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A Pathogenic Threshold of Virus Load Defined in Simian Immunodeficiency Virus- or Simian-Human Immunodeficiency Virus-Infected Macaques

PETER TEN HAAFT,[1] BABS VERSTREPEN,[1] KLAUS ÜBERLA,[2] BRIGITTE ROSENWIRTH,[1] AND JONATHAN HEENEY[1]*

Department of Virology, Biomedical Primate Research Center, 2280 GH Rijswijk, The Netherlands,[1] *and Institut für Klinische und Molekulare Virologie, Friedrich Alexander Universität, 91054 Erlangen, Germany*[2]

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To determine if a specific pathogenic threshold of plasma viral RNA could be defined irrespective of virus strain, RNA levels in the plasma of more than 50 infected rhesus macaques (*Macaca mulatta*) were measured. Animals were inoculated intravenously with either simian immunodeficiency virus (SIV) or simian-human immunodeficiency virus (SHIV) strains of known pathogenic potential (SIV_{8980}, SIV_{smm-3}, $SIV_{mac32H/J5}$, $SIV_{mac32H/1XC}$, reverse transcriptase-SHIV, $SHIV_{89.6p}$) or with attenuated strains ($SHIV_{W6.1D}$, $SHIV_{sf13}$, $SHIV_{han-2}$, $SIV_{mac\Delta nef}$, $SHIV_{sf33}$). In animals inoculated with nonpathogenic strains, shortly after the primary peak of viremia viral RNA levels declined and remained below 10^4 RNA equivalents/ml of plasma between 6 and 12 weeks postinoculation. Animals infected with documented pathogenic strains maintained viral RNA levels higher than 10^5 RNA equivalents/ml of plasma. In animals infected with strains with low virulence, a decline in plasma RNA levels was observed, but with notable individual variation. Our results demonstrate that the disease-causing potential was predicted and determined by a threshold plasma virus load which remained greater than 10^5 RNA equivalents/ml of plasma 6 to 12 weeks after inoculation. A threshold virus load value which remained below 10^4 RNA equivalents/ml of plasma was indicative of a nonpathogenic course of infection.

Infection of macaque species (*Macaca mulatta*, *Macaca fascicularis*, *Macaca nemestrina*) with various strains of simian immunodeficiency virus (SIV) originally derived from naturally infected sooty mangabeys (23) causes an immunodeficiency syndrome which closely resembles AIDS in human immunodeficiency virus type 1 (HIV-1)-infected humans (13, 17). Due to the similarities in disease symptoms, SIV infection of macaques has become a well-established primate model which is frequently used to study AIDS pathogenesis and to evaluate the efficacy of vaccine and antiviral chemotherapy strategies. Several SIV strains isolated at different primate centers (8, 10, 11, 17, 22, 28) have been well characterized with regard to their disease-causing potential. However, SIV differs somewhat from HIV-1 in terms of neutralization and cytotoxic T-lymphocyte epitopes, limiting the SIV model with regard to the evaluation of HIV-1 vaccine candidates. Testing of antiviral drugs, too, is occasionally limited by differences between SIV- and HIV-1-encoded proteins. To overcome these limitations, chimeric simian-human immunodeficiency viruses (SHIVs) were constructed. These SHIV chimeras utilize the genetic background of SIV in which either the envelope (*env*) (12, 18–20, 29, 30) or the reverse transcriptase (RT) gene of SIV has been replaced by that of HIV-1 (34). SHIV strains have already been proven to be useful for the evaluation of vaccines (2, 9, 25, 32) and antiviral drugs in macaque infection models (34). Pathogenic as well as nonpathogenic SHIV strains have been constructed and characterized (1, 5, 21, 29, 31, 34).

In HIV-1 infection, the plasma level of viral RNA has proven to be the parameter with the highest predictive value with regard to disease progression (24). In this context, quantitative determination of viral RNA load has been most useful in assessing antiviral drug therapy in patients (3, 27). Since it may be assumed that first-generation AIDS vaccines are unlikely to achieve the ultimate goal of sterilizing immunity, reduction of virus load will almost certainly be a critical parameter in the assessment of vaccine efficacy (13). Previous vaccine studies with chimpanzees (4, 33) and rhesus macaques (14, 15) suggested that virus load shortly after inoculation may be predictive for vaccine efficacy. Based on the importance of preclinical vaccine and antiviral testing in macaques and on virus load as a predictive marker, it is clear that specific virus load levels must be defined and correlated with pathogenic or nonpathogenic infections so that the efficacy of antiviral or vaccine strategies can be accurately assessed. A recent study of rhesus macaques suggested that levels of viral RNA as early as 6 weeks after inoculation were predictive for disease progression (35). In the current study, we confirm and extend the observation that predictive virus loads can be determined early after infection. Furthermore, we provide new data based on a large number of animals and a variety of different SIV and SHIV chimeras to define a specific pathogenic threshold of virus load in plasma. Such data may be critical for assessing antiviral drug or vaccine strategies for their ability to lower viral loads below this pathogenic threshold.

To measure SIV RNA levels in plasma of *Macaca mulatta* infected with various strains of SIV or SHIV, we developed a highly sensitive quantitative competitive (QC) RT-PCR assay. To compensate for sample degradation during RNA purification (as well as for variation in amplification efficiency due to copurified PCR inhibiting agents), a calibrated amount of internal standard RNA was added to the sample to be analyzed before RNA purification and was coamplified in the same reaction. For the target sequence, a highly conserved 267-bp region in the SIV *gag* gene with primer and probe regions

* Corresponding author. Mailing address: Biomedical Primate Research Centre, P.O. Box 3306, 2280 GH Rijswijk, The Netherlands. Phone: 31 15 284 2661. Fax: 31 15 284 3986. E-mail: heeney@bprc.nl.

homologous for SIV_{mac}, SIV_{sm}, and chimeric SHIV viruses was chosen. The internal standard was based on the same 267-bp target sequence, but with a 26-bp probe region that was replaced by a rearranged 26-bp sequence by using PCR. This fragment was cloned into a transcription vector, and in vitro transcripts were synthesized with T7 RNA polymerase.

To determine the sensitivity and reproducibility of the QC RT-PCR assay, viral RNA levels were measured in EDTA plasma samples from two naive, mature, outbred rhesus macaques which were infected with RT-SHIV. Blood samples were collected at weeks 0, 1, 2, 4, 6, 8, and 12 postinfection. Plasma samples from all time points were processed in quadruplicate, and the mean values over time for RNA equivalents per milliliter were plotted (Fig. 1). The maximum deviation for each sample was within ±0.4 log unit. For quantitative comparison of the resulting RNA levels, i.e., for quality control of the QC RT-PCR assay, the same samples were analyzed by the Quantiplex branched DNA (bDNA) HIV-1 assay (Chiron Corporation, Emeryville, Calif.), which recognizes HIV-1 *pol* sequences in the RT-SHIV. Figure 1 demonstrates that the kinetics of viral RNA load in plasma over time after infection as determined with both assays were highly similar. However, the dynamic range of the QC RT-PCR assay was larger and ranged to at least 4×10^7 RNA equivalents/ml compared to 8×10^5 for the bDNA assay (the dynamic range of the bDNA assay was enlarged to 5.4×10^6 for some time points by dilution of the plasma sample). Furthermore, the QC RT-PCR assay was more sensitive, with a lower detection limit of 4×10^1 RNA equivalents/ml compared to 5.6×10^2 RNA equivalents/ml for the bDNA assay. In this regard, it should also be noted that the bDNA assay requires a sample volume of 1 ml, and the QC RT-PCR requires a sample of 200 μl.

After having established sensitivity, reproducibility, and dynamic range, we used the QC RT-PCR assay to compare viral RNA load in more than 50 naive mature, outbred Indian *Macaca mulatta* which were infected with various SIV and SHIV strains. Groups of four or more animals were infected intravenously with one of the following virus strains: $SIV_{mac\Delta nef}$, SIV_{8980} (derived from SIV_{smB670} through serial in vivo passages), $SIV_{mac32H/1XC}$, $SIV_{smm\text{-}3}$, $SIV_{mac32H/J5}$, RT-SHIV, $SHIV_{89.6p}$, $SHIV_{sf13}$, $SHIV_{sf33}$, $SHIV_{han\text{-}2}$, and $SHIV_{W6.1D}$. The pathogenic capacities of the various SHIV have been evaluated and were documented (1, 5, 21, 29, 31, 34), as were those of the SIV strains $SIV_{mac\Delta nef}$ (7, 16), SIV_{8980} (14), and $SIV_{mac32H/1XC}$ (26). The plasma RNA levels of each individual animal infected with the different virus strains were plotted over time after infection (Fig. 2); lines represent the mean values for RNA equivalents per milliliter of plasma of each group. Peaks of primary viremia were highest in the animals infected with SIV strains, namely, $SIV_{mac32H/1XC}$ and SIV_{8980}, with the exception of $SIV_{mac\Delta nef}$-infected animals, which showed high levels of individual variation. The SHIV-infected animals developed lower levels of primary viremia. However, the RT-SHIV and $SHIV_{89.6p}$ chimeric virus strains replicated to high levels in vivo and more closely followed the patterns of the SIV strains with regard to peak levels of RNA in plasma. Animals infected with RT-SHIV also showed larger individual variations in peak primary viremia levels compared to those infected with other SHIV strains. When the known pathogenic potential of the SIV and SHIV strains under investigation was compared to the kinetics of virus load in plasma, and in particular after the peak of primary viremia, a highly interesting correlation became evident. In all animals which had been inoculated with nonpathogenic SHIV strains (W6.1D, sf13, han-2), viral RNA levels declined below 10^4 RNA equivalents/ml of plasma shortly after the primary peak of viremia (6 and 12 weeks postinfection). In $SHIV_{sf33}$-infected animals, large individual variation was observed at week 6. However, by 12 weeks postinfection, virus loads were below the detection limit in three of four animals. Interestingly, one animal infected with $SHIV_{sf33}$ was reported to have developed AIDS-like disease (21), suggesting that $SHIV_{sf33}$ may possess some pathogenic potential. In those animals infected with the RT-SHIV and $SHIV_{89.6p}$ strains, RNA levels in plasma remained high, though large individual variation was observed

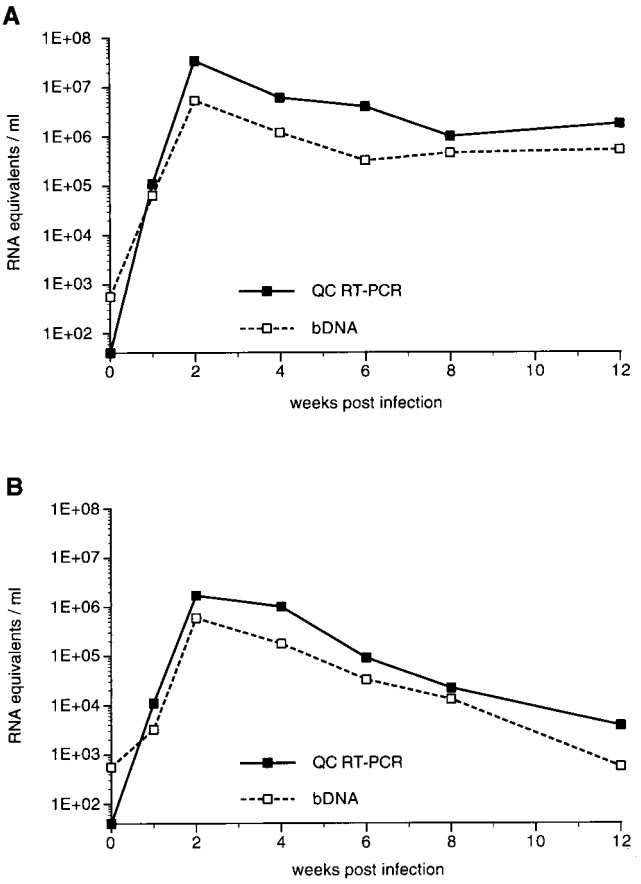

FIG. 1. Plasma viral RNA levels of two RT-SHIV-infected macaques (panels A and B) as determined by QC RT-PCR (SIV *gag*) and bDNA signal amplification (HIV-1 *pol*). Levels for weeks 2 and 8 after the infection of animal A were determined by using bDNA with 1 ml of diluted plasma to enlarge the dynamic range of the assay. For all other bDNA determinations, 1 ml of undiluted plasma was used. At week 0, both animals were negative according to both assays, and animal B was negative at week 12 as determined by bDNA testing. However, the values of the lower detection limits of the respective assays are plotted at these time points for graphical purposes. In the QC RT-PCR assay, the following 5′ biotinylated primers were used: 5′-TGGATTAGCAGAAAGCCTGTTGG-3′ (SIV_{smmH4} homology at bp 1180 to 1202) and 5′-CCTCCTCTGCCACTAGGT GGTGC-3′ (SIV_{smmH4} homology at bp 1424 to 1446). Briefly, 200 μl of plasma to be analyzed was added to 600 μl of guanidine-isothiocyanate-based lysis solution containing 300 copies of internal standard RNA. The RNA was precipitated by propanol-2 and was reverse transcribed and amplified with rTth DNA polymerase (Perkin-Elmer, Nieuwerkerk a/d IJssel, The Netherlands). The amplification products were hybridized in six fivefold dilutions to a capture probe that was covalently bound to Nucleolink microwells (Nunc A/S, Roskilde, Denmark). The amplification products were detected by a streptavidin-horseradish peroxidase-mediated colorimetric reaction. The amplified internal standard was hybridized to the rearranged 26-bp capture probe in separate microwells. The number of RNA copies in the plasma sample was calculated from the optical density of the sample wells compared to that of the corresponding internal standard well.

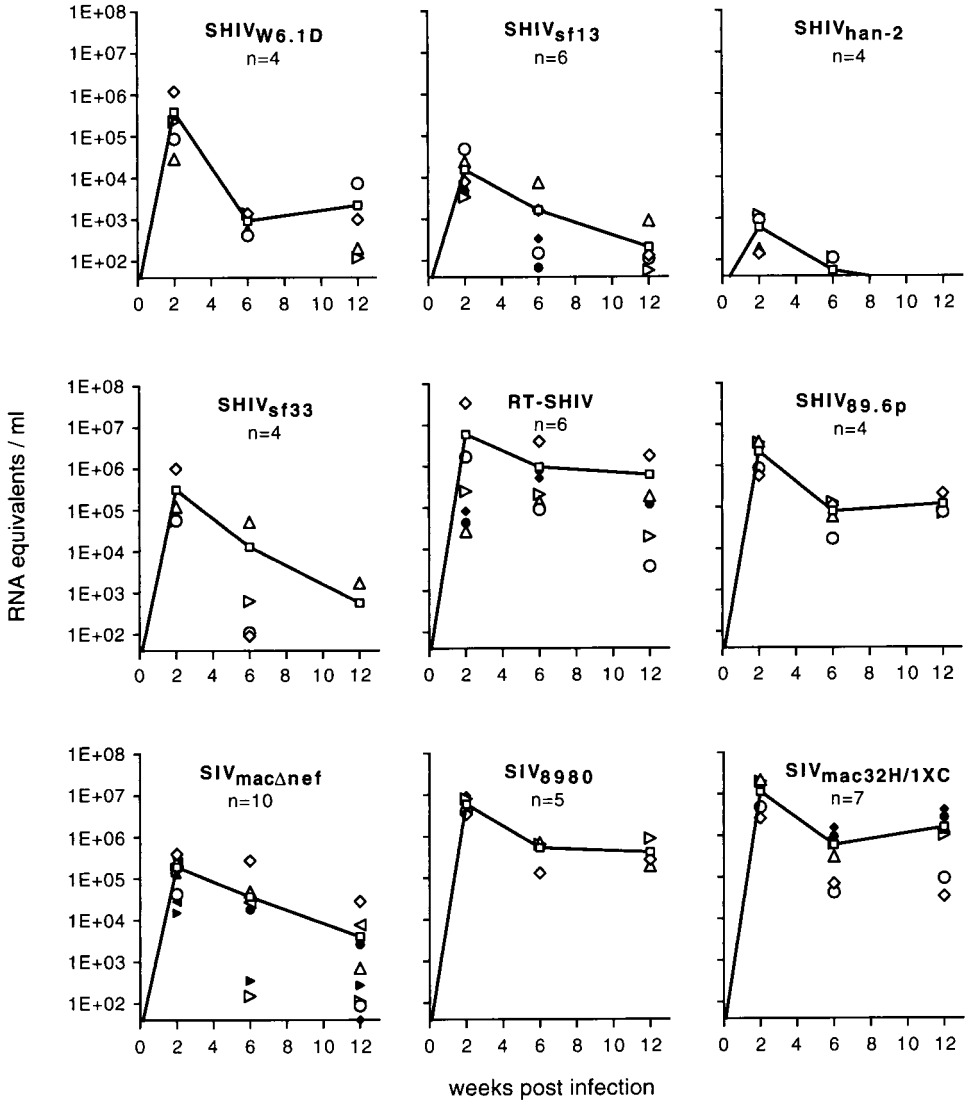

FIG. 2. Plasma viral RNA levels of individual macaques (represented by various open and filled symbols) infected with various SIV or SHIV strains and mean viral RNA levels (represented by lines and open squares) as determined by QC RT-PCR. n, number of animals used in the study.

for RT-SHIV-infected animals. These two SHIV chimeras have been reported to be able to cause disease in rhesus macaques (29, 34). A similar correlation was observed for known pathogenic SIV strains studied here (Fig. 2). Plasma RNA levels in animals infected with SIV_{8980} and $SIV_{mac32H/1XC}$ remained high, above 10^5 RNA equivalents/ml of plasma in most animals. SIV_{smm-3} and $SIV_{mac32H/J5}$ showed the same trend (data not shown). Only in $SIV_{macΔnef}$-infected animals was a decline in mean plasma RNA level observed, again with notable individual variation. $SIV_{macΔnef}$ is known to be an attenuated SIV strain with low pathogenic potential. Rarely, however, some individual animals (primarily neonates) infected with *nef* deletion mutants have been reported to develop AIDS (6).

A comparison of the mean plasma viral RNA levels illustrates the differences between the various strains (Fig. 3). All of the documented pathogenic viruses studied here induced viral RNA levels higher than approximately 10^5 RNA equivalents/ml of plasma at least up to week 12 postinfection. In contrast, infection with nonpathogenic strains typically showed a decline of mean viral RNA load to levels lower than 10^4 RNA equivalents/ml of plasma at weeks 6 and 12 postinfection. Some strains, such as $SIV_{macΔnef}$ and $SHIV_{sf33}$, appeared to have a low or intermediate pathogenic potential that may be more dependent on individual host factors which may influence virus load and progression to disease. Infection of macaques with these two strains was characterized by an intermediate pattern at week 6 with marked individual differences (Fig. 2).

From the comparison of infection with different SIV strains and SHIV chimeric strains in Indian rhesus macaques, we conclude that the disease-causing potential of a particular lentiviral strain is predictable based on the plasma virus load, which is established very early following the peak of primary viremia and seroconversion. Furthermore, we observed a threshold virus load of approximately 10^5 RNA equivalents, which correlated with the potential of an infection to progress to AIDS. Our data corroborates previous results which sug-

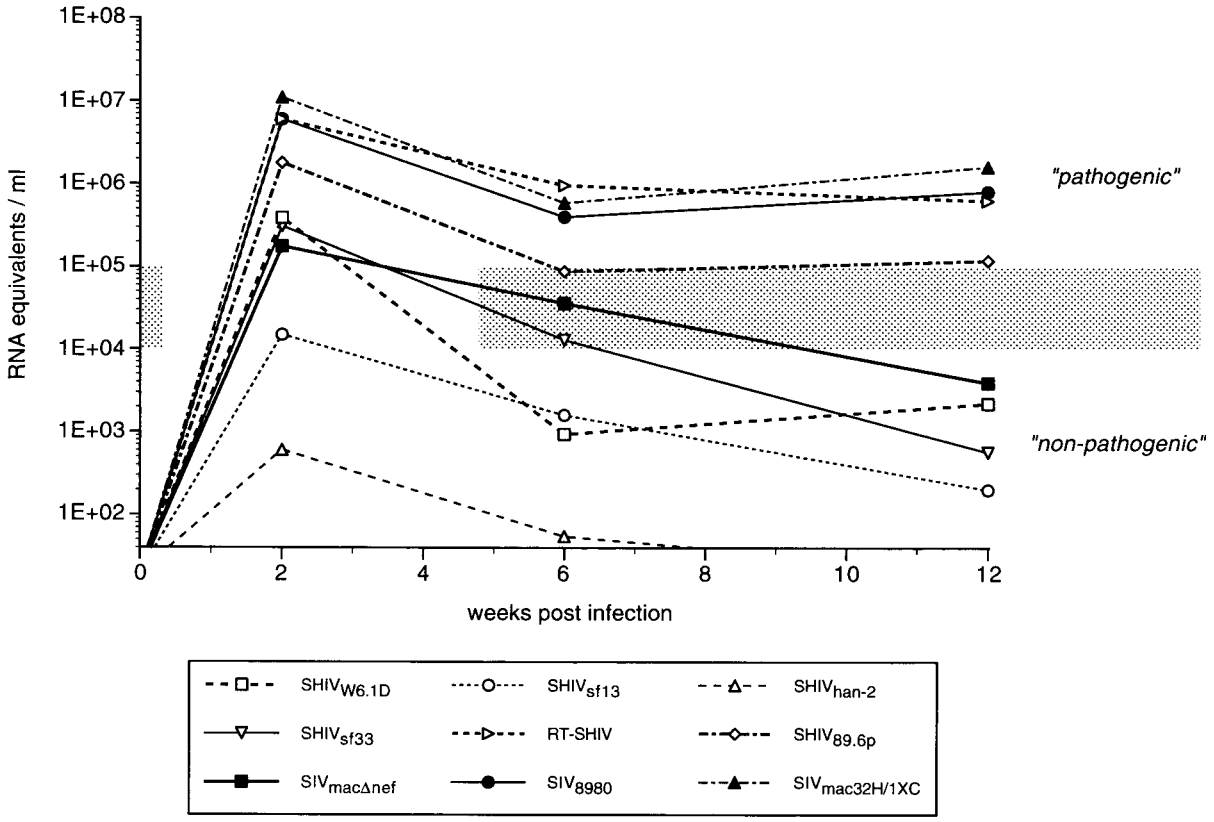

FIG. 3. Comparison of mean plasma viral RNA levels determined after infection with various SIV or SHIV strains. The shaded area represents levels of virus load in animals with infections which have pathogenic potential (i.e., the danger zone between the pathogenic and nonpathogenic threshold). One SHIV$_{89.6p}$-infected animal died within the 12-week study period due to a non-AIDS-related disease. One SIV$_{8980}$-infected animal as well as one SIV$_{mac32H/1XC}$-infected animal developed AIDS and was euthanized within the 12-week study period.

gested that the pathogenic potential of a lentivirus infection is established relatively early after inoculation when evaluated with one particular SIV strain (35). We provide new data demonstrating that irrespective of the virus strain examined, a certain threshold virus load is predictive of a pathogenic disease course. As in previous studies, we found no consistent relationship between the primary peak virus load (at approximately 2 weeks postinfection) and the disease-causing potential of the infecting strain. Clearly, only those virus strains which induced particularly high steady-state viral RNA levels ($>10^5$ RNA equivalents/ml) 6 to 12 weeks postinfection (after seroconversion) appear to readily possess pathogenic capacities in susceptible hosts. The variation in virus load in individual animals observed after infection with some viruses, such as RT-SHIV, may be indicative of the influence of particular host factors which affect individual susceptibility to disease progression (unpublished observations). Longer follow-up of these particular animals may yield further support for this assumption. Finally, infections in which virus loads remained lower than 10^4 RNA equivalents/ml 6 to 12 weeks postinfection were nonpathogenic. This level of virus load may prove to be an important nonpathogenic limit under which antiviral drug or vaccine strategies must suppress virus production.

Several of the SIV and SHIV strains studied here are currently being used to evaluate specific questions in AIDS research. This comparative study provides important information and possible targets for the evaluation of new therapeutic and vaccine strategies in this model. It will be of particular importance to evaluate the capacity of vaccines to induce protection from infection with pathogenic challenge. Moreover, if vaccines fail to induce sterilizing immunity, it will be critical to determine if immunization may at least lower virus load below the pathogenic threshold and if this will result in prolonged survival.

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