Increased Viral Burden and Cytopathicity Correlate Temporally with CD4+ T-Lymphocyte Decline and Clinical Progression in Human Immunodeficiency Virus Type 1-Infected Individuals

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The rate of clinical progression is variable among individuals infected with human immunodeficiency virus type 1 (HIV-1). Changes in viral burden which correlate with disease status have been demonstrated in cross-sectional studies; however, a detailed longitudinal study of the temporal relationship between viral burden, CD4+ T-cell numbers, and clinical status throughout the course of infection has not been reported. Multiple longitudinal blood samples were obtained from four HIV-1-infected individuals with clinically divergent profiles. Levels of HIV-1 were measured in sequential samples of peripheral blood mononuclear cells, using both end-point dilution cultures and quantitative polymerase chain reaction methods. Serial HIV-1 isolates from each case were also evaluated to determine their biological properties in vitro. For the three patients with clinical progression, a dramatic increase in the level of HIV-1 was observed concurrent with or prior to a marked drop in CD4+ T lymphocytes. This increase in viral burden was temporally associated with the emergence of a more cytopathic viral phenotype. In contrast, consistently low levels of HIV-1 were observed in the one patient who was clinically and immunologically stable for more than a decade. Moreover, viral isolates from this patient were less cytopathic in vitro compared with HIV-1 isolates from those patients with disease progression. The temporal association between increased viral burden and CD4+ T-cell decline suggests a direct role for HIV-1 in the cytopathology of CD4+ T cells in vivo. Our results indicate that the pathogenic mechanisms responsible for CD4+ T-cell depletion may be related to both quantitative and qualitative changes in HIV-1.

Following primary infection with human immunodeficiency virus type 1 (HIV-1), the majority of patients enter an asymptomatic period of variable duration, characterized by low levels of viral replication and relatively few clinical manifestations. In most patients, this period of clinical stability may extend for many years, although CD4+ T-cell numbers usually show a continuous gradual decline. In others, CD4+ T-cell counts decline rapidly, resulting in the onset of AIDS. To date, the underlying pathogenic mechanisms which govern the persistence of infection in vivo and ultimately the transition from low-level to fulminant infection are largely unknown.

We have previously demonstrated that infectious titers of HIV-1 in peripheral blood mononuclear cells (PBMC) and plasma are substantially higher in symptomatic patients than in asymptomatic individuals (11). Increased levels of infectious HIV-1 or proviral DNA have also been shown by several groups in cross-sectional studies to correlate with disease status (4, 16, 22). On the basis of limited longitudinal blood samples, Schnittman et al. (19) demonstrated an increase in viral burden in CD4+ T cells from patients with disease progression. Cumulatively, these findings suggest that increasing viral burden is associated with clinical and immunological deterioration. In other studies, progression to AIDS has been associated with the emergence of a more cytopathic viral phenotype (28-30). It is not yet clear whether changes in viral phenotype contribute to increasing

the overall viral burden or, alternatively, whether the immunological deficit that accompanies disease progression allows for selection of HIV-1 variants with increased replicative and/or cytopathic properties.

Insight into the possible pathogenic mechanisms underlying disease progression may be gained by examining the temporal relationship between changes in viral burden and phenotype, CD4+ T-cell numbers, and clinical status throughout the course of infection. To address this question, we have systematically quantified HIV-1 in sequential blood samples from four patients, three of whom exhibited a rapidly deteriorating clinical course and precipitous CD4+ T-cell decline and one of whom has remained clinically and immunologically stable 12 years after HIV-1 infection. In addition, we have evaluated the biological properties of sequential HIV-1 isolates from each of these cases to determine whether changes in viral burden and CD4+ T-cell counts are temporally associated with the emergence of a distinct viral phenotype.

MATERIALS AND METHODS

Study subjects were chosen from a cohort of 850 homosexual men monitored by the New York Blood Center since 1984 in a prospective study of AIDS (25, 26). Many of these individuals were originally enrolled in the cohort in the late 1970s as part of a hepatitis B virus vaccine study (27). About half of the individuals were HIV-1 seropositive when they entered the study in 1984, and one-fifth of these subsequently developed AIDS. Forty-six men seroconverted during the course of the study, and 13 of these have since progressed to AIDS. Other patients were retrospectively

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FIG. 1. Quantitation of HIV-1 in sequential PBMC samples from four HIV-1-infected individuals. Data are expressed as TCID₅₀ per 10⁶ CD4⁺ cells (■), HIV-1 DNA copies per 10⁶ CD4⁺ cells (▲), and CD4⁺ cell counts (●). (A to C) Results for patients A to C, who exhibited clinical progression; (D) results for clinically stable patient D. N, NSI phenotype; S, SI phenotype. ○, first positive HIV-1 antibody test; ↓, AZT treatment initiated.

documented to have seroconverted in the late 1970s, some of whom have remained clinically and immunologically stable.

Throughout the course of the study, serum, plasma and PBMC were collected from individuals at 4-month intervals and stored in liquid nitrogen. In addition, detailed clinical, laboratory, and immunological information was recorded at each visit. Four patients were selected for further study on the basis of their extreme clinical and immunological profiles. Three patients had precipitously declining CD4⁺ T-cell counts and rapid progression to AIDS. Although the clinical course of these patients is not typical of that found in most HIV-1-infected persons, the patients were selected to facilitate detection of possible temporal changes in viral burden and/or phenotype in relation to a marked CD4⁺ T-cell decline.

Patient A became HIV-1 seropositive in March 1986. Following seroconversion, he was clinically asymptomatic for approximately 2 years, and his CD4⁺ T-cell numbers remained stable within the normal range. However, over a 7-month period in 1988, his CD4⁺ T-cell counts dropped precipitously from 1,100/mm³ to less than 200/mm³ (Fig. 1A). He was clinically asymptomatic during this period. Subsequently, he developed fatigue and oral thrush and was started on zidovudine (AZT) therapy in January 1989. He was diagnosed with pneumocystis pneumonia in August 1990 and died in February 1991.

Patient B first tested positive for HIV-1 antibodies in April 1985 approximately 1 year after entry into the study. He remained clinically well and had stable CD4⁺ T-cell counts of 400 to 700/mm³ for over 3 years following seroconversion. In a 5-month period between June and November 1988, his CD4⁺ T-cell counts dropped dramatically from approxi-

mately 600/mm³ to less than 200/mm³ (Fig. 1B). He was hospitalized for pneumocystis pneumonia in May 1989, and AZT treatment was initiated 3 months later. He was hospitalized again in 1990 with recurrence of pneumocystis pneumonia and died in November 1990.

Patient C was known through retrospective testing of stored sera to have become seropositive for HIV-1 between October 1979 and February 1980. He remained clinically well, with normal CD4⁺ T-cell counts, until February 1986. Within 5 months, his CD4⁺ T-cell counts dropped from 1,050/mm³ to 485/mm³ and in the subsequent 10 months to less than 200/mm³ (Fig. 1C). This immunological decline was temporally associated with the onset of unexplained fevers. He developed pneumocystis pneumonia in September 1987, and AZT treatment was initiated 1 month later. The patient remains alive; however, his CD4⁺ T-cell count has declined further, and he has developed Kaposi's sarcoma along with a recurrence of pneumocystis infection.

Patient D became seropositive for HIV-1 between February and May 1980. In the 12 years since his first recorded positive HIV-1 antibody test, he has remained clinically well with the exception of recurrent genital herpes simplex infection. In addition, despite the long-term HIV-1 infection, his CD4⁺ T-cell counts have remained remarkably stable within the normal range (Fig. 1D). He has received no antiviral therapy for HIV-1.

Quantitative cultures of HIV-1. Aliquots of PBMC frozen in 10% dimethyl sulfoxide were thawed, and a portion of the cells were used in end-point dilution cultures (11) to determine the infectious titer of HIV-1. PBMC from each sample were serially diluted fourfold (in replicates of 4 to 10) and cocultured with 2×10^6 phytohemagglutinin-stimulated nor-

mal donor PBMC in 1.5 ml of growth medium consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 U/ml), streptomycin (100 µg/ml), and interleukin-2 (IL-2; 10 U/ml). The cultures were incubated for 24 h at 37°C and then washed three times with fresh medium. Samples of culture supernatants were tested for the presence of HIV-1 p24 antigen on days 7, 14, and 21, using a commercial enzyme-linked immunoabsorbent assay (Abbott Laboratories, North Chicago, Ill.). A culture was considered positive if the concentration of p24 was above a cutoff value of 30 pg/ml. The 50% tissue culture infective dose (TCID₅₀) was then calculated by the method of Reed and Muench (5). Titers of infectious HIV-1 in PBMC are expressed as TCID₅₀ per 10⁶ CD4⁺ T cells, using previous flow cytometric determinations of CD4⁺ T-cell numbers. This calculation is based on the assumption that all of the virus detected is from CD4⁺ T lymphocytes (20).

Quantitative PCR. DNA was extracted by standard methods (18) from the remaining PBMC in each aliquot and used to determine HIV-1 DNA copy number. Full-length HIV-1 sequences were amplified by polymerase chain reaction (PCR), using primers to the long terminal repeat-*gag* region of the HIV-1 genome (M-667-GGCTAACTAGGGAACC CACTG and M-661-CCTGCGTCGAGAGAGCTCCTCT GG). HIV-1 DNA copy number was then quantified by the method of Pang et al. (14). Briefly, 1 µg of DNA from each sample was subjected to 32 cycles of PCR, using ³²P-end-labeled oligonucleotide primers which allowed direct visualization of PCR products following separation on 8% polyacrylamide gels. Counts per minute for designated bands were measured with a Betascope 603 blot analyzer (Betagen Corp., Waltham, Mass.). The HIV-1 DNA copy number for each sample was determined by comparison with a standard curve, using DNA from 8E5 cells, which contain one integrated copy of HIV-1 per cell (8). Parallel reactions were also run with primers PC03 and PC04 (human β-globin gene) (17) as a control to ensure that equivalent amounts of DNA were analyzed in each sample. Data are expressed as HIV-1 DNA copies per 10⁶ CD4⁺ T-cells.

Viral phenotypic analyses. HIV-1 isolates obtained from the initial quantitative cultures containing 10⁶ patient cells per well were propagated by a single short-term (5 to 7 days) passage and titered in PBMC by an end-point dilution method (5). Isolates were then used to evaluate infectivity in primary macrophage and PBMC cultures and in selected T-cell lines (H9, HPB-ALL, and MT-2). Macrophages were initially separated from PBMC by plastic adherence. The adherent cells were then cultured in the absence of IL-2 or additional growth factors for 10 to 14 days, yielding a population that was >95% pure as judged by cell morphology. Macrophages, PBMC, and T cells (10⁶) were infected with 10⁴ TCID₅₀ of each HIV-1 isolate in 1.5 ml of RPMI 1640 medium supplemented with antibiotics and 10% fetal calf serum. Cultures were incubated for 24 h at 37°C, washed twice with fresh medium, and cultured for 7 days. Fifty percent medium changes were performed twice during this period, and samples of culture supernatants were collected on day 7 and assayed for the presence of HIV-1 p24. In addition, infected MT-2 cell cultures were monitored on day 7 by light microscopy for the presence of syncytia.

Selected isolates were also evaluated for their growth kinetics and cytopathic properties. Normal donor PBMC were enriched for CD4⁺ T lymphocytes by first depleting macrophages via plastic adherence and then removing CD8⁺ cells by immunomagnetic separation (Dynal, Inc., Great Neck, N.Y.). The cells were subsequently stimulated

for 48 h with phytohemagglutinin and then switched to growth medium containing IL-2 before inoculation with 10⁴ TCID₅₀ of each viral isolate. After 24 h, the cells were washed three times and resuspended in a final volume of 1.5 ml of growth medium. Samples of culture supernatants were assayed for HIV-1 p24 antigen on designated days, and cell viability was determined on the same days by trypan blue dye exclusion. Data are expressed as the mean value ± standard deviation of duplicate samples.

RESULTS

The results of quantitative studies on sequential PBMC samples are summarized in Fig. 1. Independent determinations of viral burden by quantitative culture and PCR methods demonstrated increasing levels of HIV-1 in those patients with clinical progression. Moreover, temporal changes in the levels of HIV-1 appeared to correlate inversely with CD4⁺ T-cell counts during the course of infection. The levels of HIV-1 in serial PBMC samples from patient A (Fig. 1A) showed a marked increase (up to 260-fold), which was concurrent with a sharp decline in CD4⁺ T-cell counts. A similar inverse relationship between viral load and CD4⁺ T-cell numbers was observed for patients B and C (Fig. 1B and C). In these two cases, however, the rise in HIV-1 levels generally preceded the marked decline in CD4⁺ T cells.

Infectious titers of HIV-1 in PBMC samples taken early in infection from patients A to C ranged from <1 to 300 TCID₅₀ per 10⁶ CD4⁺ T cells (Fig. 1), consistent with our previous findings in asymptomatic patients (11). Subsequently, in the period leading up to and including CD4⁺ T-cell decline, infectious titers rose substantially, reaching peak levels of 3,000 to 10,000 TCID₅₀ per 10⁶ CD4⁺ T cells. Parallel results were obtained by quantitative PCR methods. Over the same time period, the number of HIV-1 DNA copies increased dramatically in all three patients, reaching peak levels between 6,000 and 23,000 copies per 10⁶ CD4⁺ T cells (Fig. 1A to C).

In contrast, culture and PCR quantitation of HIV-1 in sequential PBMC samples from patient D revealed persistent, low levels of virus (Fig. 1D). Unlike patients A to C, this patient has remained clinically well for more than 12 years after seroconversion. During this period, CD4⁺ T-cell numbers fluctuated between 600 to 800/mm³. Levels of HIV-1 measured in PBMC from patient D were approximately 200 TCID₅₀ per 10⁶ CD4⁺ T cells and 875 DNA copies per 10⁶ CD4⁺ T cells, reflecting the mean value of eight sequential samples taken over an 8-year period (Fig. 1D).

To determine whether changes in viral burden were associated with the emergence of a distinct HIV-1 phenotype, sequential isolates from all four patients were evaluated to assess their biological properties *in vitro*. Primary cultures of normal donor PBMC, macrophages, and selected T-cell lines were inoculated with 10⁴ TCID₅₀ of each isolate, and culture supernatants were later monitored for the presence of HIV-1 p24. Seven days after virus inoculation, all PBMC and macrophage cultures were positive for p24 expression for all isolates tested (Table 1). Conversely, none of the isolates was able to establish productive infection in either H9 or HPB-ALL cells over the same time period.

Distinct differences were seen, however, in the ability of sequential isolates to infect MT-2 cells, a human T-cell leukemia virus type I-transformed T-cell line (10). Early HIV-1 isolates derived from patient A were unable to establish productive infection in MT-2 cell cultures, whereas

TABLE 1. Phenotype analysis of sequential HIV-1 isolates from patients

Patient	Date	HIV-1 replicative capacity ^a					Syncytium formation in MT-2 cells
		Normal primary cells		T-cell lines			
		PBMC	Macrophages	H9	HPB-ALL	MT-2	
A	3/86	++	+	—	—	—	—
	6/87	++	++	—	—	—	—
	4/88	++	++	—	—	++	+
	7/88	++	++	—	—	++	+
	11/88	++	++	—	—	++	+
	8/89	++	++	—	—	++	+
	5/90	++	++	—	—	++	+
B	5/85	++	++	—	—	—	—
	11/86	++	++	—	+/-	—	—
	6/88	++	++	—	+/-	++	+
	11/88	++	++	—	+/-	++	+
C	7/90	++	++	—	+/-	++	+
	5/84	++	++	—	—	—	—
	1/85	++	++	—	—	—	—
	2/86	++	++	—	—	++	+
D	7/86	++	++	—	—	++	+
	12/86	++	++	—	—	++	+
	5/87	++	++	—	—	++	+
	6/88	++	++	—	—	++	+
	11/89	++	++	—	—	++	+
	5/84	++	+	—	—	—	—
	6/85	++	++	—	—	—	—
	3/86	++	++	—	—	—	—
	6/87	++	++	—	—	—	—
	9/88	++	++	—	—	—	—
	9/90	++	++	—	—	—	—
	3/91	++	++	—	—	—	—
	5/91	++	++	—	—	—	—

^a Replication of HIV-1 isolates was determined by measuring HIV-1 p24 antigen in culture supernatants as outlined in Materials and Methods. -, <30 pg/ml; +/-, 30 to 300 pg/ml; +, 300 to 1,000 pg/ml; ++, >1,000 pg/ml.

later isolates were readily infectious in these cells (Table 1). Similar results showing this phenotypic switch were also seen with serial isolates from patients B and C (Table 1). In each case, a direct correlation was observed between infectivity and syncytium formation in the MT-2 cell cultures. Whereas earlier isolates from patients A to C were uniformly negative in inducing syncytia, later isolates established infection and induced syncytium formation in MT-2 cells. None of the isolates appeared to infect MT-2 cells in the absence of syncytium formation, suggesting that infectivity and syncytium formation may be related properties in these cells. As seen in Fig. 1, the switch from non-syncytium-inducing (NSI) to syncytium-inducing (SI) phenotype occurred prior to the onset of CD4+ T-cell decline in each of these three patients. In contrast, sequential isolates from patient D were uniformly unable to infect MT-2 cells or induce syncytium formation irrespective of the time of isolation (Table 1; Fig. 1).

Further experiments were done to evaluate the replicative and cytopathic capacity of isolates derived immediately before and after the NSI-to-SI phenotypic switch observed in MT-2 cells. For patient D, who had only NSI viruses, isolates were selected from two time points that were approximately 1 year apart. The results from these experiments are summarized in Fig. 2 and 3. For patients A to C, whose viruses exhibited an NSI-to-SI switch, the SI phenotype was in each case associated with higher levels of HIV-1

FIG. 2. Replication kinetics of selected HIV-1 isolates in CD4-enriched PBMC cultures. Paired HIV-1 isolates representing NSI (closed symbols) and SI (open symbols) phenotypes were evaluated for their replicative capacity in PBMC cultures enriched for CD4+ cells as outlined in Materials and Methods. Data are expressed as nanograms of p24 per milliliter. Growth kinetics for paired HIV-1 isolates from patients A to D are shown in panels A to D, respectively. (A) Isolates from November 1986 (●) and April 1988 (○); (B) isolates from June 1987 (●) and June 1988 (○); (C) isolates from January 1985 (●) and February 1986 (○); (D) isolates from March 1986 (●) and June 1987 (■). Data presented here and in 3 are representative of two independent experiments.

replication (Fig. 2A to C) and increased cytopathicity (Fig. 3A to C) in CD4-enriched PBMC cultures compared with the paired NSI isolate. In addition, SI isolates from patients A to C generally replicated to higher levels than did NSI isolates from patient D (Fig. 2D). Quantitation of cell viability by trypan blue dye exclusion demonstrated increased cell death in CD4-enriched PBMC cultures infected with SI isolates from patients A to C compared with cultures infected with paired NSI isolates (Fig. 3A to C). The predominant cytopathic effect was not related to syncytium formation, however, since relatively few multinucleated cells were observed. No difference in the level of cytopathicity was seen with paired NSI isolates from patient D (Fig. 3D).

DISCUSSION

In this study, we have quantified the levels of HIV-1 in serial samples from four HIV-1-infected individuals. Three of these patients had a precipitous drop in CD4+ T cells followed by rapid progression to AIDS. A significant increase was seen in the levels of HIV-1 concurrent with or prior to the CD4+ T-cell decline in these patients (Fig. 1). This is the first demonstration of a direct association between viral burden and CD4+ T-cell numbers in a detailed longitudinal study. Our findings suggest that a burst of viral replication may occur prior to or at the onset of CD4+ T-cell decline. The cause of this increase in viral burden is not known but may indirectly reflect qualitative changes in the

FIG. 3. Evaluation of single-cell killing by selected HIV-1 isolates. Cultures of CD4-enriched normal donor PBMC were inoculated with selected HIV-1 isolates as outlined in Materials and Methods. Cell viability was determined on designated days by trypan blue dye exclusion. Data are expressed as the percentage of viable cells, calculated from the number of viable cells divided by the total number of cells counted. Symbols are as for Fig. 2. Values for cell viability in duplicate uninfected control wells were 73.7% $\pm$ 2.3% (day 3), 68.0% $\pm$ 3.3% (day 5), and 50.8% $\pm$ 0.3% (day 7).

phenotypic properties of the virus. Consistent with this possibility, our results indicate that increasing levels of HIV-1 are associated with the emergence of a more virulent viral phenotype in vitro (Table 1; Fig. 2 and 3). Taken together, these observations support a central role for HIV-1 in CD4+ T-cell depletion in vivo.

In vitro correlates of HIV-1 pathogenicity have been associated in previous studies with clinical status (1, 2, 7, 28, 30). Isolates from patients with late-stage disease frequently acquire an expanded host cell range and increased cytopathicity in vitro when compared with isolates from asymptomatic individuals (1, 7, 21, 29). In sequential determinations of host cell range in vitro, we found no difference in the ability of early and late isolates from four HIV-1-infected patients to establish productive infection in primary cultures of normal donor PBMC or macrophages. In each case, high levels of supernatant HIV-1 p24 antigen were detected within 1 week of inoculation, indicating efficient viral entry and replication in these cells.

Conversely, none of the isolates tested was able to infect H9 or HPB-ALL cells within the same time period. When a more permissive T-cell line (MT-2) was used, however, clear distinctions were seen in the ability of sequential isolates to establish productive infection (Table 1). In three of the patients, infectivity in MT-2 cells in vitro was directly associated with the rapid onset of CD4+ T-cell decline. One possibility suggested by these findings is that variants of HIV-1 that are more efficient at mediating entry into susceptible cells may emerge during the course of infection, thus giving rise in part to an increase in the overall viral burden.

The temporal association between increased viral burden and CD4+ T-cell decline suggests a direct role for HIV-1 in mediating cytopathology of CD4+ T-cells in vivo. The cytopathic effects of HIV-1 infection in T cells in vitro

include syncytium formation (12, 15, 23) and single-cell killing (3, 23, 24), which is a typical feature of many primary patient isolates. In the present study, we observed a marked transition to an SI phenotype in sequential HIV-1 isolates from patients with clinical progression and CD4+ T-cell depletion, consistent with an earlier report by Tersmette et al. (29). Interestingly, SI isolates from patients with clinical progression also had increased levels of single-cell killing and enhanced viral replication in CD4-enriched PBMC cultures compared with paired NSI isolates. It is possible that this shift in pathogenicity may also reflect an increase in the replicative and cytopathic properties of the virus in vivo.

These results are further supported by the finding that similar changes in viral replication and cytopathicity did not occur with HIV-1 isolates from the one patient who has remained clinically stable. This patient has been HIV-1 seropositive for at least 12 years and has consistently maintained a low viral burden and normal CD4+ T-cell counts. Continued studies on similarly stable patients will be important for identifying specific viral and/or host factors associated with long-term survival.

Previous studies have suggested that increased levels of HIV-1 may be triggered by exogenous factors, including coinfection with other pathogens, immunizations, or transfusions (6, 9). No clinical events were identified in the patients studied here to suggest that such exogenous factors played a role in stimulating HIV-1 replication during the critical period. However, this possibility cannot be formally ruled out.

Increases in viral burden over time may also reflect the emergence of HIV-1 variants that escape immune surveillance (13). The high mutation rate of certain HIV-1 genes, coupled with immune selective pressures, may give rise to HIV-1 genotypic variants with altered antigenicity at viral determinants important for recognition by neutralizing antibodies or cytotoxic T lymphocytes. Longitudinal studies are now in progress in our laboratory to determine whether escape mutants emerge during the course of infection and whether they are associated with higher levels of HIV-1 and subsequent disease progression.

Overall, our findings suggest that the underlying pathogenic mechanisms responsible for CD4+ T-cell decline are related to both quantitative and qualitative changes in HIV-1. Increased viral load coupled with increased virulence may have a multiplicative or synergistic effect, resulting in the rapid and profound depletion of CD4+ T lymphocytes observed in three of our cases. Disruption of immune function at the time of CD4+ T-cell decline may further contribute to the establishment of a high viral burden by allowing for selection of HIV-1 variants with increased replicative and cytopathic properties. While most HIV-1-infected individuals have a more gradual decrease in their CD4+ T-cell counts, it is possible that the same pathogenic mechanisms will apply.

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