

- 10⁴ cells per well in round-bottomed, 96-well plates and incubated with effectors for 4 hours in triplicate. Supernatant (30 μ l) was removed from each well and counted in a Betaplate scintillation counter (LKB-Wallac, Turku, Finland). Maximal counts, released by addition of 6 M HCl, and spontaneous counts released without CTLs were determined for each target preparation. Percent specific lysis was calculated as 100 \times (experimental counts - spontaneous counts)/(maximal counts - spontaneous counts).
16. O. K. Röttschke *et al.*, *Nature* **348**, 252 (1990).
 17. L. A. Hawe *et al.*, unpublished observations.
 18. P. Vieira and K. Rajewsky, *Int. Immunol.* **2**, 487 (1990).
 19. J. J. Donnelly *et al.*, *J. Immunol.* **145**, 3071 (1990).
 20. Female BALB/c mice ≥ 10 weeks of age were injected intraperitoneally with 0.5 ml of pooled serum [diluted in 2.0 ml of phosphate-buffered saline (PBS)] from mice that had been injected three times with 200 μ g of NP DNA. Control mice were injected with an equal volume of pooled normal mouse serum or with pooled serum from mice that had recovered from infection with A/HK/68, also in 2.0 ml of PBS. The dose of A/HK/68 immune serum was adjusted such that the enzyme-linked immunosorbent assay (ELISA) titer of anti-NP antibody was equal to that in the serum pooled from NP DNA-injected mice. Mice were challenged unanesthetized in a blind fashion with 10⁴ median tissue culture infectious doses (TCID₅₀) of A/HK/68 2 hours after serum injection, and a further injection of an equal amount of serum was given 3 days later. Mice were killed 6 and 7 days after infection, and viral lung titers in TCID₅₀ per milliliter were determined as described (31).
 21. Viral challenges were performed with a mouse-adapted strain of A/HK/68 and maintained subsequently by *in vivo* passage in mice (I. Mbawuike, personal communication). The viral seed stock used was a homogenate of lungs from infected mice and had an infectivity titer of 5 $\times 10^8$ TCID₅₀/ml on Madin-Darby canine kidney (MDCK) cells (American Type Culture Collection, Rockville, MD). For viral lung titer determinations and mass loss studies, viral challenges were performed in blind fashion by intranasal instillation of 20 μ l containing 10⁴ TCID₅₀ on the nares of unanesthetized mice, which leads to progressive infection of the lungs with virus but is not lethal in BALB/c mice (23). In survival experiments, mice were challenged by instillation of 20 μ l containing 10^{2.5} TCID₅₀ on the nares under full anesthesia with ketamine and xylazine; infection of anesthetized mice with this dose causes a rapid lung infection that is lethal to 90 to 100% of nonimmunized mice [J. L. Schulman and E. D. Kilbourne, *J. Exp. Med.* **118**, 257 (1963); G. H. Scott and R. J. Sydskis, *Infect. Immunity* **14**, 696 (1976); (23)]. Viral lung titers were determined by tenfold serial titration on MDCK cells in 96-well plates as described (31). Data are given as the logarithm of inverse titers.
 22. J. J. Donnelly *et al.*, unpublished observations.
 23. R. A. Yetter *et al.*, *Infect. Immunity* **29**, 654 (1980).
 24. S. Parker *et al.*, unpublished observations.
 25. K. Tanaka *et al.*, *Annu. Rev. Immunol.* **6**, 359 (1988).
 26. Neutralizing (hemagglutination inhibiting) antibodies were elicited in mice, ferrets, and rhesus monkeys after injection of DNA encoding influenza A hemagglutinin (22). Antibodies to human growth hormone were generated after administration of DNA-coated gold microprojectiles into cells [D.-C. Tang *et al.*, *Nature* **356**, 152 (1992)].
 27. D. Gray and P. Matzinger, *J. Exp. Med.* **174**, 969 (1991); S. Oehen *et al.*, *ibid.* **176**, 1273 (1992).
 28. J. F. Young *et al.*, in *The Origin of Pandemic Influenza Viruses*, W. G. Lauer, Ed. (Elsevier Science, Amsterdam, 1983), pp. 129-138.
 29. B. S. Chapman *et al.*, *Nucleic Acids Res.* **19**, 3979 (1991).
 30. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P,

Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

31. T. M. Moran *et al.*, *J. Immunol.* **146**, 321 (1991).
32. PCR was performed as per instructions in the GeneAmp kit (Perkin-Elmer Cetus).
33. We thank J. Felgner (Vical) and D. DeFeo-Jones [Merck Research Laboratories (MRL)] for assistance and consultation on aspects of molecular biology, A. Williamson (MRL) and H. Zweerink (MRL) for evaluation of the manuscript, and T. Moran (Mount Sinai University School of Medicine New York) for advice on live virus challenges.

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High Levels of HIV-1 in Plasma During All Stages of Infection Determined by Competitive PCR

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Quantitative competitive polymerase chain reaction (QC-PCR) methods were used to quantify virion-associated human immunodeficiency virus type-1 (HIV-1) RNA in plasma from 66 patients with Centers for Disease Control stage I to IVC1 infection. HIV-1 RNA, ranging from 100 to nearly 22,000,000 copies per milliliter of plasma (corresponding to 50 to 11,000,000 virions per milliliter), was readily quantified in all subjects, was significantly associated with disease stage and CD4⁺ T cell counts, and decreased by as much as 235-fold with resolution of primary infection or institution of antiretroviral therapy. Plasma virus levels determined by QC-PCR correlated with, but exceeded by an average of 60,000-fold, virus titers measured by endpoint dilution culture. Quantitation of HIV-1 in plasma by QC-PCR may be useful in assessing the efficacy of antiretroviral agents, especially in early stage disease when conventional viral markers are often negative.

The natural history of HIV-1 infection is characterized by a variable clinical course, with the development of acquired immunodeficiency syndrome (AIDS) generally occurring after 7 to 11 years (1). A central paradox of HIV disease involves the progressive development of immunologic abnormalities, beginning during the early stages of infection when assays for circulating p24 antigen and culturable virus in peripheral blood suggest minimal or absent levels of viral replication (2-8). Recent studies of HIV-1 DNA and RNA in clinical samples suggest that, as compared with peripheral blood cells, lymphoid tissue represents a preferred and continuous site of viral replication, although such studies have necessarily been limited by the relative inaccessibility of the tissue compartment, especially for repeated evaluation (9).

Previous polymerase chain reaction (PCR) studies of HIV-1 RNA in plasma have generally been limited to qualitative (10) or semiquantitative analyses (11). In standard PCR methods, the absolute amount of product generated does not always bear a consistent relation to the

amount of target sequence present at initiation of the reaction, particularly for clinical specimens (12). Both the kinetics and efficiency of amplification of a target template are dependent on the starting abundance of that template and on the sequence match of the primers and target template and may also be affected by inhibitors present in the specimen (12). In PCR analysis of RNA samples, variable efficiencies in both the reverse transcription and amplification steps are potential sources of variability. For these reasons, comparison of the amount of specimen-derived PCR product to the amount of product from a separately amplified external control standard (11) does not provide a rigorous basis for absolute quantitation. Normalization based on coamplification of a heterologous "internal control" target sequence (such as β -globin or actin) does not optimally address this problem, owing to different template abundances and priming efficiencies for different primer-target combinations.

In the quantitative competitive PCR (QC-PCR) method for RNA quantitation (13) a competitive RNA template matched to the target sequence of interest, but differing from it by virtue of an introduced internal deletion, is used in a competitive titration of the reverse transcription and PCR steps, providing stringent internal control (13-15). Increasing known copy numbers of competitive template are added to replicate portions of the test specimen, and quantitation is based on determination of the relative, not absolute, amounts of the

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differently sized amplified products derived from the wild-type and competitive templates, after electrophoretic separation. To increase the sensitivity and consistency of amplification of only virion-associated RNA, we pelleted virus from plasma by ultracentrifugation, used procedures intended to maximize recovery of extracted RNA (13), and targeted a highly conserved sequence in HIV-1 *gag* (16), using oligonucleotide primers that incorporate neutral inosine residues at the few positions of recognized variability (13, 16, 17).

Sixty-six consecutively enrolled HIV-1-infected subjects representing all stages of infection [Centers for Disease Control (CDC) stages I to IV] (2) and ten HIV-1 seronegative healthy donors were evaluated for virion-associated HIV-1 RNA by QC-PCR. Infected subjects were also tested for culturable virus and for p24 antigen with both standard and immune complex dissociation (ICD) test procedures, as described (13, 17–19). For each subject, all virologic measurements were made with a single plasma sample, which was divided and frozen in replicate portions. Figure 1 illustrates QC-PCR results from a control reconstruction experiment, along with a representative experimental determination of plasma HIV-1 copy number for an infected patient. Virion-associated RNA was detected and quantified in plasma specimens from all 66 HIV-1-infected subjects (Table 1). Determined RNA copy numbers ranged from 1.00×10^2 to 2.18×10^7 HIV-1 RNA copies per milliliter of plasma (corresponding to 0.50×10^2 to 1.09×10^7 virions per milliliter). Positive signals for wild-type HIV-1 target sequences were not observed in specimens from any of ten uninfected control subjects or, when the reverse transcription step was omitted, for specimens from any of the HIV-1-infected patients.

The threshold sensitivity for RNA QC-PCR analysis was determined to be 100 copies per reaction (13, 20). Although positive signals could be detected for as few as 10 to 20 copies, results were less consistent below 100 copies per reaction, and quantitation was less reliable. QC-PCR analysis was typically performed with 0.5-ml plasma samples. However, pelleting the virus from increased volumes of plasma for specimens containing low numbers of virions did allow for increased overall sensitivity of detection. Thus, for one p24 antigen-negative, culture-negative CDC stage II patient (patient TIMI 0852, Table 1), initial QC-PCR analysis of a 0.5-ml specimen portion gave a negative result. However, analysis of virus pelleted from 2.8 ml of a replicate plasma sample allowed unequivocal detection of the low amount of virus present in this patient's plasma (100 copies per milliliter), whereas no HIV-1 RNA was

seen with analysis of comparable volumes of plasma from HIV-1 seronegative controls. In separate control studies, analysis of triplicate portions of plasma samples from six different patients, with mean HIV RNA copy numbers of 6.7×10^4 to 1.0×10^6 per milliliter, gave a mean SD of 22%. Analysis of replicate portions of the same HIV-1 RNA preparation on six different days gave an SD of 15%. This reproducibility contrasts with variability of up to 600 to 1000% reported for noncompetitive PCR procedures (11, 12, 14).

Whereas the QC-PCR method quantified virion-associated HIV-1 RNA in all 66 patients tested, virus culture and standard p24 antigen assays were much less sensitive, with positive results in 4/20 and 5/20 subjects with $CD4^+$ T cell counts >500 per cubic millimeter, 6/18 and 7/18 subjects with $CD4^+$ T cell counts of 200 to 500 per cubic millimeter, and in 22/28 and 24/28 subjects with $CD4^+$ cells fewer than 200 per cubic millimeter, respectively (Table 1). In 30 patients with negative results in standard p24 antigen assays, the use of acid treatment to dissociate p24 from immune complexes increased the frequency of detection by only four patients, although absolute measured amounts of p24 were increased in 25/36 patients with detectable levels of p24 before acid treatment.

QC-PCR-determined HIV-1 RNA levels in plasma differed between clinical stages, with levels for CDC stage II and III patients (asymptomatic or persistent lymphadenopathy) [mean, 78,200 copies per milliliter ($n = 22$)] significantly lower than for CDC stage IVC2 [AIDS-related complex (ARC)] [mean, 352,100 copies per milliliter ($n = 23$); $P \leq 0.05$] (21), and RNA levels for CDC stage IVC2 patients significantly lower than for CDC IVC1 (AIDS) patients [mean, 2,448,000 copies per milliliter ($n = 15$); $P \leq 0.05$] (21). CDC stage IVC1 (AIDS) patients had circulating virus levels comparable to those observed during the peak of viral replication in stage I (primary infection) patients [mean, 5,178,000 copies per milliliter ($n = 6$); difference not significant] (21), implying that late-stage disease is characterized by a nearly complete loss of immunological control of viral replication. There was also a significant correlation between increasing QC-PCR-determined HIV-1 RNA levels and decreasing absolute $CD4^+$ T cell counts [Spearman rank correlation coefficient $r = -0.765$, $P < 0.0001$] (22). A nonlinear regression analysis of these data yielded the equation $\log(\text{RNA}) = 4.43 + 1.77\exp[-0.0049(\text{number of } CD4^+ \text{ T cells})]$, with an r^2 value of 0.56 ($P < 0.0001$).

Figure 2 shows data for longitudinal specimens collected from three individuals

[of six studied (Table 1)], beginning at the time of presentation with acute HIV-1 infection (CDC stage I) and continuing through the establishment of chronic infection (CDC stage II to IVC2). Each patient presented with signs and symptoms of primary infection and with no detectable HIV-1-reactive antibodies as determined by enzyme-linked immunosorbent screen-

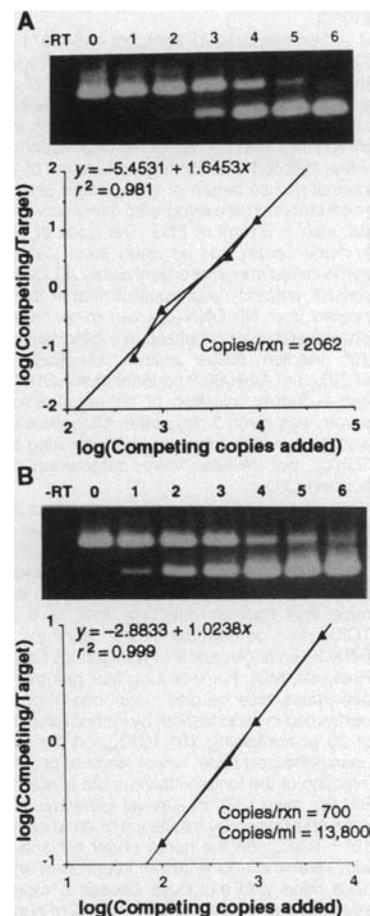


Fig. 1. QC-PCR quantitation of HIV-1 RNA. Video images are shown of electrophoretically resolved, ethidium bromide-stained PCR products derived from wild-type target sequence (upper band, 260 bp) and the competitive template (lower band, 180 bp), along with plots used to determine copy numbers of target sequence in the specimens (13, 17). (A) Reconstruction experiment with *in vitro* transcripts from plasmid (pQP1) containing full-length target sequence (260-bp product) and plasmid (pQP1Δ80) containing internally deleted competitive template (180-bp product). Actual copy number added, 2000 copies pQP1 per reaction. (B) Quantitation of virion-associated HIV-1 RNA from plasma of BECH 0171, CDC stage II (Table 1). Lanes are as follows: -RT, PCR amplification without reverse transcription (to rule out contaminating DNA); lanes 0 to 6, 0, 100, 500, 1,000, 5,000, 10,000, and 50,000 copies of competing pQP1Δ80 transcript per reaction, respectively. Note absence of signal in all -RT lanes and absence of 180-bp signal in all 0 lanes (no competitive template added).

Table 1. Virologic and clinical summary for 66 consecutively studied HIV-1-infected patients.

Patient ID	CD4 ⁺ cells* (cells/mm ³)	HIV RNA† (copies/ml)	HIV p24 Ag		Plasma culture (TCID/ml)
			ICD‡ (pg/ml)	Reg§ (pg/ml)	
<i>CDC stage I¶</i>					
HOBR 0961	920	2,617,400	386	660	125
SUMA 0874	853	1,485,000	892	1,250	625
BORI 0637	817	2,398,600	218	258	1#
INME 0632	739	2,427,900	275	360	1,000
WEAU 0575	358	355,400	258	329	0**
FASH 1057	262	21,783,600	5,072	5,406	10,000
<i>CDC stage II or III</i>					
RIPH 0179	1080	40,800	39	0	0
HIDO 1099	823	36,000	0	0	0
ATKA 0381	760	9,400	0	0	0
HOJU 0143	720	38,900	0	0	0
MAMA 0341	705	113,400	31	0	0
ADFR 0194	703	24,200	0	0	0
TIMI 0852††	678	100‡‡	0	0	0
BECH 0171	678	13,800	8	7	0
MALE 0264	644	198,800	0	0	0
ROJO 0331	640	78,700	0	0	0
BELI 1233	627	4,400	0	0	0
HAJO 0940††	624	13,800	0	0	0
ADDI 0101	616	128,300	0	0	0
FOMA 0784††	521	34,400	0	0	0
JUJA 0156	458	586,100	0	0	5
WATH 0272††	350	67,200	0	0	0
BAMA 0037	323	91,000	154	53	0
WAJO 1286	281	46,100	183	35	0
BAST 0514††	270	9,400	0	0	0
STMI 0862	257	92,900	574	350	5
STBO 1287	231	8,600	0	0	0
SLMI 0843	197	84,900	0	0	0
<i>CDC stage IVC2</i>					
ALFR 0229	615	91,800	0	0	0
DOBE 0859	533	114,500	0	0	0
CALI 0950††	456	40,800	35	5	0
ARLA 0846	435	49,100	0	0	0
WOAL 0263††	425	32,800	0	0	0
GADA 1162††	424	192,500	0	0	0
MCSE 0176	360	341,000	5,000	1,070	100
CHJI 0774	332	33,300	0	0	0
EDWI 0817	267	73,100	0	0	0
GRJO 0849	243	94,700	0	0	25
MIWI 1278	236	173,600	79	0	0
SMDO 0157††	141	2,200,000	740	284	3,125
HEMI 0562	117	223,000	123	101	125
DAJO 0306	109	36,900	361	120	0
SMST 1012	95	738,900	765	90	0
DUSE 1021	82	655,500	179	119	5
WHWI 1106	67	104,500	221	27	25
NOWR 1192	54	469,900	209	20	3,125
MIGE 1132††	29	191,800	0	0	0
DABE 0775	27	448,200	173	153	5
EMJA 0809	21	625,900	187	226	25
CLRA 0703	10	687,900	396	40	25
LARO 0833	7	479,300	823	238	5
<i>CDC stage IVC1</i>					
DEDA 0006	57	1,309,000	233	14	10,000
VAST 0181††	56	664,500	450	25	0
DODO 0116	50	1,667,000	357	385	100,000
SZHO 1173††	37	808,300	631	195	625
MILA 0284††	32	815,100	0	0	0
BIJA 0205	32	4,744,000	1,920	1,050	10,000
NAPH 0073	14	1,804,000	480	480	1,000
TIMI 0018	14	3,445,000	330	390	100
WATI 0855	7	9,300,000	606	302	625
MCM1 0063	7	232,000	221	12	625
LENA 1029	<5	2,500,000	53	0	125
FARO 1042	<5	4,800,000	199	204	625
RUTH 1145	<5	424,800	230	143	125
YOAL 0522	<5	2,600,000	205	5	125
JOJI 0070	<5	1,606,000	2,460	885	100,000

*CD4⁺ cells determined by flow cytometry within 6 weeks of sampling for virologic assessments. †HIV RNA copy number per milliliter of plasma, as determined by QC-PCR (13, 17). ‡Immune complex-dissociated HIV p24 antigen (19). §HIV p24 antigen (19). ¶Tissue culture infectious doses of virus per milliliter of plasma (18). ¶Centers for Disease Control classification system for staging of HIV-1 infection (2). #Three days previously, plasma cultures were positive at 10 TCID₅₀/ml, with HIV-1 RNA level of 1,350,600 copies/ml. **Eight days previously, plasma cultures were positive at 1,000 TCID₅₀/ml, with HIV-1 RNA level of 216,400 copies/ml (Fig. 2.). ††AZT or dideoxyinosine therapy at time of study. ‡‡Extrapolated value.

ing assay or protein immunoblot, and each subsequently seroconverted with a full spectrum of HIV-1-specific antibodies by day 50 (7). Virion-associated HIV-1 RNA levels peaked between 8 and 23 days after the onset of symptoms, reaching values between 3.55×10^5 and 2.18×10^7 copies per milliliter (corresponding to 1.78×10^5 to 1.09×10^7 virions per milliliter). Culturable virus [10 to 10,000 tissue culture infectious dose (TCID) per milliliter] and p24 antigen (258 to 5,406 pg/ml) peaked at approximately the same time as virion RNA levels, declining rapidly thereafter in parallel with virion RNA levels. Within the first 100 days after onset of symptoms, plasma RNA levels fell by between 20- and 235-fold from peak levels but, in marked contrast to p24 antigen and culturable virus, remained continuously quantifiable for the duration of follow-up in all patients.

Among three patients who presented with symptomatic acute infection (Fig. 2), there appeared to be associations between profiles of viral load in plasma over time, as determined by RNA QC-PCR, and trends in CD4⁺ T cell counts and clinical status. For example, patient SUMA (Fig. 2A) showed an initial peak of HIV-1 RNA (1.49×10^6 copies per milliliter) that decreased by a factor of nearly 1×10^2 within the first month of follow-up. Viral RNA reached a minimum (2.30×10^3 copies per milliliter) at 278 days and remained at or below 1.50×10^4 RNA copies per milliliter up to 473 days of follow-up. Plasma viral cultures and p24 assays remained negative after the initial peak. This patient maintained a normal CD4⁺ T cell count (952 to 1108 per cubic millimeter) and remained entirely asymptomatic over the period of follow-up. In contrast, patient FASH (Fig. 2B) showed the highest initial virus peak (2.18×10^7 copies per milliliter), as well as the highest persistent levels of circulating virus, in the range of 3.26×10^5 to 6.32×10^5 copies per milliliter over 301 days of follow-up. The highest CD4⁺ T cell count measured in this patient was 282 per cubic millimeter, with a subsequent progressive decline to 128 per cubic millimeter and clinical progression to CDC stage IVC2, despite antiviral therapy. Although anecdotal, these observations suggest that higher levels of circulating virus and failure to effectively control virus replication after initial infection may be associated with a negative prognosis. However, in addition to quantitative viral load, other virologic or immunopathologic factors, including a syncytium-inducing viral phenotype, likely contribute to variable rates of CD4⁺ T cell depletion and clinical outcome (23). In this regard, patient WEAU (Fig. 2C), who experienced a sharp decline in CD4⁺ cells despite a peak level of viral

RNA of only 3.55×10^5 copies per milliliter, was infected with a virus isolate that was markedly cytopathic when cultured *in vitro* (7).

Although in the present studies we did not intend to provide a comprehensive evaluation of the impact of therapeutic intervention on viral load as measured by QC-PCR, we did analyze sequential specimens from a limited number of patients, before and after initiation of treatment with azidothymidine (AZT). As shown in Table 2, treatment with AZT resulted in up to 39-fold decreases in circulating virus as measured by QC-PCR (patient JOJI 0070), with a significant treatment effect for the entire group of patients ($P < 0.0001$) (24). Despite differences between the absolute levels of virus measured by QC-PCR and by culture methods, treatment-associated decreases in plasma HIV-1 RNA levels were paralleled by decreases in culturable virus, where measured. We also evaluated in greater detail serial specimens from three previously untreated patients who were given AZT for 6 weeks, followed by a 1-week period off treatment. The observed rapid decreases in QC-PCR-determined circulating virus levels after initiation of treatment (week 1) and the rapid rebound of virus to pretreatment levels after discontinuation of treatment (week 7) reveal the dynamic nature and high levels of ongoing viral replication in these patients. Two of these patients completely lacked detectable levels of other viral markers and could only be monitored by QC-PCR, and the third had detectable levels of p24 only after ICD (Table 2). The average decline in HIV-1 RNA among the ten patients treated with AZT was 11-fold, whereas the average decline associated with resolution of the acute retroviral syndrome in six patients was 72-fold (Fig. 2 and Table 1) (20).

Using different PCR techniques, other investigators have estimated levels of HIV-1 RNA in plasma ranging from 0 to 1×10^6 copies per milliliter (11, 12, 15), values that are generally lower by a factor of 10 or more than those we observed for patients with disease of a comparable stage. The methods used by these investigators differed in various significant ways from those we describe here, including, in some instances, the use of noncompetitive PCR techniques that do not provide the level of stringent internal control that is a central feature of QC-PCR, the use of different target regions and primer sets, or direct extraction of RNA from plasma without pelleting of virions by ultracentrifugation and other steps to maximize recovery of viral RNA (13, 17).

Validation of the QC-PCR procedure with the use of known copy numbers of recombinant HIV RNA and DNA (Fig. 1)

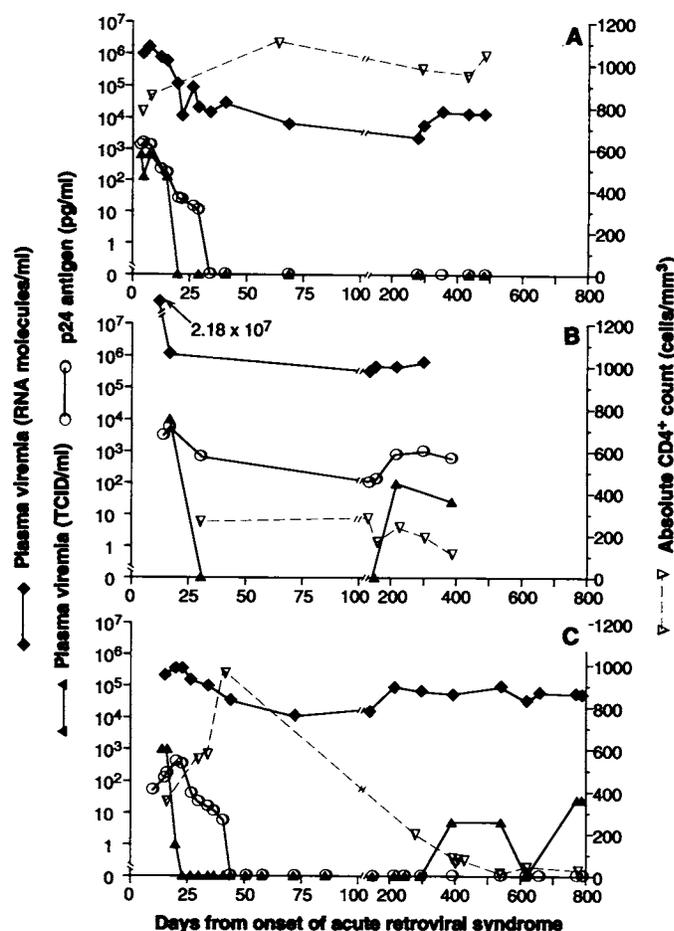


Fig. 2. Longitudinal determinations of HIV-1 RNA copy number by QC-PCR, p24 antigen levels by standard assay, culturable virus, and CD4⁺ T cell counts for patients presenting with symptomatic primary HIV-1 infection (CDC stage I). (A) Patient SUMA 0874. (B) Patient FASH 1057. (C) Patient WEAU 0575. Clinical history, partial virus culture results, and p24 antigen values [performed with a first-generation immunoassay kit (Abbott Laboratories, Chicago, Illinois)] for patient WEAU 0575 have been previously reported (7).

(13) and by measurement of viral RNA in virus preparations that had been quantified directly by electron microscopic particle counts (25) makes it unlikely that we have overestimated total virion levels in patient samples. To demonstrate conclusively that the HIV-1 RNA quantified by QC-PCR was virion associated, we fractionated samples of HIV-1-containing culture supernatant and plasma from infected patients by using buoyant density centrifugation on continuous (20 to 60%) sucrose gradients. The HIV-1 RNA peaks corresponded precisely to the peaks of HIV-1 p24 antigen, both of which localized to fractions of the expected specific gravity for HIV-1 particles (26). For the banded virus, the ratio of virions (assuming two HIV-1 RNA molecules per virion) to p24 antigen (in picograms) was approximately 10⁴:1, in good agreement with estimates based on other biophysical studies of HIV-1 virions (25). Similar ratios were seen for virus pelleted from plasma (Table 1), with QC-PCR-determined virus RNA levels for most subjects exceeding p24 levels (measured in picograms) by the expected 1,000- to 10,000-fold.

Circulating levels of plasma virus determined by QC-PCR also correlated with, but exceeded by an average of nearly

60,000-fold (Table 1) (27), titers of infectious HIV-1 determined by quantitative endpoint dilution culture of identical portions of plasma. Several virologic and immunologic factors already identified in HIV-1 infection, including neutralizing antibody (28), viral envelope shedding (29), deterioration of other viral components (25), and genotypically defective virus (30) likely contribute to the differences in levels of circulating virus determined by QC-PCR and titers of culturable virus. However, the minimum requirements for establishment of productive infection of primary mononuclear cells are not known. If more than one intact viral particle is required to attain productive infection of a host cell, this would exaggerate the discrepancy observed between viral titers in plasma determined by QC-PCR as compared with those determined by endpoint dilution culture. For HIV-1 propagated *in vitro*, total virions have been reported to exceed culturable infectious units by factors of 10⁴ to 10⁷ (25), ratios similar to those we observed in plasma.

The evidence provided here for continuous viral replication throughout all stages of infection and the demonstration of significant associations between HIV-1 RNA levels and both disease stage and CD4⁺ T

Table 2. AZT treatment: Virologic and clinical data summary. Table shows values for patients before and after treatment with AZT for the indicated periods. ND, not determined.

Patient ID	HIV RNA (copies/ml)	HIV p24 Ag*		Plasma culture (TCID ₅₀ /ml)	AZT	Time on treatment (weeks)
		ICD (pg/ml)	Reg (pg/ml)			
MCSE 0176	341,000	5,000	1,070	100	-	
	118,000	5,025	1,530	1	+	17
NOWR 1192	469,900	209	20	3,125	-	
	82,400	41	0	ND	+	1
EMJA 0809	625,900	187	226	25	-	
	75,500	195	216	ND	+	6
TIMI 0018	3,450,000	330	390	100	-	
	235,000	35	35	0	+	2
NAPH 0073	1,800,000	480	480	1,000	-	
	182,000	320	320	100	+	17
MCM1 0063	232,000	221	12	625	-	
	134,300	160	7	ND	+	6
JOJI 0070	1,606,000	2,460	885	100,000	-	
	40,800	263	58	10	+	20
SLMI 0843	84,900	0	0	0	-	Week 0†
	18,000	0	0	ND	+	Week 1
	33,500	0	0	ND	+	Week 2
	28,100	0	0	ND	+	Week 6
	72,700	0	0	0	-	Week 7
ARLA 0846	49,100	0	0	0	-	Week 0
	7,300	0	0	ND	+	Week 1
	6,500	0	0	ND	+	Week 2
	11,200	0	0	ND	+	Week 6
	58,400	0	0	0	-	Week 7
MIWI 1278	173,600	79	0	0	-	Week 0
	21,900	28	0	ND	+	Week 1
	10,900	24	0	ND	+	Week 2
	9,200	31	0	ND	+	Week 6
	136,300	47	0	25	-	Week 7

*Parameters as for Table 1. †For kinetic analysis of viral load by RNA QC-PCR over a 6-week period of treatment with AZT, patients were studied before initiation of treatment (week 0), after 1, 2, and 6 weeks of treatment, and 1 week after temporary discontinuation of treatment (week 7).

cell counts argue strongly for a direct role for HIV-1 replication in the pathogenesis of HIV disease. These results confirm and extend work by Coombs (5), Ho (5), Pantaleo (9), Schnittman (31), and Michael (32) who, by alternative approaches, also found evidence of continuous viral replication related to clinical stage and disease progression. The finding of a large proportion of circulating virus that is not culturable raises the possibility that genetically defective (30) or otherwise noninfectious virus may contribute importantly to HIV-1 pathogenesis, in keeping with precedents in animal retrovirus systems (33). Numerous mechanisms by which noninfectious particles might contribute to the pathogenesis of HIV-1 infection have been proposed (34). Furthermore, recent observations have documented the presence of HIV-1-reactive cellular immune responses in patients who have been exposed to HIV-1 by sex or use of shared needles but who are not demonstrably infected with the virus (35). Exposure to an inoculum consisting largely of noninfectious particles might produce such a result, even without the establishment of productive infection of the exposed individual.

Finally, our data suggest that determination of virion-associated HIV-1 RNA levels in plasma by QC-PCR represents a marker

of viral replication with potential for widespread applicability in assessment of the activity of antiretroviral therapy. There is currently a widely recognized need for new markers that allow timely assessment of the in vivo antiviral activity of new therapeutic approaches and agents and that better predict the ultimate clinical efficacy of new treatments (36). Ideally, such markers should bear a biologically plausible relation to the disease process, be present in most or all patients, change rapidly in response to effective therapy, be derived from readily obtained clinical specimens, and correlate directly with eventual clinical outcome. Quantitation of HIV-1 virion levels in plasma as determined by QC-PCR satisfies the first four of these requirements. Future studies making use of QC-PCR methods will help to determine the relation between virion-associated HIV-1 RNA levels and clinical outcome and the role of persistent viral replication and viremia in the pathogenesis of HIV infection and AIDS.

REFERENCES AND NOTES

1. R. Anderson, *AIDS* 1, 241 (1988); A. Munoz et al., *Am. J. Epidemiol.* 130, 530 (1989); P. Bacchetti and A. R. Moss, *Nature* 338, 251 (1989).
2. *Morbidity Mortal. Weekly Rep.* 35, 334 (1986).
3. A. S. Fauci, *Science* 239, 617 (1988); A. S. Fauci,

- S. M. Schnittman, G. Poli, S. Koenig, G. Pantaleo, *Ann. Intern. Med.* 114, 678 (1991); H. C. Lane et al., *N. Engl. J. Med.* 313, 79 (1985); A. Amadori et al., *AIDS Res. Hum. Retroviruses* 6, 581 (1990).
4. G. Pantaleo, C. Graziosi, A. S. Fauci, *N. Engl. J. Med.* 328, 327 (1993).
5. R. W. Coombs et al., *ibid.* 321, 1626 (1989); D. D. Ho, T. Moudgil, M. Alam, *ibid.*, p. 1621.
6. M. S. Saag et al., *J. Infect. Dis.* 164, 72 (1991).
7. S. J. Clark et al., *N. Engl. J. Med.* 324, 954 (1991).
8. E. S. Daar, T. Moudgil, T. Meyer, D. D. Ho, *ibid.*, p. 961.
9. G. Pantaleo et al., *Proc. Natl. Acad. Sci. U.S.A.* 88, 9838 (1991); C. Graziosi, G. Pantaleo, D. P. Kotler, A. S. Fauci, *Clin. Res.* 40, 333 (1992).
10. M. Ottmann et al., *J. Virol. Methods* 31, 273 (1991); G. H. Murakawa et al., *DNA* 239, 295 (1988).
11. M. Holodniy, M. A. Winters, T. C. Merigan, *Bio-Techniques* 12, 36 (1992); M. Holodniy, D. A. Katzenstein, D. M. Israelski, T. C. Merigan, *J. Clin. Invest.* 88, 1755 (1991); S. Aoki et al., *AIDS Res. Hum. Retroviruses* 8, 1263 (1992).
12. K. E. Noonan et al., *Proc. Natl. Acad. Sci. U.S.A.* 87, 7160 (1990); R. E. Dickover et al., *J. Clin. Microbiol.* 18, 2130 (1990).
13. M. Piatak, K.-C. Luk, B. Williams, J. D. Lifson, *BioTechniques* 14, 70 (1993).
14. G. Gilliland, S. Perrin, K. Blanchard, H. F. Bunn, *Proc. Natl. Acad. Sci. U.S.A.* 87, 2725 (1990); G. Gilliland, S. Perrin, H. F. Bunn, in *PCR Protocols: A Guide to Methods and Applications*, M. A. Innis, D. H. Gelfand, J. J. Sninsky, T. J. White, Eds. (Academic Press, San Diego, CA 1990), pp. 60-69; M. Becker-André and K. Hahlbrock, *Nucleic Acids Res.* 17, 9437 (1989).
15. M. Stieger, C. Demoliere, L. Ahlborn-Laake, J. Mous, *J. Virol. Methods* 34, 149 (1991); S. Menzo et al., *J. Clin. Microbiol.* 30, 1752 (1992); S. Jurriaans, J. T. Dekker, A. De Ronde, *AIDS* 6, 635 (1992); D. T. Scadden, Q. Wang, J. Groopman, *J. Infect. Dis.* 165, 1119 (1992); P. Bagnarelli et al., *J. Virol.* 66, 7328 (1992).
16. *Human Retroviruses and AIDS. A Compilation and Analysis of Nucleic Acid and Amino Acid Sequences*, G. Myers, B. Korber, J. A. Berzofsky, R. F. Smith, G. N. Pavlakis, Eds. (Los Alamos National Laboratory, Los Alamos, NM, 1991).
17. All patients were evaluated at the University of Alabama, Birmingham, where approval from the human subjects review board and informed consent were obtained. Blood specimens were collected in acid-citrate-dextrose and processed within 3 hours of phlebotomy. After centrifugation (200g for 15 min), plasma was collected and centrifuged again (1000g for 15 min) to ensure cell-free specimens. Replicate portions of plasma were used immediately for virus culture or stored at $\leq -70^{\circ}\text{C}$ until further analysis. For extraction of virion-associated RNA, plasma samples were thawed and subjected to ultracentrifugation (Beckman type 70.1 rotor, 40,000 rpm, 1 hour) to pellet virions. Pellets were resuspended in solution [20 mM tris (pH 7.5), 150 mM NaCl, 2 mM EDTA], adjusted to contain 1 mg of proteinase K per milliliter and 0.1% SDS, and incubated at 37°C for 1 hour. After repeated extraction with phenol:chloroform:isoamyl alcohol (24:24:1) followed by one extraction with chloroform, samples were adjusted to contain ~ 40 μg of glycogen per milliliter (as carrier) and 10 ng of 7.5-kb synthetic RNA per milliliter (BRL, Bethesda, MD) (as carrier and to normalize total RNA content in the reverse transcription and PCR steps). RNA was then precipitated with ethanol at -20°C for 48 hours and pelleted by ultracentrifugation to maximize recovery. The RNA pellets were partially dried and then dissolved in 100 μl of sterile, ribonuclease-free water. Samples were stored at -70°C until subsequent analysis. The primers GAG04 (CATCTATTGTTCTGAAGGGTACTAG) and GAG06 (GCITTAGCCCGAAGTIATACCCATG) have been described previously (13) and were designed to amplify an internal fragment of either 260 bp (from wild-type HIV-1 target sequences) or 180 bp (from the pQP1 Δ 80 competitive template). To maintain

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equivalent and competitive priming efficiency with divergent sequences, primers incorporated inosine residues at the few positions where divergence from the conserved consensus sequence has been reported (13, 16). For QC-PCR analysis, two plasmids were prepared, one containing the target sequence (pQP1) and the other containing the identical sequence except for an 80-bp internal deletion (pQP1Δ80), sufficient to allow the derived PCR products to be readily resolved by electrophoresis (13). In vitro RNA transcripts were prepared with commercially available kits. Final preparations in water were determined to be essentially free of degradation products by Northern (RNA) blot analysis and were quantified by measurement of absorbance at 260 nm. Portions were stored at -70°C until needed. PCR reaction conditions and protocols were generally similar to those found in commercially available kits (Perkin-Elmer, Norwalk, CT). Each test sample was divided into eight replicate portions and analyzed in the presence of 0 to 50,000 copies per reaction of competitive template. The initial reaction was performed in a total volume of 30 μl and contained 5 μl of test RNA (corresponding to 5% or less of the total specimen), 5 μl of competing RNA preparation or water, and 30 U of cloned Moloney virus reverse transcriptase (BRL, Bethesda, MD). One portion from each specimen was analyzed without reverse transcription and in the absence of competitive template. After 10 min at room temperature to allow for partial extension and stabilization of random hexamer primers, conversion of RNA into cDNA was allowed to continue for 30 min at 42°C. This reaction was then adjusted to contain primers and additional buffer in a total volume of 60 μl. Amplification was performed as described (13), with 45 cycles (94°C for 1 min, 50°C for 2 min, and 72°C for 1 min), followed by a final incubation at 5°C for 5 min. After amplification, approximately 7% of each reaction product mixture was separated by electrophoresis in composite 2% Synergel (Diversified Biotech, Newton Center, MA)-1% agarose (FMC Bioproducts, Rockport, ME) gels in 20 mM Tris acetate (pH 7.8) and 1 mM EDTA. Gels were stained with ethidium bromide for visualization under ultraviolet illumination. Quantitation of fluorescence of both wild-type and competitive template product bands was performed on a Lynx 4000 molecular biology workstation with matched custom software (Applied Imaging, Santa Clara, CA), as described (13). Competition equivalence points were determined by interpolation on plots of the logarithm of the calculated ratio of signal for the competitive template-derived product over the signal for the wild-type target sequence-derived product (corrected for molar ratio) versus the logarithm of the copy number of added competitive template (Fig. 1) (13).

18. Endpoint dilution cultures of plasma for cell-free infectious virus were determined for fresh specimens, generally in duplicate or quadruplicate, as described (6, 7). Plasma samples were not filtered (to eliminate the possibility of inadvertent loss of virus).
19. Regular and ICD p24 antigen determinations were performed in duplicate with the Coulter Diagnostics kit assay, according to the manufacturer's recommendations (Coulter, Hialeah, FL).
20. M. Piatak *et al.*, unpublished observations.
21. Analysis of variance, Duncan's multiple range test, and Tukey's Studentized range (HSD) test as calculated by the SAS statistical analysis software package (SAS Institute, Cary, NC). Plasma RNA data were subjected to logarithm transformation before statistical analysis.
22. Spearman correlation coefficients as calculated by the SAS statistical analysis software package. Plasma RNA data were subjected to logarithm transformation before statistical analysis.
23. M. Tersmette *et al.*, *Lancet* **335**, 983 (1989); M. Tersmette *et al.*, *J. Virol.* **63**, 2118 (1989); D. S. Stein, J. A. Korvick, S. H. Vermund, *J. Infect. Dis.* **165**, 352 (1992).
24. Paired *t* test analysis on logarithm-transformed data set.

25. S. P. Layne *et al.*, *Virology* **189**, 695 (1992); A. S. Bourinbaier, *Nature* **349**, 111 (1991); J. A. McKeating, J. P. Moore, *ibid.*, p. 660.
26. F. Barré-Sinoussi *et al.*, *Science* **220**, 868 (1983); M. Popovic, M. G. Sarngadharan, E. Read, R. C. Gallo, *ibid.* **224**, 497 (1984); J. A. Levy *et al.*, *ibid.* **225**, 840 (1984).
27. To calculate ratios of total virions determined by RNA QC-PCR analysis to culturable virus, we assigned specimens that did not contain culturable virus a TCID value of 1 to avoid denominators of 0. The ratios calculated therefore represent a lower estimate of the true ratios of total circulating virions to culturable virus.
28. R. A. Weiss *et al.*, *Nature* **316**, 69 (1985); M. Robert-Guroff, M. Brown, R. C. Gallo, *ibid.*, p. 72; P. L. Nara, R. R. Garrity, J. Goudsmit, *FASEB J.* **5**, 2437 (1991).
29. J. P. Moore, J. A. McKeating, R. A. Weiss, Q. J. Sattentau, *Science* **250**, 1139 (1990).
30. Y. Li *et al.*, *J. Virol.* **65**, 3973 (1991); Y. Li *et al.*, *ibid.* **66**, 6587 (1992); J. Meyerhans *et al.*, *Cell* **58**, 901 (1989).
31. S. M. Schnittman, J. J. Greenhouse, H. C. Lane, P. F. Pierce, A. S. Fauci, *AIDS Res. Hum. Retroviruses* **7**, 361 (1991).
32. N. L. Michael, M. Vahey, D. Burke, R. R. Redfield, *J. Virol.* **66**, 310 (1992).
33. J. Overbaugh, P. R. Donahue, S. L. Quackenbush, E. A. Hoover, J. I. Mullins, *Science* **239**, 906 (1988); D. C. Aziz, Z. Hanna, P. Jolicœur, *Nature* **338**, 505 (1989); S. K. Chattopadhyay, H. C. Morse III, M. Makino, S. K. Ruscetti, J. W. Hartley, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3862 (1989).
34. J. B. Margolick, D. J. Volkman, T. M. Folks, A. S. Fauci, *J. Immunol.* **138**, 1719 (1987); D. L. Mann

et al., *ibid.*, p. 2640; M. R. Shalaby *et al.*, *Cell. Immunol.* **110**, 140 (1987); L. O. Arthur *et al.*, *Science* **258**, 1935 (1992); L. Imberti, A. Sottini, A. Bettinardi, M. Puoti, D. Primi, *ibid.* **254**, 860 (1991); C. Terai, R. S. Kornbluth, C. D. Pauza, D. D. Richman, D. A. Carson, *J. Clin. Invest.* **87**, 1710 (1991); A. G. Laurent-Crawford *et al.*, *Virology* **185**, 829 (1991); J. C. Ameisen and A. Capron, *Immunol. Today* **12**, 102 (1991); H. Groux *et al.*, *J. Exp. Med.* **175**, 331 (1992); M. K. Newell, L. J. Haughn, C. R. Maroun, M. H. Julius, *Nature* **347**, 286 (1990); L. Meyaard *et al.*, *Science* **257**, 217 (1992); R. S. Mittler and M. K. Hoffman, *ibid.* **245**, 1380 (1989); G. P. Linette, R. J. Hartzman, J. A. Ledbetter, C. H. June, *ibid.* **241**, 573 (1988).

35. M. Clerici, J. A. Berzofsky, G. M. Shearer, C. O. Tacket, *J. Infect. Dis.* **164**, 178 (1991); M. Clerici *et al.*, *ibid.* **165**, 1012 (1992).
36. J. Cohen, *Science* **258**, 388 (1992).
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Requirement of the Carboxyl Terminus of a Bacterial Chemoreceptor for Its Targeted Proteolysis

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The bacterium *Caulobacter crescentus* yields two different progeny at each cell division: a chemotactically competent swarmer cell and a sessile stalked cell. The chemotaxis proteins are synthesized in the predivisional cell and then partition only to the swarmer cell upon division. The chemoreceptors that were newly synthesized were located at the nascent swarmer pole of the predivisional cell, an indication that asymmetry was established prior to cell division. When the swarmer cell differentiated into a stalked cell, the chemoreceptor was specifically degraded by virtue of an amino acid sequence located at its carboxyl terminus. Thus, a temporally and spatially restricted proteolytic event was a component of this differentiation process.

Cell divisions that yield two different progeny cells are fundamental to developmental programs in all organisms (1). In *Caulobacter crescentus*, the generation of two distinct cell types at each cell division is due, in part, to the asymmetric distribution of proteins in the cell before it has divided. The predivisional cell assembles a flagellum and several pili at one pole, which are then partitioned to the swarmer cell progeny. In addition to bearing a flagellum and polar pili, the swarmer cell is chemotactically competent. The chemoreceptor McpA,

which is one of the family of MCPs (methyl-accepting chemotaxis protein), is synthesized only in the predivisional cell (2). Thus, the swarmer cell inherits McpA from the predivisional cell (3).

We now describe our studies of the spatial distribution of the newly synthesized chemoreceptor in the predivisional cell and its subsequent fate throughout the cell cycle. Assays in vitro have shown that the methyl-accepting activity of the chemoreceptors, and the activities of the methyltransferase and methylesterase are lost during the transition of a swarmer cell into a stalked cell (4). The McpA chemoreceptor is positioned at the flagellated pole of the swarmer cell (3), and we now show that it is degraded during the transi-

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