1 infection have a very low viral burden in the blood that is associated with the presence of high levels of anti-HIV-1-specific CTLm. Anti-HIV-1 CTLm therefore appear to be important determinants of host control of virus replication in initial, primary HIV-1 infection, as shown by other investigators (1, 18), and in chronically infected nonprogressors, as shown in this study.

We next assessed circulating effector CTL (CTLe) capable of directly lysing HIV-1 antigen-expressing targets without requiring in vitro prestimulation (40) because of the reported association of decreased anti-HIV-1 CTLe responses with small numbers of $CD4^+$ T cells in HIV-1-infected subjects (15, 27). Fresh PBMC from the blood used in the CTLm and virus studies were assayed for direct anti-HIV-1 cytotoxicity by previously described methods (38). Data were expressed as lytic units (LU) per 10^7 effector cells, with one LU defined as the number of effector cells required for 10% lysis of 10^4 target cells (30). The number of LU provides the most accurate measure of lysis for the entire range of effector-to-target-cell ratios (42). Concurrent assay of blood from HIV-1-seropositive subjects that was split into duplicate samples showed that there was $<10\%$ variation in CTLe responses to the HIV-1 proteins and in NK cell reactivity against K562 cells.

CTLe responses specific for the three structural HIV-1 proteins, the Gag/Pol/Env triplex, and the two regulatory HIV-1 proteins were detected at comparable levels in the three cohorts (Fig. 3) (P = not significant; Kruskal-Wallis test, chi-square approximation). Levels of NK cell lysis of K562 cells were also similar in the three groups. Cell enrichment studies showed that $CD8^+$ cells accounted for approximately 90% of the Gag- and Pol-specific CTLe responses and 25% of the Env-specific CTLe lytic activity. This supports previous reports that a major portion of the Env-specific CTLe response is mediated by antibody-armed $CD8^-$ NK cells (21, 33, 41).

The lack of association of circulating anti-HIV-1 CTLe with disease progression in our study concurs with previous results from our laboratory (32) and others (3, 12). The sensitivity of our CTLe assay was equal to those of other studies (3, 12, 15, 17) and was therefore not the basis for this lack of association. Thus, we conclude that direct lysis mediated by peripheral blood CTLe is not a significant correlate of disease progression in HIV-1 infection. The anti-HIV-1 CTLe activity still evident in the blood of advanced progressors likely evolves from residual memory or naive T cells and is of questionable significance as a measure of host immunity.

Our results suggest that a reservoir of anti-HIV-1 $CD8^+$ CTLm persists in the circulation and presumably in the lymphoreticular tissues of long-term nonprogressors and that it is important in limiting HIV-1 replication and disease. It is likely that this reservoir of CTLm evolves from a portion of activated anti-HIV-1 CTL that survive after the initial HIV-1 infection is controlled. Because CTLm are noncytolytic (36), they must periodically proliferate and develop into CTL with anti-HIV-1 lytic properties in response to HIV-1 antigens on antigen-presenting cells.

Evolution of HIV-1 antigenic variants that are no longer sensitive to CTL (7, 29) or replicative variants that are inherently more virulent (31, 37) or insensitive to host antiviral cytokines (8) and enhanced HIV-1 replication regulated by other, proinflammatory and immunoregulatory cytokines (39) are among the factors that could lead to the high levels of HIV-1 noted in progressors. We hypothesize that this may result in selective elimination of HIV-1-specific $CD8^+$ CTLm by apoptosis that is mediated by excess HIV-1 antigen in the antigen-presenting cells of lymphoreticular tissues. Similar mechanisms of clonal exhaustion due to high viral burden have been proposed for loss of anti-lymphocytic choriomeningitis virus CTLm activity (25, 35). This hypothesis would predict that the depletion of such CTLm activity is HIV-1 specific. This prediction is supported by the finding that CTLm specific for Epstein-Barr virus are still detectable when anti-HIV-1 CTLm are no longer evident in persons with late-stage HIV-1 infection (3). Our study of long-term nonprogressors and progressors therefore suggests that the efficacy of treatment for HIV-1 infection will depend on the maintenance of high anti-HIV-1 CTLm activity and a low viral burden.

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