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Plasma Viremia in Macaques Infected with Simian Immunodeficiency Virus: Plasma Viral Load Early in Infection Predicts Survival†

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A reliable method for the quantitation of plasma viremia in nonhuman primates infected with simian immunodeficiency virus (SIV) and related viruses is described. This method is based on an established quantitative-competitive PCR format and includes a truncated control for internal assay calibration. Optimization of assay conditions has significantly improved amplification specificity, and interassay variability is comparable to that of commercially available assays for human immunodeficiency virus (HIV) quantitation. This procedure was used to monitor viral loads in a group of *Macaca mulatta* animals that were infected with SIVsmE660 for over 2 years. Highly diverse profiles of plasma viremia were observed among animals, and high viral loads were associated with more rapid disease progression. Spearman rank correlation analyses were done for survival versus three parameters of viral load: plasma viremia, p27 core antigen, and frequency of infected peripheral blood mononuclear cells. Plasma viremia had the strongest overall correlation and was significantly ($P < 0.05$ to $P < 0.01$) associated with survival at 10 of the 13 time points examined. Plasma viremia did not correlate with survival during the primary viremia phase; however, the strength of this correlation increased with time postinfection and, remarkably, viremia levels as early as week 6 postinfection were highly predictive ($P < 0.01$) of relative survival. These findings are consistent with the available clinical data concerning viral load correlates early in HIV infection, and they provide further support for the view that disease outcome in lentiviral infection may be largely determined by events that occur shortly after infection.

The infection of nonhuman primates such as Asian macaques with simian immunodeficiency virus (SIV) or human immunodeficiency virus type 2 (HIV-2) results, in many cases, in an immunodeficiency disease which resembles that caused by HIV-1 infection in humans. These animal models of human AIDS have played a central role in the understanding of lentiviral pathogenesis and in the evaluation of vaccine strategies, potential antiviral agents, and novel treatment approaches (8, 15, 16, 32).

An important step to realizing the full potential of primate models in AIDS research is to establish that the relationship between viral load and clinical disease observed in HIV-1 infection is represented in infected monkeys. Clinical investigations into the nature of this relationship in HIV infection have been facilitated by the development of practical assays to measure HIV viral loads in plasma (3, 20, 23, 25). These assays have found wide application in clinical studies and have helped to establish that higher viral loads are associated with more advanced disease, that stratification of viral loads among individuals occurs early in infection, and that higher viremia levels are prognostic of more rapid disease progression (12, 21, 22, 25, 28, 29, 31). Of all of the clinical markers examined to date, the plasma viral load has shown the strongest association with disease progression (22).

A more controversial finding has been that plasma HIV levels at the time of seroconversion are predictive of progression to AIDS (11, 18, 21). This has not been observed in all studies (17), and the discordant findings have been attributed to factors such as the sample type, the patient cohort studied, and the sampling interval after seroconversion. This situation serves to illustrate the difficulty in conducting studies of early infection in a clinical setting. Nonhuman primate models, however, have inherent advantages, since the time of infection is known and other important variables such as the frequency of sample acquisition can be carefully controlled.

The available methods to measure viral loads in nonhuman primates include quantitative viral culture, quantitation of infected cells, and measurement of core (p27) antigen in plasma. These procedures are either cumbersome, associated with significant technical limitations, or poorly predictive of disease progression (3, 10). To circumvent these limitations, we and others (6, 7, 13) have developed SIV nucleic acid-based viral load assays to directly quantitate viremia in the plasma of macaques infected with SIV and related viruses.

Presented here is a detailed description of an internally controlled PCR-based assay (quantitative-competitive PCR [QC-PCR]) to quantitate plasma SIV RNA. This assay was used to evaluate the correlation between plasma viremia, p27 antigen level, and infected-cell frequency with survival in macaques infected with SIVsmE660. Plasma viremia showed the strongest association with survival, and the strength of the correlation was dependent on the time postinfection (p.i.). Plasma viremia levels as early as week 6 p.i. were found to be highly predictive of survival time.

MATERIALS AND METHODS

Animals. The animals used in this study were 10 macaques (*Macaca mulatta*) infected with SIVsmE660 (50 50% monkey infectious doses, intravenously) and were controls in a passive-immunity study (9). Six of the control macaques received a placebo (normal macaque immunoglobulin; two 170-mg/kg doses [24 h and 2 weeks p.i.] intravenously), and four were untreated. The animals were housed in the BL2/3 nonhuman primate holding facility in the Wexler Institute for Pediatric Research at Children's Hospital, Columbus, Ohio. During the study

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† This paper is dedicated to C.A.W.

six animals were euthanized to eliminate pain and discomfort caused by the preterminal illnesses described below. One died without intervention, and one was euthanized prematurely for reasons including a questionable tuberculosis test reaction unrelated to AIDS. This latter animal was excluded from the survival analyses. The following were the clinical signs and the necropsy findings. Animal 192 was euthanized at week 18 p.i. due to rapid weight loss and depression. Animal 184 was euthanized at week 42 p.i. due to wasting, dehydration, and ataxia; necropsy findings included involution of lymphoid tissue in the lymph nodes and spleen and multiple pleural adhesions. Animal 104 was euthanized at week 43 p.i. after cough developed with a poor response to antibiotic therapy; necropsy findings included moderate to marked peripheral and mesenteric lymphadenopathy, lungs mottled, deep red, and firm (interpreted as consolidation secondary to pneumonia), and multiple pleural adhesions. Animal 88 was euthanized at week 53 p.i. after weight loss, diarrhea, and dehydration; gross necropsy findings included dehydration and a lack of subcutaneous adipose tissue. Animal 182 was euthanized at week 78 p.i. and had an elevated peripheral leukocyte count of 99,400 cells/μl, typed as B-cell leukemia; necropsy findings included lymphoma involving the stomach, intestine, mesenteric nodes, liver, diaphragm, and omentum. Peripheral and tracheobronchial lymph nodes were not involved. Additional findings included pulmonary arterial thrombosis with pulmonary infarction. Animal 195 was found dead in the cage at week 65 p.i. with no premonitory signs of illness; necropsy findings included embolus in the pulmonary trunk and large thrombus arising in the right ventricle and extending through the pulmonic valve. Additionally, there were multiple pleural adhesions and marked lymphoid follicular hyperplasia in the lymph nodes and spleen. Animal 213 was euthanized at week 30 p.i. due to wasting, chronic diarrhea, and dehydration with inguinal rash with secondary bacterial infection; necropsy findings include involution of all lymphoid tissue.

Plasmid (pCon-1) construction. To construct a plasmid containing the truncated internal control, noncontiguous regions of SIVmne *gag* DNA were ligated to produce a deletion of 82 bp. To do this, two mutagenic PCRs (MUT-A and MUT-B) were first performed with SIVmne DNA (GenBank accession number M32741; the *gag* sequences are underlined in the mutagenic primers below) as a template to produce two products from the *gag* gene. MUT-A used the following two primers: 5′-CCGGCGAAGCTTGAGAAACTCCGTCTTGTCAGGGAAG-3′ (*gag* homology at bp 541 to 565) and 5′-GCGCGTGGTACCGAATGCACCAGATGACGCAGACAG-3′ (*gag* homology at bp 774 to 797). Product MUT-A contained native SIVmne *gag* gene sequence from bp 541 to 797 and was modified during PCR to add cloning sites on each end (*Hin*dIII at the 5′ end and *Asp*718 at the 3′ end). MUT-B used the following two primers: 5′-CGCCCAGGTACCGCCAAAAACAAGTAGACCAACAGC-3′ (*gag* homology at bp 886 to 909) and 5′-CCAATTGGATCCATCTCCTGTAAATGTTGC-3′ (*gag* homology at bp 1311 to 1340). Product MUT-B contained native SIVmne *gag* gene sequence from bp 886 to 1334 and was modified during PCR to add a cloning site on one end (*Asp*718 at the 5′ end). These PCR products were then ligated together such that 88 bp (bp 798 to 885) was replaced by a 6-bp *Asp*718 recognition sequence, resulting in a net 82-bp deletion. The product of this ligation contained bp 541 to 797 adjacent to bp 885 to 1329, with a PCR-generated *Asp*718 site joining them. To construct the pCon-1 plasmid, the truncated SIVmne *gag* DNA was cloned into plasmid pSP73 downstream for the T7 RNA polymerase promoter element to permit transcription of RNA competitors.

Preparation of pCon-1 RNA competitor. The pCon-1 plasmid containing the internally deleted SIV *gag* insert was linearized by digestion at the *Bam*HI cloning site downstream from the T7 promoter, treated with proteinase K (50 μg/ml) at 37°C for 30 min, extracted twice with phenol-chloroform-isoamyl alcohol (24:24:1) (Gibco BRL, Grand Island, N.Y.), and precipitated with ethanol. The dried pellets were dissolved in TE (EDTA, 0.1 mM; Tris, 10 mM; pH 7.4) and analyzed on 1% agarose gels to confirm linearity, and the DNA concentration (optical density at 260 nm) was determined. RNA transcripts were made by using an RNA transcription kit and procedures recommended by Stratagene (La Jolla, Calif.). RNA transcripts were purified from the DNA template by digestion with RNase-free DNase (1 U/μg of DNA), extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (24:24:1) and once with chloroform, and fractionated on an RNase-free G50 column [Select D (RF); 5-Prime-3-Prime, Boulder, Colo.] according to the manufacturer's instructions. Eluted RNA was precipitated with ethanol, dissolved in diethyl pyrocarbonate-treated water, and analyzed on 1% agarose gels to confirm digestion of plasmid DNA. The purified pCon-1 RNA was quantitated by optical density at 260 nm and by fluorimetry with ethidium bromide.

Preparation of plasma viral RNA. The following procedure is modified from the QC-PCR assay described by Piatak et al. (25). Whenever possible the procedures described below were carried out in a biosafety hood. Fresh or frozen EDTA-anticoagulated plasma samples were precleared in a Microfuge (Beckman no. 5415C; 10,000 × *g*, 15 min, 4°C). Aliquots of plasma (0.25 ml) were diluted 1:8 in phosphate-buffered saline, and virus was pelleted at 40,000 rpm for 70 min in a Beckman type 50.3 rotor (115,200 × *g*) with Beckman polycarbonate thick-walled tubes (1/2 by 2 1/2 in.). (A comparative analysis of ultracentrifugation versus a Microfuge-based centrifugation [14,000 rpm, 4°C, 90 min; Beckman Microfuge no. 5402] [6] has been conducted with plasma from HIV-2_{287}-infected macaques. Results from [i] replicate analyses of a single plasma sample, [ii] analysis of a plasma sensitivity panel, and [iii] an evaluation of optimal Microfuge times showed that the Microfuge procedure gave results equivalent to those with ultracentrifugation [data not shown]. The Microfuge-based procedure has advantages derived from the use of capped disposable tubes, fewer sample transfer steps, and compatibility with heating blocks allowing elevated-temperature [56°C] proteinase K digestions [see below].) The pellets were vortex resuspended in 300 μl of 20 mM Tris-HCl (pH 7.5)–150 mM NaCl–2 mM EDTA containing 1 mg of proteinase K per ml and 0.1% sodium dodecyl sulfate and incubated at 37°C for 1.5 to 2 h. (In Microfuge-based procedures and with capped tubes, equivalent results are obtained if digestions take place at 56°C for 30 min instead of at 37°C for 90 min.) Following transfer to 1.7-ml polypropylene Microfuge tubes, samples were vigorously extracted three times with 300 μl of phenol-chloroform-isoamyl alcohol (24:24:1) and once with 300 μl of chloroform, with the lower organic layer being removed at each extraction. To precipitate the RNA, the upper aqueous layer was transferred to a new tube and adjusted to contain 40 μg of glycogen/ml and 10 ng of 7.5-kb synthetic RNA/ml (Gibco BRL), 10 mM Tris (pH 7.5), and 1 mM EDTA by using a buffer containing 10× concentrations of these reagents. Ethanol (900 μl) was then added, and following storage for at least 16 h at −20°C, RNA was pelleted in a Microfuge (14,000 rpm, 30 min, 5°C). (We have established in three separate experiments that quantitative RNA recovery can be accomplished after only 30 min of ethanol precipitation at −20°C.) The ethanol was carefully aspirated, and the pellet was dissolved in 25 μl of DEPC-treated water and stored at −70°C. Prior to analysis, the RNA preparations were routinely treated to remove possible DNA contamination. DNase (2 U; amplification grade; Gibco BRL) and 10× DNase buffer (3 μl; Gibco BRL) were added to each RNA preparation and incubated for 37°C for 30 min, and then 20 mM EDTA (3.3 μl) was added and the reaction mixture was heated 65°C for 10 min. Samples were then stored at −70°C prior to analysis.

RT and PCR. The viral RNA samples were serially diluted in a 96-well PCR microplate into a reaction buffer containing a fixed copy number of competitor RNA and were subjected to reverse transcription (RT) and then PCR. The following primers were used for the RT and PCR to detect SIV (nucleotide positions are from the SIVmne *gag* sequence): 5′ primer (5G), nucleotides 675 to 698, AAAGCCTGTTGGAGAACAAAGAAG; 3′ primer (3Diii), nucleotides 993 to 1011, AATTTTACCCAGGCATTTA. (With primers 3Diii and 5G the assay has been used to quantitate plasma viremia in macaques infected with SIVmne, SIVmneE11S, SIVsmE660, SIVmac, and chimeric simian-human immunodeficiency virus constructs.) For the RT reaction, viral RNA (3 μl) was serially diluted (1:4) into buffer containing a known number of copies of internal competitor RNA (usually 100 copies), placental RNase inhibitor (20 U; Boehringer Mannheim, Indianapolis, Ind.), Moloney murine leukemia virus reverse transcriptase (50 U; Gibco BRL), the 3′ primer (3Diii) (0.5 μM final concentration), and magnesium (to 6 mM final concentration) in a volume of 30 μl. All serial dilutions, RT reactions, and PCRs were carried out in Perkin-Elmer thin-walled tubes in a 96-well format designed for the Perkin-Elmer 9600 thermocycler. The RT reaction was conducted at 42°C for 15 min followed by inactivation at 99°C for 5 min. PCR was performed by adding to each well a master mix (70 μl) containing 10× PCR buffer (Boehringer Mannheim), *Taq* polymerase (2.5 U; Boehringer Mannheim) mixed 1:1 with Taq Start antibody (Clontech, Palo Alto Calif.) (see below), 5′ primer (5G or 5Gii; 0.15 μM final concentration), and magnesium (3.5 mM final concentration). (Mispriming and formation of primer-dimers are problems that both reduce the sensitivity of the QC-PCR assay and increase the ambiguity of assigning equivalence points. In our hands, "hot-start" procedures using wax barriers were only partially effective in reducing these artifacts and were technically cumbersome. The use of Taq Start antibody to block *Taq* polymerase activity prior to the first denaturation step virtually eliminated primer-dimers and mispriming and increased the assay sensitivity about fivefold [see Fig. 1].) Optimal thermocycling conditions in a Perkin-Elmer 9600 thermocycler have been established to be as follows: 95°C for 1 min, followed by 45 cycles of 95°C for 10 s, 55°C for 30 s, and 72°C for 30 s, followed by extension at 72°C for 9 min. Amplified products were separated on 30-well 3.0% agarose HT gels (ISS Corp) containing 0.25 μg of ethidium bromide per ml. The gels were illuminated at 254 nm and photographed with Polaroid type 57 film. The internal RNA control contains a deletion of 82 bp which enables discrimination between viral (336-bp) and internal control (254-bp) amplified products. The equivalence point is the dilution of sample RNA which gives a signal visually equivalent to that of the internal RNA control. It has been our experience that the visual determination of the equivalence point from photographs of the gels is sufficiently obvious that video imaging is unnecessary. Once the equivalence point has been assigned, the RNA equivalents per milliliter are calculated by using an Excel spreadsheet (Microsoft Corp., Redmond, Wash.), in which a correction is applied to adjust for the theoretical differences in ethidium bromide fluorescence resulting from the different molecular sizes of the viral DNA band (336 bp) and the internal control band (254 bp). Therefore, a factor of 1.32 (336/254) is used to reduce the visually assigned equivalence point prior to calculation of RNA equivalents per milliliter.

Assay validation and sample quantitation procedures. Positive and negative controls are included in all analyses. The positive control is a plasma sample from an infected monkey and is stored at −70°C as subaliquots. To qualify the assay, a positive control is included in every batch of plasma samples tested. If the positive control equivalence point is greater than one dilution (1:4) from the historically established mean equivalence point, the assay is repeated. The negative control is phosphate-buffered saline and controls principally for PCR contaminations and correct amplification of the internal control RNA. To determine

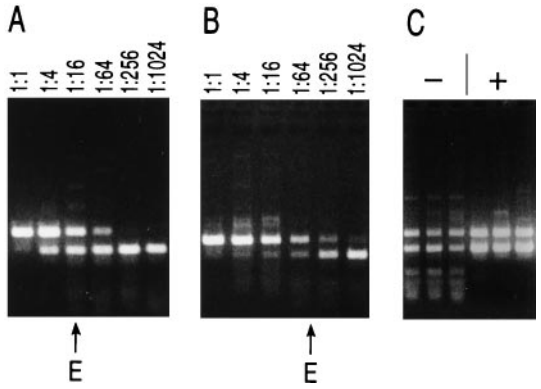

FIG. 1. QC-PCR analysis of plasma SIV. Shown are examples in which the equivalence point (E) falls on a sample RNA dilution (A) or between dilutions (B). (C) Three replicate analyses of an SIV RNA sample amplified in the absence (−) or presence (+) of a *Taq* polymerase-inhibiting antibody. In these assays, 500 copies of competitor template were used, and PCR products were analyzed on 3.0% agarose HT gels containing 0.25 μg of ethidium bromide per ml. The gels were illuminated at 254 nm and photographed with Polaroid type 57 film. The upper band (336 bp) is viral; the lower band (254 bp) is the competitor.

if viral RNA preparations are contaminated with viral DNA, control reactions in the absence of reverse transcriptase can be run. We did not include this control in the assays described here because all viral RNA preparations were DNase treated prior to analysis. During the development of the QC-PCR assay methodology, when samples were analyzed in the absence of reverse transcriptase, and following DNAse treatment, no evidence for the presence of contaminating viral DNA was obtained. For sample analysis, the viral RNA in each plasma is extracted twice. Both RNA extracts are analyzed in one RT-PCR experiment. If the equivalence points of the duplicate analyses differ by more than one serial dilution (1:4), a third extraction of RNA is done. All three samples are simultaneously analyzed in RT-PCR, and the two equivalence points with a difference equal to or less than 1:4 are used to calculate RNA equivalents per milliliter, as described above. In a review of our RNA extraction records for 750 plasma samples over a 2-year period, a third extraction was required in 13 cases. This corresponds to only 1.7% of plasma samples for which duplicate determinations of the equivalence point differed by more than fourfold.

The assay described here can detect 100 copies of RNA internal competitor per reaction. This establishes the lower limit of assay quantitation. Adjusting for sample volumes and dilutions and including the ethidium bromide fluorescence correction factor due to the different molecular weights of the viral and competitor amplified DNAs, this translates to a lower limit of quantitation of 3,333 RNA copies/ml of plasma.

p27 core antigen assay. Plasma samples were assayed for p27 antigen by the Coulter SIV Core Ag procedure (Coulter Corporation, Hialeah, Fla.) with standards provided in the kit.

Assay of infected-cell frequency. Serial fivefold dilutions of previously frozen macaque peripheral blood mononuclear cells (PBMC) starting at 10^6 cells/well were cocultivated with fresh human PBMC as described previously (14), with the exception that $CD8^+$ cells were depleted from human PBMC prior to stimulation with phytohemagglutinin A. Cultures were incubated for 14 days, and the presence of virus was detected by using an HIV-2 p27 antigen capture assay. The virus load was calculated as the number of infectious units per 10^6 input PBMC.

Statistical analyses. The relationship between survival and viral load parameters (plasma viremia, p27 antigen, and frequency of infected cells) during the course of infection was evaluated by using Spearman's rank correlation procedure. Only p27 assay values of above 0.1 ng/ml were used calculate the rank order. The significance levels of correlation coefficients were determined by using a standard table of critical values of *r*. The curve fit in Fig. 4 was generated by Cricket Graph III V1.5.2.

RESULTS

Assay performance. Typical analyses of SIV RNA are shown in Fig. 1A and B. The equivalence point is assigned to be the point where the dilution of the viral RNA sample gives a band on agarose gel analysis with an intensity equivalent to that of the competitor band. When the equivalence point falls between dilutions (for example, 1:64 and 1:256 in Fig. 1B), then it is assigned the intermediate dilution (1:128).

One of the most successful improvements introduced was the inclusion of an antibody that blocks low-temperature activity of *Taq* polymerase. Figure 1C shows a comparison of replicate analyses of a single SIV plasma RNA done in the presence and absence of *Taq* binding antibody. In the presence of the antibody, highly specific amplifications are obtained and both viral and competitor bands are more intense, while the low-molecular-weight bands usually associated with primer-dimers have been essentially eliminated. This has resulted in more easily assigned equivalence points and an increase in assay sensitivity. In the present assay configuration, the lower limit of assay quantitation is 3,333 RNA equivalents/ml of plasma.

While formation of primer-dimers has been effectively eliminated, samples in which multiple bands with molecular weights higher than that of the viral band are observed are still encountered (data not shown). These bands are detected in the absence of the truncated competitor, and, although mispriming is a possible cause, modifications such as increasing the annealing temperature have not eliminated them. We suspect that these bands are heteroduplexes and may be a consequence of amplifying complex viral populations.

Interassay variability. The RT reactions and PCR amplifications of the plasma viremia assay are often regarded as the steps with the highest potential for introducing assay variation. To examine the variability inherent to these reactions, a purified SIV RNA preparation was replicate assayed 16 times in a multiwell PCR plate. This procedure was repeated on two separate days. Figure 2A shows that the interassay and interday variations in equivalence points obtained were small (day 1 geometric mean = 38.05, 95% confidence interval = 33.49 to 43.24; day 2 geometric mean = 49.35, 95% confidence interval = 33.8 to 72.04).

The assay variation associated with replicate analyses of a single plasma sample over time was also assessed by retrospective analysis of the positive control data acquired over a period of more than 5 months. Thirty-three data points were available during this period, which resulted in a mean value of 2.63×10^5 RNA equivalents/ml, a standard deviation of $\pm 1.03 \times 10^5$ RNA equivalents/ml, and a 39.1% coefficient of variation (CV). These data are comparable to those for the SIV plasma viremia assay recently reported by Hirsch et al. (CV = <25%) (13) and commercial plasma viremia assays (Amplicor RT-PCR assay [Roche Molecular Systems], CV = 52 to 82%; Quantiplex bDNA HIV-1 assay [Chiron Corp], CV = 15 to 26%) (30).

Relationship between input RNA and detected RNA levels. Since the assay for measurement of viral RNA is a multistep procedure, it was important to confirm that a linear relationship existed between the input viral load in plasma and the RNA level calculated from the equivalence point. To address this, a plasma sample from a macaque infected with SIVmne was serially diluted (1:3) into normal macaque plasma, and the viral RNA was extracted and assayed by standard procedures. This experiment was repeated on three separate occasions, and the combined results are shown in Fig. 2B. The log of the observed RNA levels was linearly related to the log of the expected RNA levels over a wide range of viral RNA input. In the three experiments, the correlation coefficients (*r*) between the two parameters were 0.993, 0.984, and 0.988. Note that the lowest levels of RNA input were close to the limit of assay detection.

Plasma viral load profiles in macaques infected with SIVsmE660. Plasma viremia profiles have been monitored in 10 *M. mulatta* animals infected with SIVsmE660 for over 2 years. Figure 3 shows data from six macaques selected to illustrate the wide diversity of plasma viremia profiles observed.

A

day 1 equivalence	day 2 points
16	32
32	32
32	64
32	64
64	64
32	64
64	64
64	32
32	64
32	64
64	64
32	64
32	32
32	64
64	32
32	32
mean 38.05	49.35
95% CI. 33.49-43.24	33.8-72.04

B

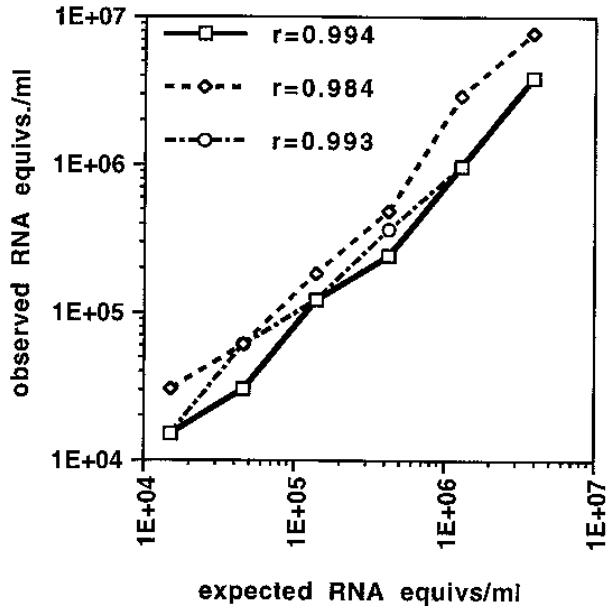

FIG. 2. Plasma viremia assay performance characteristics. (A) Variation in equivalence points obtained after replicate analyses of an SIV RNA sample on two separate days. The geometric means and associated 95% confidence intervals (CI) are calculated. (B) Results of three separate experiments using a serially diluted positive control plasma, where the relationship between the calculated plasma RNA levels (expected) and the measured RNA levels (observed) were compared.

The general profile characteristics include a peak of primary viremia occurring around day 14, followed by a decline of variable magnitude and duration and then an extended viremia rebound. Some animals, however, showed more extreme profiles. For example, in animal 192, the plasma viremia was the highest of the group at day 7 p.i. and continued to increase until euthanasia at week 18. In contrast, animal 200 had plasma virus detectable only at one time point, day 14 p.i. Subsequently, plasma viremia declined below the level of assay detection and remained there for the duration of monitoring.

Relationship of plasma viral load, p27 antigen, and infected-cell frequency with survival. To date, 2 of the 10 *M. mulatta* animals infected with SIVsmE660 are still alive. Six animals were euthanized, and one animal died without intervention due to AIDS-associated disease. One animal was euthanized prematurely for reasons unrelated to AIDS, and data from this animal were excluded from the survival analysis (see Materials and Methods). When the relationships between survival time and the parameters used to monitor viral load (plasma viremia, p27 antigen, and infected-cell frequency) were analyzed by using Spearman's rank correlation procedure, plasma viremia showed the strongest association, with a correlation that was statistically significant at 10 of the 13 time points examined (Table 1).

The correlation between plasma viremia and length of survival was dependent on the stage of infection. Plasma viral load during the peak of primary viremia (day 14) was not correlated with length of survival (Table 1 and Fig. 4A). As viremia declined and levels stratified between animals, the correlations improved, and the log of plasma viremia levels from week 6 onward was linearly associated with survival (Table 1 [$P < 0.05$ to $P < 0.01$] and Fig. 4B). Plasma viremia levels in samples obtained at or close to the time of euthanasia or death were also significantly correlated with survival.

In contrast, the association between p27 values and survival was significant at only two time points, week 28 p.i. and the time of euthanasia. Similarly, a significant correlation between survival and results from the infected-cell frequency assay was found only at week 12. In the case of the p27 assay, the weak correlation could be a consequence of inaccurate quantitation of p27 in plasma due to SIV-specific antibodies inhibiting the immunoassay. While we have no direct evidence for this, it is supported by the finding that in three of the seven monkeys, the detection of anti-Gag antibodies was temporally associated with an abrupt loss of p27 antigen detection (data not shown). As infection progressed, however, p27 antigen was again detected, and it was present in the plasma samples from six of the seven animals at the time of euthanasia.

DISCUSSION

Described here is an assay for the quantitation of plasma viremia in macaques infected with SIV and related viruses. This assay was used to measure plasma viremia in a group of 10 *M. mulatta* animals infected with SIVsmE660 for over 2 years. A highly significant correlation between plasma viral load and survival was found.

Of particular interest is that plasma viremia levels as early as 6 weeks after infection were highly predictive of survival. This most likely reflects the relative ability of each animal to mount an immune response capable of exerting control over viral replication. In support of this are the findings of Haigwood et al. (8) and Hirsch et al. (13), who have reported that augmenting the immune response early in infection either by administration of high-titer SIV immune globulin at days 1 and 14 p.i. or by prior immunization with a trivalent SIV-recombinant vaccinia virus is associated with reduced viral loads, sustained low plasma viremia, and extended survival.

This link between early viral load and survival is also consistent with the results of a prospective study by Mellors et al. (21), who examined 62 HIV-infected patients with documented seroconversion and found that the presence of plasma viremia levels of greater than 10^5 RNA equivalents/ml at the seroconversion visit was a powerful predictor of more rapid progression to AIDS. This relative risk association has also been demonstrated by other clinical analyses (11, 18), although a study by Jurriaans and colleagues (17) failed to find a similar correlation. An insight into the reasons for this inconsistency may be obtained from the correlations in Table 1: the power of

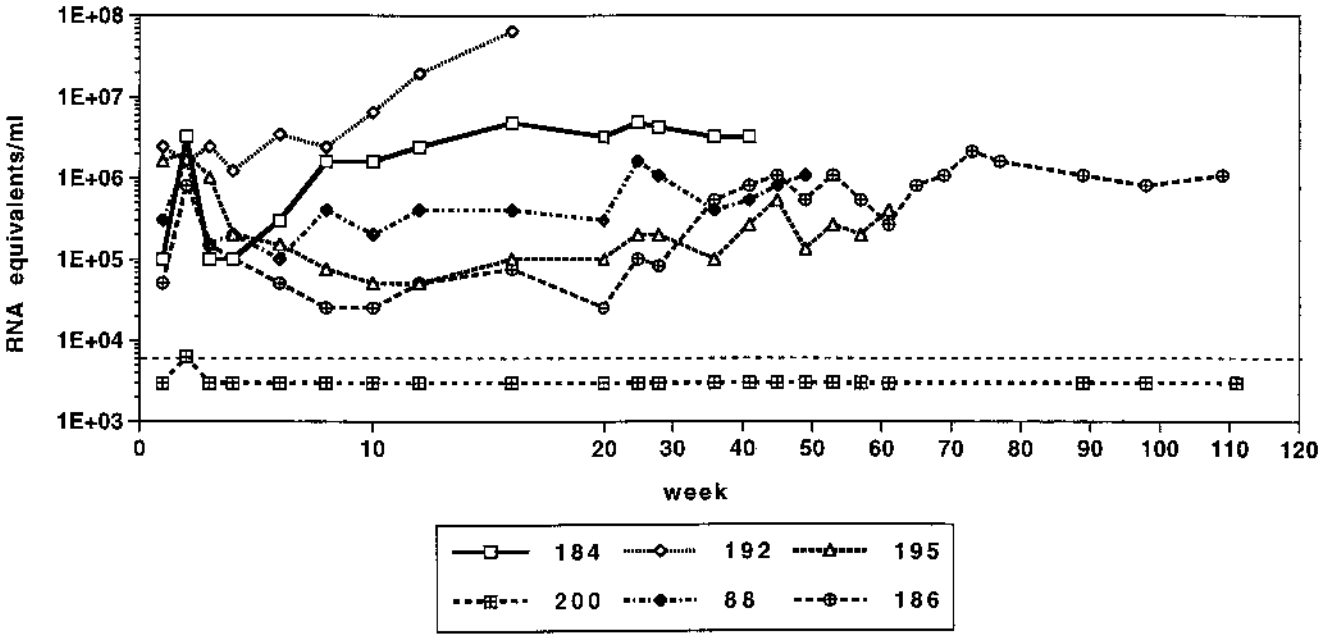

FIG. 3. Plasma viremia profiles of *M. mulatta* animals infected with SIVsmE660. Presented are results from six animals monitored either until the time of euthanasia or up to week 111 p.i. The scale to week 20 is expanded to display initial changes in viremia. Viremia was analyzed by using an earlier version of the QC-PCR assay in which the lower limit of assay quantitation was 6.3×10^3 RNA equivalents/ml (dashed line). The RNA levels for animal 200 were below the limit of detection after day 14 and were arbitrarily assigned the value of 3×10^3 RNA equivalents/ml for graphing purposes.

plasma viremia as a prognosticator of survival develops with time p.i. Since Jurriaans et al. (17) used more frequent patient monitoring than Mellors et al. (21) (3 versus 6 months) to identify the seroconversion point, it is possible that their seroconversion samples were taken before viremia levels had fully subsided following primary viremia.

A significant correlation between plasma viremia levels (and p27 levels) at euthanasia and time to euthanasia was observed (Table 1). This is in contrast to expectations from clinical data from HIV infection, where plasma viremia levels cluster according to disease stage; more advanced disease is associated

TABLE 1. Spearman rank correlations between survival and plasma virus load, p27 core antigen, and infected-cell frequencies[a]

Week p.i.	Spearman rank correlation between survival and:		
	Plasma viral load (RNA equivalents/ml)	p27 antigenemia (ng/ml)	Infected cells/ 10^6 PBMC
2	0.270	0.437	−0.604
3	0.580	0.437	0.280
4	0.645	0.333	0.604
6	0.895**	0.333	0.250
8	0.910**	0.666	0.729
10	0.958***	0.666	0.770
12	0.958***	0.708	0.937***
16	0.958***	0.708	0.770
20	1.000***	0.875	0.600
28	1.000***	1.000***	−0.542
36	1.000***	0.916	0.708
41	1.000***	0.916	ND
Euthanasia	0.791*	0.958***	ND

[a] The data are from seven *M. mulatta* animals euthanized as a result of AIDS-associated disease following infection with SIVsmE660. Significance levels of correlations: *, $P < 0.05$; **, $P < 0.02$; ***, $P < 0.01$. ND, not determined.

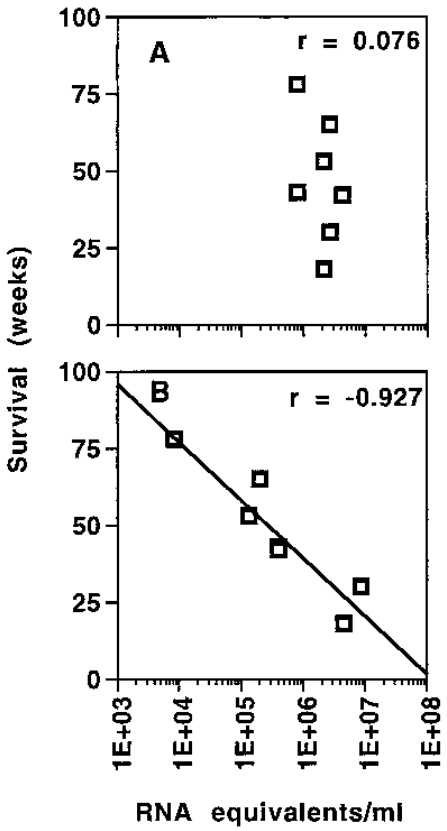

FIG. 4. Survival is linearly related to the log of plasma viremia levels. Shown are the results obtained by analyzing plasma viremia data obtained at day 14 (A) and at week 6 (B) p.i. The graph for week 6 has values from two animals superimposed. The best-fit line and correlation coefficients (r) were calculated by using Cricket Graph III V1.5.2.

with higher viral loads in most patients (1, 2, 25). While this discrepancy may reflect differences in the pathogenic courses of SIV and HIV infections, it may also be due to the policy to intervene and euthanize SIV-infected primates to avoid prolonged suffering. In contrast, the objective in HIV infection is to prolong life, and therefore clinical AIDS may represent a more advanced stage of immunodeficiency disease in which the severely compromised immune system is less able to control the viral load.

Neither p27 antigen (except at the time of euthanasia) nor infected-cell frequency showed strong statistical correlations with survival. In the case of p27 antigen, this was presumably due to core-specific antibodies interfering with antigen quantitation. It is less clear why the results from the infected-cell frequency assays were less predictive, since experience over a number of years with this assay suggests that it is a useful index of viral load. Although a number of factors have been recognized to influence this type of assay (3, 10), our inability to demonstrate a statistical correlation may be more a consequence of using frozen and stored PBMC. In contrast to the case for human PBMC (10), optimal and consistent viral recovery from macaque cocultures requires that freshly isolated PBMC be used.

The survival data for this study were obtained from seven animals that were euthanized or died due to AIDS-associated disease. Two healthy animals, animals 200 and 186 (Fig. 3), remain and have been infected for over 112 weeks. Consistent with this extended survival, animal 200 has maintained a very low viral load, undetectable plasma viremia, and stable $CD4^+$-cell counts, a profile which is reminiscent of that for HIV-infected long-term nonprogressors (4, 24, 26). In contrast, animal 186 has had a significant plasma viral load ($\sim 10^6$ RNA equivalents/ml) since week 40 p.i. (Fig. 3). The unexpected length of survival of this animal underscores the fact that viral load is a major but not exclusive determinant of the rate of disease progression, as has also been reported for HIV infection (27). It is important to note that animal 186 is not disease free, and we have observed persistent secondary infections, a gradual $CD4^+$-cell decline, and the emergence of extensive diversity in viral DNA samples from PBMC and lymph nodes (8), all of which are factors consistent with underlying disease progression. Possible viral and host factors which may delay disease are under investigation. For example, the association of *nef* deletions with reduced pathogenic potential in vivo is well established (5, 19). Whether *nef* region variants have arisen in this animal is not yet known; however, modifications in *nef* are generally associated with low viral loads in vivo, which is inconsistent with the observations for animal 186. Other factors also exist that may contribute to the absence of overt disease: a founder effect may have resulted in infection by less pathogenic viral variants, or attenuated viral phenotypes may have been selected and/or may have evolved during infection. Host factors such as the ability to maintain an equilibrium over viral cytopathic effects may also play a critical role.

The results presented here provide additional evidence that the immunodeficiency disease observed in macaques experimentally infected with SIV closely resembles the disease caused by infection of humans with HIV-1. Our study, together with the results of Hirsch et al. (13), demonstrates that SIV and HIV-1 infections have a number of features in common: a distinct phase of primary viremia followed by a decline of varying magnitude, the stratification of viremia levels between individuals, and plasma RNA levels that usually fall between 10^3 and 10^8 equivalents/ml. These studies also show that during infection with SIV and HIV, a close association between plasma viral levels, disease progression, and survival exists. In addition, our findings that a risk relationship is established between survival and plasma viremia levels early in SIV infection are also supported by clinical studies of HIV-1 infection. Taken together, these data provide a compelling argument for the validity of using primate models for the study of AIDS, and, in particular, they highlight the usefulness of such models in investigations of the events occurring early in infection.

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ADDENDUM

SIV RNA levels in matched plasma samples have recently been measured by using the QC-PCR assay and the Chiron branched-DNA assay. Plasma samples from eight chimeric simian-human immunodeficiency virus- and four SIVmac251-infected *M. mulatta* animals (kindly provided by Keith Reimann, Beth Israel Hospital, Boston, Mass.) were analyzed in a blinded fashion with both assays. There was a strong correlation between the results of the two assays ($r = 0.96$; $P < 0.001$) as determined by Pearson's correlation coefficient. Furthermore, the geometric mean levels of SIV RNA as determined by the two assays differed by only 0.053 $\log_{10}$ unit (1.13-fold). This difference was not significant as determined by the *t* test for dependent samples ($P = 0.61$).

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