Published in final edited form as:

AIDS. 2009 July 31; 23(12): 1453–1460. doi:10.1097/QAD.0b013e32832b43d9.

Monitoring HIV vaccine trial participants for primary infection: studies in the SIV/macaque model

James B. Whitney^{a,b}, Corinne Luedemann^a, Saran Bao^c, Ayako Miura^a, Srinivas S. Rao^c, John R. Mascola^c, and Norman L. Letvin^{a,b,c}

^aDivision of Viral Pathogenesis, Department of Medicine, Beth Israel Deaconess Medical Center, Boston, Massachusetts

^bHarvard Medical School, Boston, Massachusetts

^cVaccine Research Center, National Institutes of Allergy and Infectious Diseases, Bethesda, Maryland, USA

Abstract

Objectives—The ability to detect and quantify acute HIV-1 infection prior to seroconversion would be an important tool for use in HIV vaccine clinical efficacy trials. We have utilized the SIV/rhesus monkey model to evaluate whether samples more easily obtained than peripheral blood might be used for intensive monitoring of vaccine trial participants.

Methods—We have evaluated viral loads in peripheral blood, saliva, feces, and urine of five rhesus monkeys during primary SIV_{mac251} infection by quantitative real-time PCR. As an alternative to the direct monitoring of frozen samples, we have also developed a fully quantitative viral load assay utilizing dried blood spots.

Results—Although all compartments were found to harbor viral RNA during primary infection, viral RNA could be detected in the peripheral compartments only when levels of plasma viremia exceed a threshold value of 10⁴ RNA copies/ml. We found no direct correlation between viral burden in plasma and saliva, feces, or urine viral loads. Importantly, both dried saliva and whole blood spots can be used for viral detection. Quantitative whole blood or plasma spotting correlated well with viral burden in plasma during both the acute and set point phase of infection.

Conclusion—Dried blood spots are amenable to rapid quantitative viral load testing. Whole blood spotting has a significant logistical benefit as it requires low blood volumes and no blood processing. Saliva or dried saliva spots or both are potential candidates for acute phase diagnostic screening. These studies indicate the feasibility of intensive monitoring of HIV-1 vaccine trial participants for virus acquisition in resource-limited settings.

Keywords

acute HIV infection; HIV-1 infection monitoring; simian immunodeficiency virus

Introduction

The ability to detect and quantify HIV-1 in humans prior to their seroconversion would contribute to the evaluation of vaccine efficacy in international HIV vaccine trials. Although the practical application of nucleic acid testing in HIV diagnostics has been well established [1,2], most currently employed methods for viral detection and quantification rely on venipuncture and processed blood plasma. These methods require trained personnel and modern, well equipped laboratory facilities that are not routinely available at resource-limited vaccine trial sites [3,4].

A strategy for screening at-risk human populations for HIV-1 infection using specimens from easily sampled compartments would provide a valuable tool for monitoring large cohorts of vaccinees. Numerous studies have shown the presence of viral RNA in a variety of compartments during the course of HIV infection, including the gut, saliva, breast milk and semen [5–10]. However, limited information is available regarding the correlation during acute HIV infection between viral RNA levels in the blood and in other compartments. Moreover, the presence, magnitude and duration of measurable viral RNA in various compartments (i.e. saliva, urine and feces) during acute HIV infection are not well described. Similarly, the predictive value of viral RNA levels in these different compartments has not been defined. Information of this type is critical for the development of rapid, high-throughput monitoring technologies for high-risk populations or vaccinees in HIV vaccine trials in resource-limited settings [3].

In the current study, we have employed the SIV/rhesus monkey model to determine if alternatives exist to routine venipuncture for monitoring HIV-1 infection in humans. Our aim was the detection and quantification of SIV RNA in simultaneously obtained samples of blood, saliva, urine and feces during the first 12 weeks following SIV_{mac251} infection. To evaluate an alternative to using frozen samples for analysis, we also developed a quantitative SIV assay using dried sample spots [11].

Methods

Animals and SIV infection

This study enrolled and monitored five adult male rhesus monkeys (*Macaca mullata*) that were 4–6 years of age. All animals were screened and found to be negative for SIV and simian retrovirus type D prior to the initiation of this study. The monkeys were housed under Biosafety level-2+ conditions. All experiments were conducted in accordance with Institutional Animals Care and Use Committee standards. Prior to, and routinely after SIV infection, all animals were monitored by physical exam, complete blood count with differential, and T cell subset analysis.

All animals were experimentally injected with a 1 ml bolus of SIV_{mac251} (2.11×10^5 viral RNA copies), directly into the saphenous vein. Productive infection of each monkey was confirmed by demonstration of seroconversion by western blot analysis (not shown).

Sample collection and processing

Viral RNA levels were monitored in peripheral blood, saliva, feces and urine for the first 12 weeks after infection. Specimens were collected twice weekly for the first 6 weeks; all samples thereafter were collected every second week for a total duration of 12 weeks. Blood was collected into EDTA anticoagulant. Saliva was collected by cotton roll Salivette (Sarstedt, Nümbrecht, Germany). Both feces and urine were obtained by early morning clean pan collection.

Blood was routinely processed by Ficoll-Hypaque overlay (Pharmacia, Uppsala, Sweden). Saliva was centrifuged immediately after collection at 4°C and according to the manufacturer's recommendations (Sarstedt). Fecal samples were immersed into equal amounts (w/v) of *RNAlater* (Ambion, Inc., Austin, Texas, USA) and homogenized by vortexing. Both fecal supernatants and urine were clarified by low-speed centrifugation and filtered through 100 µmol/l filters (BD Falcon, Franklin Lakes, New Jersey, USA) prior to aliquoting.

Samples were spotted onto Whatman 903 protein saver cards. Forty microliters of saliva or 50 µl of whole unprocessed blood were placed as individual spots onto each card. Spots were dried for 2–3 h prior to being packaged in sealable sandwich bags and stored in the dark at room temperature, with desiccant until required for RNA extraction. Liquid samples were immediately frozen and maintained at –80°C until use. Dried sample spots were cut (with a 1–2 mm clean filter paper border) using disposable scissors. This method allowed extraction of the entire 50 µl spotted sample.

Viral RNA isolation, quantitative real-time PCR, and assay evaluation

Viral RNA was routinely isolated from 200 µl or 400 µl of cell-free blood plasma, clarified saliva, urine or feces using the NucliSENS Isolation Kit (Biomerieux, Lyon, France) following the manufacturer's protocol. Excised sample spots were also processed using the NucliSENS Isolation Kit, with the exception that spots were incubated in NucliSENS lysis buffer for 2 h at room temperature with rocking, prior to further purification steps. To remove any contaminating cellular DNA, sample spot RNA preparations were DNase treated for 20 min at 37°C followed by heat inactivation at 75°C for 10 min prior to reverse transcription of template RNA.

The SIV RNA standard was transcribed from the pSP72 vector containing the first 731 bp of the SIV_{mac239}-Gag gene using the Megascript T7 kit (Ambion, Inc.). RNA was isolated by phenol-chloroform purification followed by ethanol precipitation. All purified RNA preparations were quantified by optical density. RNA quality was determined by Agilent bioanalyzer RNA chip (Agilent, Inc., Santa Clara, California, USA).

Quantitative real-time PCR (qRT-PCR) was conducted in a two-step process. First, RNA was reverse transcribed in parallel with an SIV-*gag* RNA standard using the gene-specific primer sGag-R 5'-CACTAGGTGTCTCTGCACTATCTGTTTTG-3' under the following conditions: the 50 µl reactions containing 1× buffer (250 mmol/l Tris-HCl pH 8.3, 375 mmol/l KCl, 15 mmol/l MgCl₂), 0.25 µmol/l primer, 0.5 mmol/l dNTPs (Roche, Nutley, New Jersey, USA), 5 mmol/l dTT, 500 U Superscript III RT (Invitrogen, Carlsbad, California, USA), 100 U

RnaseOUT (Invitrogen) and 10 µl of sample. Real time conditions were 1 h at 50°C, 1 h at 55°C and 15 min at 70°C. All samples were then treated with RNase H (Stratagene) for 20 min at 37°C. All real-time PCR reactions used EZ RT-PCR Core Reagents (Applied Biosystems, Foster City, California, USA) following the manufacturer's suggested instructions under the following conditions: the 50 µl reactions containing 1× buffer (250 mmol/l bicine, 575 mmol/l potassium acetate, 0.05 mmol/l EDTA, 300 nmol/l passive reference 1, 40% (w/v) glycerol, pH 8.2, 0.3 mmol/l each of dATP, dCTP, dGTP, 0.6 mmol/l dUTP, 3 mmol/l Mn (OAc)₂, 0.5 U uracil *N*-glycosylase, 5U r*Tth* DNA polymerase, 0.4 µmol/l of each primer and 10 µl of sample template. PCR reagents were assembled at room temperature and spun briefly to eliminate air bubbles. Following 2 min at 50°C, the polymerase was activated for 10 min at 95°C, and then cycling proceeded at 15s at 95°C and 1 min at 60°C for 50 cycles. Primer sequences were adapted from those described by Cline *et al.* [12], forward primer s-Gag-F: 5'-GTCTGCGTCATCTGGTGCATTC-3', reverse primer s-Gag-R: 5'-CACTAGGTGTCTCTGCACTA TCTGTTTTG-3' and the probe s-Gag-P: 5'-CTTCCTCAGTGTGTTTCACTTTCTCTCTGCG-3', linked to Fam and BHQ (Invitrogen). All reactions were carried out on a 7300 ABI real-time PCR system (Applied Biosystems) in triplicate according to the manufacturer's protocols.

Preliminary experiments were done to evaluate the quantitation of blood plasma, saliva, feces and urine viral RNA levels and their correlation with known quantities of viral RNA added to compartment specimens sampled from SIV-uninfected monkeys (data not shown). To determine the linear range of the assay, RNA extraction efficiency, potential inhibition of reverse transcriptase activity, and the presence of compartment-specific RNase activity, known quantities of serially diluted SIV_{mac251} viral stock were spiked into samples of peripheral blood, feces, urine or saliva that were recovered from three SIV-naïve monkeys. No significant differences in extraction efficiency were observed between the blood and samples derived from other compartments (data not shown). Furthermore, using the extraction methods described above, we observed no significant inhibition of RT activity or diminution of input RNA as a consequence of RNase activity present in any of the virus-spiked specimens (data not shown). All correlations between compartments were statistically significant. Correlations between blood and saliva ranged between 0.9843 and 0.9994 (Pearson's *r*), *P* = 0.006 to less than 0.0001, blood and urine, *r* = 0.9627–0.9991, *P* = 0.086 to less than 0.0001 and blood and feces, *r* = 0.9812–0.9965, *P* = 0.010 to less than 0.0003 as measured over eight orders of magnitude. These findings indicated, therefore, that it was appropriate to employ this technique to isolate viral RNA from study samples of peripheral blood, saliva, feces and urine from the newly infected rhesus monkeys.

The analytical sensitivity of our quantitative RT-PCR assay was determined by using a series of purified *gag* RNA transcripts serially diluted and tested eight times; each individual run was conducted in triplicate. In these experiments, all dilutions containing four or more RNA copies could be detected in 100% of the samples. The assay detection limit was determined to be 60 copies of viral RNA when using 200 µl of blood plasma for the RNA extraction and transferring 10 µl RNA from 30 µl of extraction eluate. Similarly, the assay detection limit for 200 µl of saliva, urine or clarified feces was determined to be 125 viral RNA copies.

Analysis of SIV-specific antibody responses

Binding antibodies to SIV proteins were analyzed by western blot. Commercially available SIV western blot strips (ZeptoMetrix, Buffalo, New York, USA) were probed with plasma samples and developed according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was conducted using commercially available software GraphPad Prism version 4.0b (GraphPad Software, Inc., San Diego, California, USA). Linear regressions were conducted to determine the significance of correlations. Significance of differences between groups was determined using Kruskal–Wallis analysis of variation (ANOVA) as appropriate. Probability values of P less than 0.05 were interpreted as significant.

Results

qRT-PCR analysis of SIV-RNA showed that all compartments of the monkeys were viral RNA-positive during the acute phase of infection. The kinetics of viral RNA expression during this time was characterized by a period of undetectable RNA levels (below 60 copies/ml, triplicate samples), followed by a peak of viral RNA production, and then a set point nadir in the first 5 weeks after infection. The largest amount of viral RNA in the saliva, feces and urine was observed during the first 4 weeks of viral replication, coincident with the rise and fall of viral RNA levels observed in the peripheral blood (Fig. 1).

The median peak in viral RNA levels in the blood of these five monkeys (day 14 after infection) was 10^7 vRNA copies/ml of plasma in the peripheral blood, 10^5 vRNA copies/ml for both saliva and feces and 10^3 vRNA copies/ml in the urine. SIV RNA reached detectable levels in the peripheral blood as early as day 3 following infection. Viral RNA was detected in saliva in two of the five animals by day 10 and all animals by day 14. Both feces and urine of all animals had readily detectable levels of viral RNA by day 14. However, both feces and urine viral RNA levels fell to undetectable levels by 4 weeks postinfection.

Viral RNA was detected in the peripheral blood and saliva of the monkeys over a prolonged period of time. Saliva viral RNA levels varied in each monkey, consistent with the plasma viral RNA set point values. During the postacute phase of infection (after day 35), viral RNA levels in saliva were diminished significantly. Occasional positive saliva samples were, however, observed in animals with high set point plasma viral RNA levels.

To determine the total amount of detectable viral RNA in each of the sampled compartments, we calculated the total viral burden during acute viremia by area under the curve analysis (AUC). We found a significant difference ($P = 0.003$) between the AUC values in these monkeys in the peripheral blood and the other compartments. Blood plasma RNA levels reached 10^8 viral RNA copies/ml whereas the other compartments were found to be, on average, two orders of magnitude lower. We found that the levels of viral RNA did not differ significantly in saliva, feces or urine (Fig. 2).

In view of the established importance of plasma RNA levels for clinical disease progression in HIV-infected individuals, we were interested in exploring the association between this

value and viral RNA levels in each of the other monitored compartments in these monkeys. No direct correlation was observed between plasma viral RNA levels and viral RNA levels measured in saliva, feces or urine (Fig. 3). Interestingly, however, these data demonstrate that viral RNA is detectable in the saliva, feces and urine only when plasma viral loads are in excess of 10^4 RNA copies/ml in the blood. This observation suggests that total body viral RNA levels must surpass a threshold prior to their detection in saliva, feces and urine. The viral RNA in these compartments may even represent 'spillover' from the blood.

To complement these studies, we were also interested in exploring the utility of assessing viral RNA levels in an easily and inexpensively stored format. We, therefore, evaluated the use of saliva and whole blood spotting onto Whatman 903 cards for monitoring and quantification of viral RNA. Dried sample spotting obviates the need for sample processing and requires low sample volumes. Moreover, control experiments assessing viral RNA extracted from dried blood spots (DBSs) up to 6 months after spotting showed that this method allowed for stable room temperature sample storage (data not shown). Similar results regarding the stability of DBS specimens have also been reported by other groups [11,13]. Although the risk of carrying over contamination from cutting devices is low [14], we chose to avoid this altogether and cut out the entire dried sample spot with a 2 mm border. This also provided the benefit of an exact sample volume for extraction (i.e., 50 μ l) resulting in very low sample-to-sample variation in viral RNA quantification.

In contrast to other sampling methods, the monitoring of viral RNA levels from dried saliva spots would allow the rapid and simple screening of large cohorts of vaccinees. Therefore, we monitored SIV RNA levels using dried saliva spots (Fig. 4a) and detected viral RNA as early as day 14 in three of the five animals, in all five of the animals on day 17 and in four of the 5 animals on day 21. We then compared these values to those obtained from the monitoring of frozen saliva samples. The association of spotted saliva viral RNA levels with the viral RNA levels obtained from frozen saliva was modest ($r = 0.6253$, $P < 0.0001$) (Fig. 4b.). These results likely reflect not only the diminished total viral RNA levels present in this compartment, but also the smaller sample volumes used in saliva spotting (50 μ l) than in the monitoring of frozen samples (200 μ l).

In contrast, dried blood spotting provided a robust method to evaluate both SIV-infection status and viral RNA levels. The longitudinal monitoring of SIV RNA levels from dried blood spotting from these monkeys is shown in Fig. 5a. In this format, SIV RNA was detectable in four of the five animals by day 7 and all of the five animals by day 10. The median viral load as measured from dried blood spotting was 0.2 log lower than values obtained from matched samples of processed frozen plasma (Fig. 5b). Importantly, the dried blood spotting based monitoring had a dynamic range comparable to monitoring in frozen plasma, from 10^3 to more than 10^8 viral RNA copies/ml of plasma. Moreover, the correlation of DBS viral load versus plasma viral load measurements was quite good (Fig. 5c) ($r = 0.8319$, $P < 0.0001$).

Therefore, between days 10 and 28 following infection, when plasma viral RNA levels were at least 10^4 copies/ml in all evaluated monkeys, we found that all blood spots were RNA positive. By comparison, 80% of saliva, 70% of feces and 50% of urine samples were SIV

viral RNA-positive during this period. Detection of SIV RNA as dried saliva spots was approximately half (44%) compared with screening methods using fresh frozen saliva.

The detection of SIV RNA after the acute phase peak of viremia (i.e. set point) dropped to near or completely undetectable in all compartments with the exception of the peripheral blood. During this period, dried blood spotting based methods were capable of detecting SIV acquisition in 90% of samples. The 10% of samples that scored negative were from a single animal that strongly controlled set point viremia. This animal routinely had peripheral blood plasma viral RNA levels of less than 500 copies/ml after day 50 of infection.

Discussion

Vaccine studies in the SIV-macaque and simian human immunodeficiency virus-macaque models suggest that control of viral replication during primary infection is prognostic of long-term immunologic control of virus and clinical disease course. These studies show that the clinical effect of a T cell-based vaccine is most apparent during the first days following infection [15–17]. These studies demonstrate that diminished peak plasma viral RNA levels in vaccinated monkeys are associated with prolonged survival. Moreover, these studies indicate that peak plasma virus levels have greater prognostic value than set point values [16]. Monitoring of vaccinees in human HIV vaccine trials to capture data during peak viral replication would therefore be of enormous value [18].

However, obtaining such viral load data in humans during this early peak of viral replication has proven difficult. Clinical trial protocols include few time points over a large time span for infection determination, and detection of acute viremia is therefore easily missed. Sampling is particularly difficult in field trials in the developing world where vaccine recipients have typically been monitored only once every 3–6 months. The monitoring of vaccinees would have to be more intensive to capture the brief burst of viral replication during the course of primary HIV-1 infection. Furthermore, plasma specimens have been used for monitoring infection status in human clinical trials, and these specimens have been obtained by venipuncture. As these procedures require large numbers of highly trained personnel and an extensive laboratory infrastructure, they are very expensive [3].

Perhaps most importantly, human vaccinees, who have acquired HIV-1 often begin antiretroviral therapy immediately after diagnosis of infection. Set point viral load data from such individuals would be of limited value for judging the clinical outcome of vaccination. These individuals must therefore be dropped from any evaluation of the clinical utility of the vaccine intervention. In contrast, intensive monitoring for acute HIV-1 acquisition would allow early determination of infection status as well as valuable vaccine efficacy endpoints.

The data in the present study regarding compartment-specific SIV RNA levels closely parallel well described SIV RNA levels in the peripheral blood and gut-associated lymphoid tissue during primary SIV infection. The increased levels of viral RNA measured in these compartments may represent a combination of local virus production and trafficking of infected cells into these mucosal compartments during acute infection [10].

A number of further modifications of these methodologies would be needed to employ these technologies in human clinical trials. Adopting this methodology for use in trials of human vaccine candidates would require extensive evaluation to avoid problems associated with diverse viral subtype detection [19]. An additional caveat is that the frequency of sample collection to detect acute HIV acquisition would need to be quite high. Thus, determination of the optimal collection frequency for DBS and dried saliva spot would need to be tailored to the specific size and location of each vaccine candidate trial.

Sample frequency would be particularly critical if HIV monitoring was done using only saliva. The dynamic range of the viral RNA assay using saliva or dried saliva format was substantially less than the blood-based assays and likely would need improving.

Finally, it is conceivable that the duration of exposure of viral RNA to host proteases and RNases *in vivo* might be longer than that modeled in the present experiments, and, therefore, the results of these assays for human volunteers may be less sensitive.

The present studies suggest that monitoring human vaccine recipients for a quantitative determination of peak viral replication during primary infection is feasible even in resource-limited settings. Both saliva and blood appear to be useful compartments for this monitoring. Moreover, dried saliva and dried whole blood spots should provide easily obtained specimens for quantitative acute phase HIV detection during acute infection or viral load testing during chronic infection. The cost of utilizing dried samples for HIV detection as compared with processing, storing and shipping frozen samples makes this format attractive for field vaccine trials. This study underscores the need to develop rapid, inexpensive technologies for quantitative viral RNA measurement using such dried specimens. DBSs, in particular, are becoming accepted as a method for viral load monitoring [13,20–25]. However, the present study indicates the feasibility of intensive, yet cost-effective monitoring of vaccinees for acute HIV-1 acquisition.

Acknowledgments

This work was supported by the Intramural Research Program of the Vaccine Research Center, NIAID. J.B.W. was the recipient of a postdoctoral fellowship from the Canadian Institutes of Health Research (CIHR). J.B.W., S.S.R., J.R.M., and N.L.L. designed the study. J.B.W., C.L., S.B., A.M. and S.S.R. conducted the experiment and collected all experimental data. J.B.W. and N.L.L. analyzed the data. J.B.W., J.R.M. and N.L.L. wrote the manuscript.

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Fig. 1. SIV RNA levels in various compartments of rhesus monkeys during the first 90 days following infection

Viral RNA in plasma (a), saliva (b), feces (c) and urine (d) was measured by qRT-PCR and is expressed as $\log_{10}$ copies of RNA per ml. The limit of detection for blood plasma for each compartment is indicated (red dashed line). Circles are individual data points.

Fig. 2. SIV burden in various compartments during primary infection

Viral burden during the first 4 weeks following SIV infection is expressed as $\log_{10}$ area under the curve (AUC). AUC was calculated for SIV RNA levels in plasma, saliva, feces and urine between days 3 and 35 after SIV infection. The comparison was analyzed using the Kruskal–Wallis test. Circles are individual data points.

Fig. 3. Association between SIV RNA levels in blood plasma and other compartments

Associations are shown between levels in blood plasma and clarified saliva ($r = 0.5311$, $P < 0.0001$) (a), clarified feces ($r = 0.4886$, $P < 0.0001$) (b) and clarified urine ($r = 0.4002$, $P < 0.0001$) (c). Circles are individual data points.

Fig. 4. Monitoring of SIV RNA dried saliva spots during the first 90 days following infection
 Viral RNA was extracted from 50 μ l DSS and measured by qRT-PCR (a). A correlation is shown between SIV RNA measurements in clarified saliva and DSS ($r = 0.6253$, $P < 0.0001$) (b). DSS, dried saliva spot; qRT-PCR, quantitative real-time polymerase chain reaction. Circles are individual data points.

Fig. 5. Monitoring of SIV RNA in dried blood spots during the first 90 days following infection Viral RNA was extracted from a 50 μ l DBS and measured by qRT-PCR (a). Median log₁₀ SIV RNA measured in blood plasma (inverted triangles) and in DBS (open circles) is shown (b). A correlation is shown between blood plasma and DBS SIV RNA measurements ($r = 0.8319$, $P < 0.0001$) (c). DBS, dried blood spot.