Plasma viremia as a sensitive indicator of the antiretroviral activity of L-697,661

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ABSTRACT   L-697,661 is a non-nucleoside analogue with potent, selective inhibitory activity against the reverse transcriptase of human immunodeficiency virus type 1 (HIV-1). The present study evaluated the potential role of this compound in the treatment of HIV-1-infected patients in a double-blinded, placebo- and zidovudine-controlled trial using plasma viremia as a marker of antiviral activity and real-time phenotypic evaluation of viral isolates for the emergence of resistance. Participants received 12 weeks of either placebo, 25 mg twice a day, 100 mg three times a day, or 500 mg twice a day of L-697,661, or zidovudine, 100 mg five times a day. Mean logarithmic reciprocal titers of plasma virus in patients taking either L-697,661 or zidovudine decreased by week 4 of therapy; for L-697,661 recipients these changes were dose-dependent and, at the highest dose tested, were comparable in magnitude to those seen with zidovudine. Viral suppression induced by L-697,661 persisted through 8 weeks of treatment but decreased by week 12. This rebound paralleled emergence of viral isolates showing resistance to L-697,661. We conclude that although L-697,661 has potent antiretroviral activity *in vivo*, its utility may be compromised by rapid emergence of L-697,661-resistant virus. Plasma viremia is a highly sensitive technique affording considerable utility in the early testing of such agents.

In this second decade of the human immunodeficiency virus type 1 (HIV-1) pandemic, considerable uncertainty still remains concerning the appropriate timing for introduction of antiretroviral therapy in patients with only mild to moderate immunodeficiency. One major reason for this has been the paucity of established surrogates of clinical efficacy in patients at an early stage of their illness. Patients with more advanced HIV-1 infection are not only more likely to display abnormalities in conventional laboratory markers of disease activity but they are also substantially more likely to develop clinical endpoints of disease. In contrast, earlier-stage patients are often asymptomatic individuals with negative conventional virologic studies in whom peripheral immune system function, though impaired, nonetheless may remain relatively well-preserved over short windows of observation.

It may be more difficult to demonstrate significant alterations in standard antiviral parameters in early patients and to establish clinical correlation for these changes. Larger study cohorts with longer periods of active participation and follow-up are often required to compensate for the insensitivity of current techniques of monitoring. Paradoxically, early and moderate-stage patients are those who may derive the greatest benefit from antiretroviral interventions because of their presumed greater capacity for immune reconstitution

during effective viral suppression. It is clear that newer techniques must be applied in the study of these patients if rapid evaluation of promising new therapies is to proceed in a timely fashion and also if patients are not to be continued on potentially ineffective treatments for longer than is absolutely necessary to determine comparative efficacy.

In the present study, we chose to evaluate the role of one such technique, quantitative plasma viremia, in an early patient population within the context of a randomized, double-blinded, placebo-controlled phase I/II trial of a putative new oral antiretroviral, L-697,661 (3-{[(4,7-dichloro-1,3-benzoxazol-2-yl)methyl]amino}-5-ethyl-6-methylpyridin-2(1H)-one). L-697,661, a substituted pyridinone derivative, is a member of a family of non-nucleoside analogues with potent, specific activity against HIV-1 (1). Because *in vitro* studies suggested that it might also be an agent capable of inducing rapid emergence of resistant virus (2), it seemed ideally suited for monitoring by a sensitive technique geared to measuring early shifts in plasma viral burden.

METHODS

Patients. Eligible participants were asymptomatic HIV-1-infected individuals without AIDS-defining infections or malignancy other than strictly mucocutaneous Kaposi sarcoma. For an initial phase I pharmacokinetic study, only individuals having CD4$^+$ counts >500 cells per mm^3 were enrolled. In the subsequent randomized phase II comparative trial, a CD4$^+$ count ≥200 cells per mm^3 and a positive plasma virus culture at screening were required. Phase I participants were permitted to enroll in phase II if they met all criteria except for a positive plasma culture. Patients were required to discontinue antiretroviral therapy 1 month prior to study entry and were excluded if they had a prior cumulative history of zidovudine use exceeding 6 months.

Study Design. *Phase I.* Sixteen participants each received single oral doses of either 25 mg, 50 mg, 100 mg, 200 mg, or 500 mg of L-697,661 or oral placebo in a double-blinded format; patients were monitored serially for pharmacokinetics and safety.

Phase II. Based upon phase I pharmacokinetics, three doses of L-697,661 were selected for a randomized, double-blinded placebo-controlled trial of L-697,661 versus zidovudine. Patients were assigned to at least 12 weeks of treatment with one of the following five oral regimens: L-697,661 at 25 mg twice a day (BID), 100 mg three times a day (TID), or 500 mg BID, zidovudine at 100 mg five times a day, or placebo

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Abbreviations: HIV, human immunodeficiency virus; BID, twice a day; TID, three times a day.
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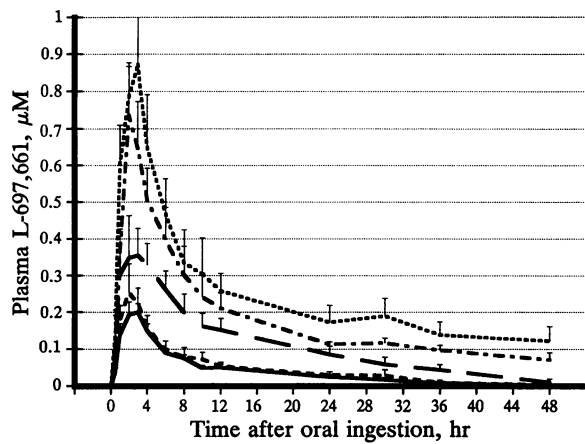[‖]Present address: Sandoz Pharmaceutical Co., East Hanover, NJ 07936.

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FIG. 1. Mean serial plasma concentrations (μM) of L-697,661 following ingestion of single oral doses of 25 mg (——), 50 mg (—-—), 100 mg (— —), 200 mg (— - —), or 500 mg (-------) of L-697,661. Each time point represents the mean plasma concentration (+ SE in vertical hatched bars) in six individuals at any given dose.

five times a day. No adjustments in either dose or schedule of study medication were permitted during this 12-week period. Serial measurements of surrogate markers of antiviral efficacy were performed at least twice as baseline and then repeated every 2–4 weeks thereafter for the duration of study participation. Sensitivity testing of viral isolates to L-697,661 and zidovudine was performed on a subset of patients using paired samples obtained at baseline and at week 12.

Drugs. L-697,661, placebo, and commercially purchased zidovudine were formulated to be identical in appearance and taste and were dispensed in a system of four separate color-coded bottles whose contents varied according to each patient's assigned treatment arm. Participants were instructed to remove their study medications from these bottles according to a generic routine that blinded them to active study medication versus placebo.

Laboratory Monitoring. Lymphocyte subset enumerations were performed as described (3). Serum β_2-microglobulin levels and immune-complex dissociated serum p24 antigen were measured by commercial immunoassay (3, 4). Plasma L-697,661 concentrations were measured by RIA at Merck Sharp & Dohme.

Plasma virus quantitation was performed on plasma (from heparinized samples of whole blood) inoculated within 1 hr of collection into tissue culture containing fresh phytohemagglutinin-stimulated peripheral blood mononuclear cells from HIV-1-seronegative donors (5). Serial 3-fold dilutions of patients' plasma were prepared from each sample. Cultures were monitored serially for p24 antigen production by ELISA. Results were recorded as the reciprocal titer of the last positive dilution during 30 days of incubation.

Resistance Testing. Viral isolates derived from plasma culture were tested for changes in drug sensitivity by mea-

suring growth in normal peripheral blood mononuclear cells in the presence of different concentrations (0–10 μM) of L-697,661 or zidovudine. Cultures were monitored for p24 antigen production by ELISA on days 6 and 12 after infection. Ninety percent inhibitory concentrations (IC_{90}) of drug against virus were determined based upon comparative growth of isolates in untreated control cultures.

Statistical Analysis. For efficacy assessments, all patients were analyzed according to their original randomly assigned treatment arm; analysis of surrogate changes within each group was restricted to those patients for whom at least 12 weeks of data were available. Serum p24 and plasma viremia titer values were logarithmically transformed, base e and base 3, respectively, prior to analysis to make their distributions more symmetric. Negative plasma cultures were assigned a dilution titer 1 $\log_3$-fold lower than neat (i.e., 0.3) to facilitate parametric analysis. Analysis of covariance adjusting for baseline levels was performed for all parameters (6). For plasma viremia, intergroup differences were evaluated by Fisher's exact test and the Armitage test for trend in proportions (7).

RESULTS

Phase I. Mean peak plasma concentrations ($\pm$SE) of L-697,661 were 0.200 ± 0.024, 0.254 ± 0.078, 0.355 ± 0.074, 0.737 ± 0.129, and 0.872 ± 0.150 μM for the 25, 50, 100, 200, and 500 mg doses, respectively (Fig. 1). At doses of 100 mg or more, plasma concentrations of L-697,661 remained above 0.1–0.15 μM [i.e., the *in vitro* 95% inhibitory concentration (IC_{95}) of L-697,661] for up to 24 hr after a single oral dose, peaking 2–3 hr following oral ingestion. Observed adverse events consisted only of occasional mild headaches that were not dose-dependent and were indistinguishable from placebo.

Phase II. Eighty-four participants were enrolled, whose demographics are summarized in Table 1. Participants (15 of 84, 17.9%) with negative plasma cultures at entry had higher baseline CD4$^+$ counts (831 versus 539 cells per mm^3) and CD4$^+$ percentages (41.4% versus 30.8%), as well as lower levels of serum β_2-microglobulin (2.7 versus 3.1 mg/liter), relative to plasma viremic individuals. Overall, 69 (82%) of 84 enrollees were plasma viremic at entry and 35 (42%) had detectable serum p24 antigen. Thirty-four (97.1%) of 35 patients with detectable p24 antigen were also plasma viremic at study entry, whereas in only 34 (49.3%) of the 69 plasma viremic individuals was antigen detected. Therefore, although having detectable serum p24 antigen was strongly predictive of the likelihood of also being plasma viremic, successful virus recovery from plasma also captured a substantial number of individuals below the threshold of antigen detection.

Non-plasma viremic individuals were randomly distributed throughout all five groups, as were zidovudine-naive participants (Table 2). Data were analyzed for all 58 participants completing a minimum of 12 weeks on study. The remaining individuals either dropped off study because of toxicity prior

Table 1. Demographics of the study population

Status of plasma viremia at entry	No. enrolled	Sex of patients	Age of patients,* years	CD4$^+$,* %	Total CD4$^+$ count,* cells per mm^3	Serum p24 positive, no. of patients	Serum p24 antigen,* pg/ml	Serum β_2-microglobulin,* mg/liter	Prior use of zidovudine, no. of patients
Negative	15	14 ♂ 1 ♀	35.6 ± 1.5	41.4 ± 2.2	830 ± 69	1	0	2.7 ± 0.1	5
Positive	69	64 ♂ 5 ♀	35.3 ± 0.9	30.8 ± 1.3	539 ± 28	34	387 ± 129	3.1 ± 0.1	21
Total	84	78 ♂ 6 ♀	35.3 ± 0.8	32.6 ± 1.2	588 ± 28	35	323 ± 109	3.0 ± 0.1	26

*Mean ± SE.

Table 2. Randomization of patients into treatment arms

Assigned treatment arm	Initial randomization	Completed ≥12 weeks of treatment	Plasma viremic	p24 positive	Prior zidovudine
Placebo five times a day	17	12	11	6	2
L-697,661 25 mg BID	17	11	8	5	4
L-697,661 100 mg TID	17	12	9	6	6
L-697,661 500 mg BID	17	12	8	5	4
Zidovudine 100 mg five times a day	16	11	8	2	4
Total	84	58	44	24	20

to week 12 (3 patients) or had not yet completed a full 12 weeks (23 patients) when, based upon identification of the early emergence of resistance, the study was terminated by the pharmaceutical sponsor.

Clinical Status. All patients remained asymptomatic from their HIV-1 infection during their study participation; no AIDS-defining illnesses were reported in study participants.

Efficacy Assessments. Group-wise comparison by analysis of variance showed no statistically significant differences (at $P \leq 0.05$ level) between the five treatments in terms of baseline values for any of the major virologic or immunologic surrogates of antiviral activity.

Changes in mean $CD4^+$ percentages are depicted in Fig. 2. Mean $CD4^+$ percentages for zidovudine and 500 mg BID L-697,661 recipients rose slightly relative to baseline over the 12-week study period; however, these differences were of insufficient magnitude to achieve statistical significance. Changes in absolute $CD4^+$ counts also did not change significantly during this period (data not shown).

The number of participants with detectable serum p24 antigen in each group was too low to assess statistical changes in this parameter over time (data not shown). In the zidovudine treatment arm, for example, only 2 of 11 (18%) recipients entered with detectable antigen.

Fig. 3 shows the effect of either L-697,661 or zidovudine therapy upon serum β_2-microglobulin levels. Zidovudine recipients showed statistically significant differences ($P < 0.001$) in mean β_2-microglobulin levels by 8 weeks relative to placebo and to low-dose (25 mg BID) L-697,661 recipients. These differences persisted through week 12. Similarly, by week 8 the mean β_2-microglobulin level for high-dose (500 mg BID) L-697,661 recipients also began to show differences ($P = 0.07$) from the placebo group; by week 12, these differences also became highly statistically significant with respect to placebo ($P = 0.006$) and to low-dose L-697,661 ($P = 0.005$)
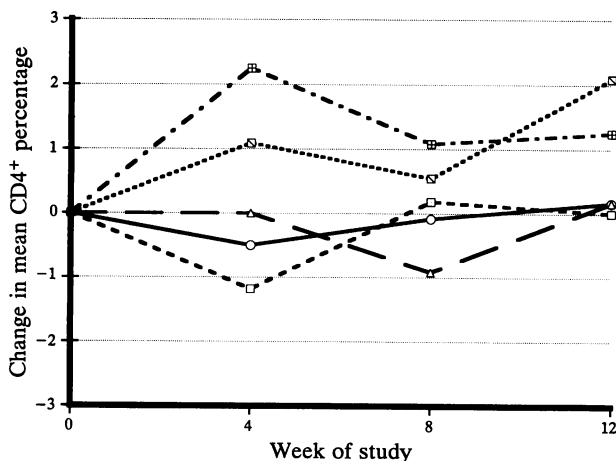
recipients. A lesser degree of change was observed in intermediate-dose (100 mg TID) L-697,661 recipients.

Comparisons of quantitative titers of plasma viremia in the five treatment arms are shown in Fig. 4. Relative to placebo recipients, the mean logarithmic titer of plasma virus declined significantly ($P = 0.009$) in zidovudine-treated patients by week 4 of therapy and remained suppressed throughout the full 12 weeks of study. Patients receiving 25 mg BID L-697,661 had a moderate drop in mean logarithmic titer relative to placebo by week 4; by week 12, however, the mean titer was not statistically significantly different from that of placebo recipients. Patients receiving intermediate dose L-697,661 demonstrated similar suppression of virus early in the study but that persisted at least through week 12; by week 8, these differences approached but did not quite reach statistical significance ($P = 0.09$) relative to placebo. In contrast, patients receiving high-dose L-697,661 manifested significant drops ($P = 0.02$) in mean logarithmic viral titer by week 4 comparable to the steepest drop induced by zidovudine; these changes persisted through week 8 at the same level of significance. By week 12, however, this suppression in quantitative titer declined relative to zidovudine, although clearly still distinguishable from the changes seen with placebo or lower doses of L-697,661. Cultures obtained at week 13 (obtained 1 week off study medications) showed similar trends (data not shown), arguing that these antiviral effects were not simply due to carry-over of active drug from plasma into viral cultures.

Table 3 summarizes drug sensitivity testing of 36 paired viral isolates from a randomly chosen subset of patients from the five treatment arms. Five of 5 (100%) isolates obtained from recipients of high-dose L-697,661 showed a high degree of L-697,661 resistance by 12 weeks of therapy; the mean entry $CD4^+$ count for these 5 individuals was 624 cells per mm^3 (range, 365–1053 cells per mm^3). In contrast, 2 of 4 (50%)

FIG. 2. Relative changes in mean $CD4^+$ percentage for patients receiving placebo (○——), L-697,661 25 mg BID (□——), L-697,661 100 mg TID (△——), L-697,661 500 mg BID (⊞——), or zidovudine 100 mg five times a day (◨·······) for 12 weeks. Changes are shown normalized to baseline (week 0).
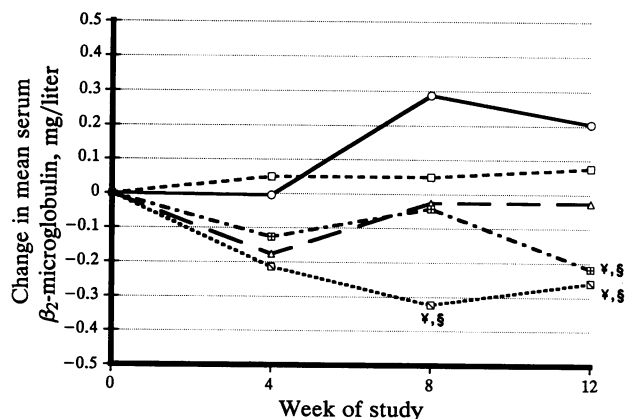

FIG. 3. Relative changes in mean serum β_2-microglobulin levels for patients receiving placebo (○——), L-697,661 25 mg BID (□——), L-697,661 100 mg TID (△——), L-697,661 500 mg BID (⊞——), or zidovudine 100 mg five times a day (◨·······) for 12 weeks. Changes are shown normalized to baseline (week 0). "¥" and "§" indicate changes statistically different from the corresponding values for the placebo or the 25 mg BID L-697,661 treatment arms, respectively.
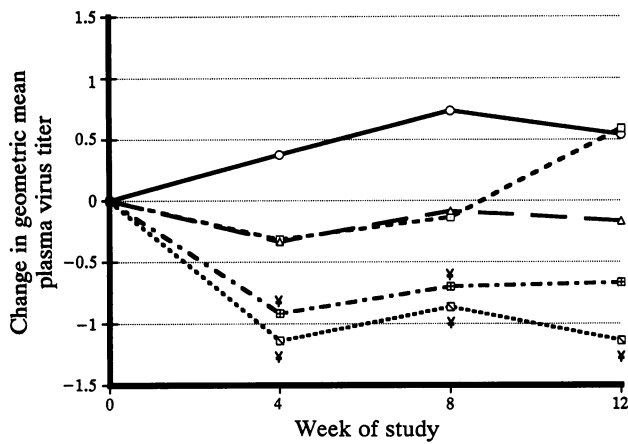
FIG. 4. Relative changes in mean $\log_3$ plasma virus titer for patients receiving placebo (O——), L-697,661 25 mg BID (◻———), L-697,661 100 mg TID (△———), L-697,661 500 mg BID (⊞———), or zidovudine 100 mg five times a day (⊡-------) for 12 weeks. Changes are shown normalized to baseline (week 0). "¥" indicates a change statistically different from the corresponding value for the placebo group.

comparably resistant isolates were isolated from patients on the intermediate dose of L-697,661, and none was isolated from low-dose L-697,661, zidovudine, or placebo recipients. Only one viral isolate from this cohort showed relative zidovudine resistance by week 12 of study; this occurred in a zidovudine recipient who entered the study with a history of several months of prior zidovudine use.

DISCUSSION

Development of more potent, specific, and less toxic inhibitors of HIV-1 remains a major goal of current antiretroviral strategies. One of the most promising developments over the past 2 years was the introduction into clinical testing of several different nonnucleoside reverse transcriptase inhibitors (8, 9). These agents do not require metabolic activation and, in certain cell lines, are up to 100-fold more potent than zidovudine in inhibiting propagation of laboratory strains of HIV-1 (1). They also display antiretroviral synergy with nucleoside analogues, suggesting a possible role in combina-

tion therapy in addition to their potential as monotherapeutic agents (10).

Appearing to fulfill this *in vitro* promise initially, L-697,661 treatment in the present study was associated with a significant reduction in mean quantitative plasma virus titers as early as 4 weeks after initiation of therapy. Some reduction in titer was observed at each L-697,661 dose and, at the highest dose tested, the changes observed in this sensitive marker were of a magnitude comparable to those seen with zidovudine. In contrast, a more conventional virologic marker such as immune-complex dissociated serum p24 antigen was of insufficient sensitivity for use in this early patient population. Further, changes in other conventional but indirect surrogates of antiviral activity, such as $CD4^+$ cell numbers or serum β_2-microglobulin levels, were either delayed or of insufficient magnitude to reach statistical significance.

Unfortunately, therapy with L-697,661 selects for rapid emergence of mutant strains of HIV-1 with decreased sensitivity to its antiviral effects. This occurred within only 12 weeks of therapy, was associated with a lessened effect on plasma virus suppression, and may also have been more common at the higher doses of L-697,661 tested. The latter argues for the emergence of resistant HIV-1 under direct selective pressure from the drug. Previous *in vitro* studies have suggested that stable mutations in one or two key amino acids (i.e., positions 103 and 181) of the reverse transcriptase of HIV-1, distinct from those associated with zidovudine or didanosine resistance, likely confer this resistance pattern (2).

Our findings also dampen hopes that, as in the case of zidovudine resistance (11), viral resistance to L-697,661 perhaps might be slower to develop in early patients than in those with more advanced disease. It also argues against the notion that a prolonged period of silent viral "latency" exists in such patients; in contrast, there must exist a rapidly replicating pool of virus capable of responding quickly to selective pressures induced by drug therapy. Presumably resistance patterns derived from plasma virus provide only a small measure of corresponding changes in viral turnover and mutation occurring within lymph nodes and other major sites of viral replication (12).

It is conceivable that with better formulations or higher doses of non-nucleoside analogues, steady-state plasma concentrations of drug could be achieved that remain above the

Table 3. IC_{90} levels of L-697,661 and zidovudine

Treatment arm	Patient	L-697,661 IC_{90}, μM		Zidovudine IC_{90}, μM	
		Pre-study	≥Week 12	Pre-study	≥Week 12
Placebo five times a day	A	0.1–1	<0.1	0.1–1	0.1–1
	B	0.1–1	<0.1	0.1–1	0.1–1
	C	<0.1	<0.1	<0.1	<0.1
L-697,661 25 mg BID	D	<0.1	<0.1	<0.1	0.1–1
	E	0.1–1	<0.1	0.1–1	0.1–1
	F	<0.1	<0.1	<0.1	0.1–1
L-697,661 100 mg TID	G	0.1–1	1–10*	0.1–1	0.1–1
	H	<0.1	<0.1	<0.1	<0.1
	I	<0.1	0.1–1	0.1–1	0.1–1
	J	<0.1	>10*	<0.1	<0.1
L-697,661 500 mg BID	K	0.1–1	>10*	0.1–1	<0.1
	L	0.1–1	1–10*	0.1–1	0.1–1
	M	<0.1	>10*	<0.1	<0.1
	N	<0.1	1–10*	0.1–1	<0.1
	O	<0.1	1–10*	<0.1	<0.1
Zidovudine 100 mg five times a day	P	0.1–1	<0.1	0.1–1	1–10*
	Q	<0.1	<0.1	<0.1	0.1–1
	R	<0.1	<0.1	<0.1	<0.1

*Significant viral resistance to drug *in vitro* ($IC_{90} > 1$ μM).

IC$_{90}$ levels of even resistant virus. The therapeutic value of combining these agents with conventional nucleoside analogues also merits continued exploration. Accordingly, the possibility of a potential therapeutic role for these agents still exists. Regardless of the outcome of these efforts, however, the present study remains instructive in several respects. To begin with, it clearly demonstrates the value of real-time drug sensitivity monitoring in the early testing of putative antiretrovirals. In this case this information not only provided *in vivo* corroboration of previous *in vitro* findings but also had a direct influence on the outcome of the clinical trial.

It also demonstrates that quantitative plasma viremia may facilitate the evaluation of such agents along an accelerated timeline. Plasma culture may have particular value in studies involving early-stage patients, in whom only a minority may have other markers such as detectable p24 antigen by which to gauge a therapeutic response to antiretroviral therapy. Similar to plasma PCR methodology, it may also provide a more immediate picture of HIV-1 replicative activity than quantitative lymphocyte cocultivation techniques, inasmuch as the latter presumably represent past and present states of viral infection. This distinction may be especially critical if the antiviral effects of an agent are short-lived, as presumably (though not conclusively) would have been the case for L-697,661.

As the alarming toll of HIV-1 infection only continues to grow, the search for newer agents with the promise of enhanced potency assumes an ever greater importance. As such agents are identified, it becomes a legitimate concern of patients and physicians alike that, without sacrificing rigor, early virologic assessment of these drugs is completed as rapidly as technically feasible, hastening into expanded clinical testing those therapies that appear well-tolerated and that provide some early indications of possible efficacy. Quantitative plasma culture offers promise in terms of improved sensitivity and in applicability to an expanded population base, especially one in whom strict reliance upon more traditional endpoints has come under increasing challenge.

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