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Blocked early-stage latency in the peripheral blood cells of certain individuals infected with human immunodeficiency virus type 1

THIKKAVARAPU SESHAMMA*, OMAR BAGASRA*, DIDIER TRONO†, DAVID BALTIMORE‡, AND ROGER J. POMERANTZ*§

*The Dorrance H. Hamilton Laboratories, Division of Infectious Diseases, Department of Medicine, Jefferson Medical College, Thomas Jefferson University, 329 Jefferson Alumni Hall, 1020 Locust Street, Philadelphia, PA 19107; †The Salk Institute, Infectious Disease Laboratory, 10010 N. Torrey Pines Road, La Jolla, CA 92037-1099; and ‡Rockefeller University, 1230 York Avenue, New York, NY 10021-6399

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ABSTRACT **Human immunodeficiency virus type 1 (HIV-1) infections of humans have a natural history characterized by a variable but usually slow progression to an immunodeficient state. We have described a molecular model of HIV-1 proviral latency in certain cell lines, characterized by extremely low or undetectable levels of unspliced genomic HIV-1-specific RNA but significant levels of multiply spliced HIV-1-specific RNA. We have utilized a quantitative reverse transcriptase-initiated polymerase chain reaction to measure the levels of various HIV-1 RNA species in peripheral blood mononuclear cells. The median level of multiply spliced HIV-1 RNA was dramatically higher than the median level of unspliced viral RNA in asymptomatic individuals. In addition, HIV-1 RNA patterns characterized by at least a 10-fold excess of multiply spliced to unspliced viral RNA were significantly more common in asymptomatic individuals than in patients with the acquired immunodeficiency syndrome. We suggest that asymptomatic clinical HIV-1 infection is characterized by a preponderance of HIV-1-infected peripheral blood cells blocked at an early stage of HIV-1 infection. This viral expression pattern, which we have called blocked early-stage latency, may constitute a reservoir of latently infected cells in certain HIV-1-infected persons.**

Infection of people by human immunodeficiency virus type 1 (HIV-1) is usually marked by three general stages of disease progression (1–6). After initial infection, the individual undergoes a state of rapid viral replication and seroconversion to a variety of HIV-1 antigens (1–6). Within a few weeks or months, this initial viremia subsides, leading to the second phase of clinical infection characterized by a variable, but usually prolonged, period of relatively quiescent infection, with low viremia, but a slow depletion of CD4-positive T lymphocytes. During this virtually latent clinical stage of HIV-1 infection, the replicative state of the virus is unknown. As the host's immune system gradually deteriorates, this clinically latent state of infection evolves into a third stage of increasing viral replication, with increasing numbers of peripheral blood cells harboring the HIV-1 genome and extensive CD4-positive lymphocyte depletion (4–6).

The U1 monocytoid and the ACH-2 T-lymphocytic cell lines harbor HIV-1 in a latent form, in that the cells, although infected, constitutively produce very small quantities of HIV-1 (7, 8). Virus production can be induced in these cells by treatment with a variety of agents (7–10). The HIV-1-specific RNAs may be separated into three classes, according to their sizes. The multiply spliced (2 kilobases) species encode the regulatory proteins Tat, Rev, and Nef. The singly spliced (4.3 kilobases) species encode at least the envelope proteins, and the unspliced (9.2 kilobases) HIV-1 RNA species function as both the viral genomic RNA and as mRNA for the Gag–Pol and Gag proteins (11–13). We have shown (11), and it has been confirmed (12), that latently infected cell lines, in the unstimulated state, express viral RNA that consists of singly and multiply spliced HIV-1 mRNA species with little or no unspliced HIV-1 RNA. Stimulation causes a dramatic upregulation in the rate of viral RNA transcription and a subsequent switch to predominant synthesis of the unspliced form of HIV-1 RNA (11, 12). This switch from predominantly spliced HIV-1 RNAs to increasing levels of unspliced HIV-1 RNA also occurs during an acute productive infection of T lymphocytes and monocyte/macrophages and has been described as a switch from the early to the late stage of retroviral infection (13). Thus, the unstimulated latently infected lines behave like early-stage infected cells but are blocked from progression to the late stage unless externally stimulated. We have proposed a molecular model of proviral latency in these cells lines, in which the low levels of viral production are a consequence of their inability to switch to the late stage of infection (11). This blocked early-stage latency may be due to low levels of transcription and, thus, a low level of the viral-encoded protein Rev, which is required for the accumulation of unspliced HIV-1 RNA (14).

Amounts of viral RNA per cell are difficult to measure because the number of infected cells and the distribution of viral RNA content in different cells must be known. The pattern of viral RNA is easier to ascertain and it provides average sampling of viral RNA but will be dominated by cells with a relatively high content of viral RNA. We have speculated that clinical HIV-1 latency during a human infection might involve a predominance of blocked early-stage cells that harbor a latent infection, like that of the latently infected cell lines (11). In this paper, we report a test of this possibility, utilizing a quantitative HIV-1 reverse transcriptase (RT)-initiated polymerase chain reaction (PCR) to separately determine the levels of unspliced and multiply spliced HIV-1 RNA species.

METHODS

Patients and RNA Samples. Heparinized blood specimens were obtained from 52 HIV-1-seropositive adults, all untreated for at least 1 month with anti-retroviral agents. Blood specimens were also drawn from 8 HIV-1-seronegative individuals. All specimens were evaluated in a blinded fashion. The HIV-1-infected individuals were classified by their clinical state of disease, using the modified CDC HIV classification scheme (15). Twenty individuals were classified as asymptomatic (stage 2), 13 had solely persistent generalized

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Abbreviations: HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; PBMC, peripheral blood mononuclear cell.
§To whom reprint requests should be addressed.

lymphadenopathy (stage 3), and 19 had the acquired immunodeficiency syndrome (AIDS) (stages 4 A–D). Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll/Hypaque gradient centrifugation. Total cellular RNA was harvested from these unstimulated PBMCs by using a one-step acid guanidinium method, as described (16).

Quantitative RT-PCR. A RT-PCR technique, as described (17), was modified to obtain precise quantitation of the copies of selected HIV-1 RNA species in each sample (18). Quantities of total cellular RNA were adjusted to be within the linear amplification ranges of the standard curves (see below). The 3′ primers were annealed and extended as described (17). A tube without RT was used as a negative control to test for DNA contamination in all experiments. The upstream primers were radioactively labeled using [γ-^{32}P]ATP. Thus, a hybridization step using a labeled probe was not necessary (17, 18). Samples were resolved on a nondenaturing 8% polyacrylamide gel. Specific bands were excised and radioactivity was measured using liquid scintillation.

A major modification of this protocol, which allowed precise quantitation of each HIV-1 RNA species in the samples, was the use of standard curves of *in vitro* transcribed RNAs (18). The concentrations of the *in vitro* transcribed standard RNAs were measured spectrophotometrically and the copy number of transcripts present in 1 μl of solution was calculated, using the molecular weight of the transcript and Avogadro's number. Serial 1:2 dilutions of the RNA standards were made. Precise standard curves could, therefore, be generated in parallel. In the linear amplification range of these curves, the copies of *in vitro*-transcribed RNAs were plotted against the cpm of radioactivity of the amplified fragments. By using these dilution curves of *in vitro*-transcribed RNA, the copy numbers of unspliced and multiply spliced HIV-1 RNA, per microgram of total cellular RNA in a sample, could be calculated by extrapolation to these standard curves. Primers selected for this PCR procedure were in extremely conserved regions of the HIV-1 genome to facilitate detection of the various strains of HIV-1 found in clinical specimens (18, 19). The primers for multiply spliced RNA were located in the first and second exons of *tat* and *rev* and the primers for unspliced RNA flanked the major splice donor site (18).

Statistical Analyses. The Wilcoxon signed rank test was utilized to compare differences in the median levels of viral RNA species within each stage of disease. This nonparametric test was used as the normality of the distributions could not be assumed. Fischer's exact test was used to compare differences in viral RNA expression patterns between groups of patients.

RESULTS

By utilizing serial dilution curves, productively HIV-1-infected H9 lymphocytic cells gave approximately equal amounts of multiply spliced and unspliced HIV-1 RNA (Fig. 1*A* and *B*), as expected for most productive infections (11). Samples were quantitated by extrapolation to a standard curve, by using *in vitro*-transcribed RNA from genomic HIV-1 DNA and multiply spliced HIV-1 cDNA fragments (Fig. 1 *C* and *D*). Three representative patient samples are presented in Fig. 1*E*. Lanes 1 and 2 show a productive pattern with approximately equal amounts of these two types of HIV-1 RNA. Lanes 3 and 4 show a 20-fold excess of multiply spliced HIV-1 RNA. Lanes 5 and 6 show an extreme case, with multiply spliced but no detectable unspliced HIV-1 RNA. Lanes 7 and 8 show that an uninfected individual's cells are negative in both assays. Lanes 9 and 10 show that uninduced U1 cells gave the expected pattern of analysis,

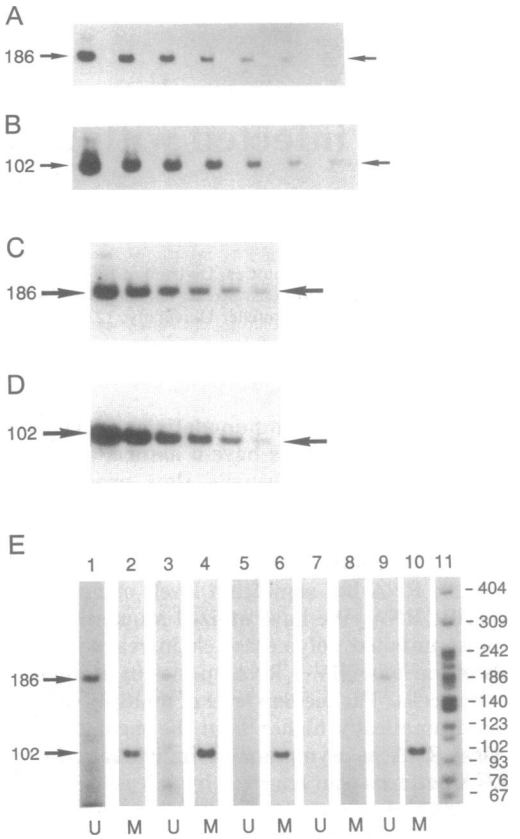

FIG. 1. Quantitation of unspliced and multiply spliced HIV-1-specific RNA species by a RT-PCR. (*A* and *B*) Autoradiographs of serial 1:2 dilutions of total cellular RNA isolated from productively HIV-1-infected H9 cells, after RT-PCR. (*A*) Unspliced HIV-1 RNA. (*B*) Multiply spliced HIV-1 RNA. (*C* and *D*) Autoradiographs of serial 1:2 dilutions of *in vitro* transcribed HIV-1 RNA standards, after RT-PCR. (*C*) Unspliced HIV-1 RNA. (*D*) Multiply spliced HIV-1 RNA. (*E*) Representative autoradiographs of total cellular RNA, harvested from PBMCs and subjected to RT-PCR (M, multiply spliced HIV-1 RNA; U, unspliced HIV-1 RNA). Lanes: 1 and 2, productive pattern (unspliced to multiply spliced ratio, 1:1); 3 and 4, blocked early-stage latent pattern (unspliced to multiply spliced ratio, 1:20); 5 and 6, blocked early-stage latent pattern, with no unspliced HIV-1 RNA detected; 7 and 8, HIV-1-seronegative control; 9 and 10, U1 cells (unstimulated); 11, DNA marker lane (pBR322, *Msp* I digest). Arrows point to specific amplified PCR products. Molecular sizes in base pairs are shown.

with significantly more multiply spliced than unspliced HIV-1 RNA (11).

For all 52 HIV-1-infected individuals, classified by the modified CDC HIV classification scheme (15), HIV-1 RNA was detectable in their PBMCs, by using the quantitative HIV-1 RT-PCR (Table 1), in agreement with a recent report (20). None of the HIV-1-seronegative controls were positive for any HIV-1 RNA species. Although the levels of HIV-1 RNAs detected varied extensively, the median levels of HIV-1-specific RNA species in certain disease stages were informative (Table 2). The most obvious trend was that the median ratio of multiply spliced to unspliced HIV-1 RNA in the stage 2 (asymptomatic) individuals was 85 and the median ratio in the stage 3 (persistent generalized lymphadenopathy) and stage 4 (AIDS) individuals was 3 to 5 (Table 2). The difference between the median levels of unspliced and multiply spliced HIV-1 RNA, in stage 2 individuals, was highly significant ($P < 0.001$, Wilcoxon signed rank test). The differences between median levels of unspliced and multiply spliced viral RNA were not significant in stage 3 or stage 4 patients. The relative excess of multiply spliced HIV-1 RNA

Table 1. Levels of HIV-1-specific RNA species in PBMCs from HIV-1-infected individuals by a quantitative RT-PCR

Stage	Patient	HIV-1-specific RNA: Multiply spliced	HIV-1-specific RNA: Unspliced	Total CD4-positive cells, no./mm³
2 (*n* = 20)	1	11	UD	408
	2	12	UD	349
	3	5	UD	992
	4	4	0.2	256
	5	10	5	ND
	6	22	UD	ND
	7	18	3	ND
	8	97	UD	ND
	9	100	UD	429
	10	85	UD	ND
	11	64	UD	ND
	12	62	UD	ND
	13	210	26	405
	14	23	UD	ND
	15	140	6	1050
	16	44	7	ND
	17	3	11	ND
	18	1	0.4	339
	19	238	2	612
	20	23	0.8	572
3 (*n* = 13)	21	1	UD	39
	22	3	1	5
	23	45	3	21
	24	9	UD	ND
	25	4	0.5	168
	26	23	0.6	ND
	27	45	25	84
	28	29	UD	584
	29	UD	1	28
	30	UD	1	ND
	31	0.4	1	ND
	32	UD	1.5	ND
	33	28	34	334
4 (*n* = 19)	34	2	3	164
	35	2	0.5	60
	36	6	0.9	ND
	37	10	UD	80
	38	160	20	6
	39	6	1	37
	40	169	18	ND
	41	640	280	32
	42	60	5	448
	43	130	15	3
	44	2	3	107
	45	3	0.8	104
	46	12	1	226
	47	42	7	13
	48	UD	30	144
	49	25	8	63
	50	UD	44	188
	51	112	114	21
	52	UD	1	209

HIV-1-specific RNA is expressed as copies ($\times 10^{-6}$) per μg of total cellular RNA. UD, undetectable; ND, not determined. The lower limit of detection sensitivity for this quantitative RT-PCR was 1×10^4 copies for both multiply spliced HIV-1 RNA and unspliced HIV-1 RNA. Two samples from each patient were tested, in duplicate, in at least two experiments. Standard deviations between experiments and samples, for both species of HIV-1 RNA in the PBMCs from each individual evaluated, were always <10%.

in the asymptomatic individuals is consistent with a high fraction of blocked early-stage latently infected cells in the peripheral blood of these individuals.

Table 2. Median levels of HIV-1-specific RNA species in PBMCs from HIV-1-infected individuals

Stage	HIV-1-specific RNA: Multiply spliced	HIV-1-specific RNA: Unspliced	Multiply spliced/ unspliced HIV-1 RNA
2 (*n* = 20)	23 (1–238)	0.15 (<0.1–26)	85/1
3 (*n* = 13)	9 (<0.1–45)	1 (<0.1–34)	3/1
4 (*n* = 19)	10 (<0.1–640)	7 (<0.1–280)	5/1

Median level of HIV-1-specific RNA is expressed as copies ($\times 10^{-6}$) per μg of total cellular RNA. The range is in parentheses. The individual ratios of multiply spliced to unspliced viral RNA levels, in each clinical stage, underwent logarithmic transformation prior to ranking the ratios for computation of the median ratio in each group.

A more informative way to view the data is to classify the various HIV-1 RNA patterns and examine the distribution of the classes as a function of the clinical stage of infection. In previous work, this group (11, 13) and others (12) have demonstrated that during acute productive HIV-1 infections, in cell cultures, the patterns of HIV-1 RNA expression are ones in which multiply spliced HIV-1 RNA levels and levels of unspliced genomic HIV-1 RNA do not dramatically differ. If we consider that any sample with a 10-fold excess of multiply spliced RNA can be classified as a state of "predominantly blocked early-stage latency," then 70% of the stage 2 individuals fit this definition (Table 3). By contrast, 38% of the stage 3 individuals and only 16% of the stage 4 patients display a pattern indicative of predominant latency. Thus, most of these latter patients' PBMCs fit the criterion of "significant productive cells" [Table 3: the difference between stage 2 and stage 4 samples was highly statistically significant ($P < 0.001$), by Fisher's exact test].

There were two extreme patterns of HIV-1 RNA, no detectable unspliced RNA or solely unspliced RNA. Of the stage 2 individuals, 50% had no unspliced HIV-1 RNA detected whereas 23% of the stage 3 and only 5% of the stage 4 showed this pattern [Table 3: the difference between stage 2 and stage 4 samples was highly significant ($P < 0.001$)]. This is presumably due to a vast excess of blocked early-stage latently infected cells in asymptomatic patients. The opposite pattern, only unspliced HIV-1 RNA, was found in no stage 2 individuals but in six stage 3 and stage 4 patients. It could be indicative of a vast excess of productively infected cells in these individuals, as multiply spliced HIV-1 RNA will gradually decrease in a productive infection in cell cultures, possibly secondary to feedback from the viral-encoded protein Rev (10). These data suggest that a viral RNA pattern consistent with blocked early-stage latency is more commonly demonstrated in asymptomatic HIV-1-infected patients, as compared to patients with AIDS.

DISCUSSION

This study was undertaken to determine whether a specific form of proviral latency, blocked early-stage latency, might be a common state of HIV-1 in cells of individuals who are in the asymptomatic phase of the infection. For the word "latency" to be relevant does not require that there be no viral expression, only that there be little virus production relative to a full-blown productive state. A variety of animal lentiviruses, to which HIV-1 is closely related, have been demonstrated to maintain a state of proviral latency for variable lengths of time (22).

Latency is a word often used in connection with HIV-1 infection (6); it implies that the viral genome is present but is not extensively expressed. This could refer to the entire infected individual, where some cells harbor the viral genome but little virus is expressed in the circulation, or it could be

Table 3. Patterns of HIV-1-specific RNA species

Stage	Individuals, no.			
	Productive pattern of HIV-1 RNA	Blocked early-stage latent pattern of HIV-1 RNA	No unspliced HIV-1 RNA detected	No multiply spliced HIV-1 RNA detected
2 (n = 20)	6 (30)	14 (70)	10 (50)	0 (0)
3 (n = 13)	8 (62)	5 (38)	3 (23)	3 (23)
4 (n = 19)	16 (84)	3 (16)	1 (5)	3 (16)

Blocked early-stage latent HIV-1 RNA pattern was defined as a 10-fold or higher level of multiply spliced HIV-1 RNA, as compared to unspliced HIV-1 RNA (see text). The number of individuals in each HIV classification stage expressing a particular HIV-1 RNA pattern, in their PBMCs, is presented in this table. The percentage of individuals in each stage expressing a particular HIV-1 RNA pattern, in their PBMCs, is shown in parentheses. Of note, those individuals expressing no detectable unspliced HIV-1 RNA and those expressing no detectable multiply spliced HIV-1 RNA are considered subgroups of the latent and productive patterns of HIV-1 RNA, respectively. Those samples in which no unspliced HIV-1 RNA was detected, using the original primers, also demonstrated the same HIV-1 RNA expression pattern using *gag* primers (21). These *gag* primers consistently detected unspliced HIV-1 RNA in those samples in which the original primers, for unspliced HIV-1 RNA, yielded positive results (data not shown). Thus, by this test, heterogeneity of the HIV-1 genome did not appear to account for those PBMC samples in which no unspliced HIV-1 RNA was detected.

in cells of the HIV-1-infected person, in which the viral genome is present but there is little virus production. Latency could come about through a variety of means. It could result if the viral genomes were incompletely or completely replicated and unintegrated but maintained until the infected cell received an activation signal, as has been suggested for infection of resting T lymphocytes (23, 24). While the RNA patterns we found in patient samples are indicative of blocked early-stage latency, they could have other interpretations. For instance, they could be due to defective viral genomes or defective cells that are unable to progress to later stages of infection. Thus, these viral RNA patterns may correlate with various strains of HIV-1 isolates (25).

Our data agree with reports in which HIV-1 RNA could be detected in the PBMCs of most individuals with HIV-1 infections (20, 21). It is evident that in the vast majority of HIV-1-seropositive individuals, regardless of disease stage, significant numbers of cells are transcribing HIV-1 RNA. Nevertheless, our studies demonstrate significant differences in the patterns of HIV-1-specific RNA, based on clinical stage of disease. In a study utilizing a nonquantitative RT-PCR technique, Gag-encoding HIV-1 RNA was less frequently detected in PBMCs of patients with high CD4-positive lymphocyte counts than in patients with low CD4-positive lymphocyte counts (21).

It is important to fit these observations into a general conception of the evolution of an HIV-1 infection in a newly infected individual. A newly HIV-1-infected individual experiences an initial productive infection, involving extensive viral proliferation over numerous weeks (1, 2). Thereafter, the immune system would seem to partially control the HIV-1 infection, reducing the circulating titer of virus and productively infected cells to very low levels (4, 5, 26). At this stage, a preponderance of CD4-positive lymphocytes harboring blocked early-stage latent infections would exist in the peripheral blood of asymptomatic HIV-1-infected individuals. The final stage of infection probably comes about when CD4-positive lymphocyte function has been so compromised that the humoral and cellular immune control of the infection fails, with an outgrowth of productively infected cells.

We derive from these data a picture of clinical HIV-1 infection characterized by at least two types of HIV-1-infected cells. One type of cell is productively infected and is rare in asymptomatic HIV-1-infected individuals and in patients with AIDS, and the second type of HIV-1-infected cell is blocked in the early stage of infection. The productively infected cells were detected using *in situ* hybridization and were determined to be in 1 of 10,000 to 1 of 100,000 total lymphocytes (27). Recent analyses have demonstrated that the number of cells harboring HIV-1 proviruses is vastly in excess of the number producing HIV-1 RNA detectable by the *in situ* hybridization technique (4, 21, 28). Many of these cells may be blocked early-stage latently infected cells. Importantly, preliminary data suggest that latently infected cells may be less common in lymphoid tissue than in PBMCs (29).

The predominance of blocked early-stage latently infected cells may have important clinical significance. The viral RNA expression pattern of blocked early-stage latency in peripheral blood cells will be important to further correlate with disease progression and, thus, may potentially be useful as a surrogate marker to follow during the course of HIV-1 infection in patients (30). Additional long-range follow-up studies of HIV-1 RNA expression patterns in HIV-1-infected patients are ongoing. Our data also suggest that HIV-1-infected individuals have a reservoir of cells whose infected state will be maintained even in the face of treatment with anti-RT compounds. Such drugs can only prevent new infections of cells; they cannot clear HIV-1 latently infected cells.

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