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Quantification of Simian Immunodeficiency Virus Cytotoxic T-Lymphocyte Escape Mutant Viruses

Liyen Loh and Stephen J. Kent

Abstract

Escape from cytotoxic T-lymphocyte (CTL) pressure is common in HIV-1 infection of humans and simian immunodeficiency virus (SIV) infections of macaques. CTL escape typically incurs a fitness cost as reversion back to wild-type can occur upon transmission. We utilized sequence-specific primers and DNA probes with real-time polymerase chain reaction (PCR) to sensitively and specifically track wild-type and escape mutant viremia at the Mane-A*17-restricted SIV $Gag_{371–379}$ epitope AF9 in pigtail macaques. The generation of minor escape mutant populations is detected by the real-time PCR 2 weeks earlier than observed using standard sequencing techniques. We passaged the AF9 CTL escape mutant virus into two naïve Mane-A*17-negative pigtail macaques and showed that reversion to wild-type was rapid during acute infection and then slowed considerably at later stages of the infection. These data help refine our understanding of how CTL escape mutant viruses evolve.

Introduction

IMMUNE ESCAPE from immunodominant $CD8^+$ cytotoxic T-lymphocyte (CTL) responses is widely documented in both HIV-1 and simian immunodeficiency virus (SIV) in humans and macaques, respectively.[1–6] The impact of these CTL escape variants on viral fitness has also been demonstrated *in vivo*, illustrating reversion of escape mutant (EM) viruses upon transmission to MHC-mismatched hosts.[4,7–9] The generation and selection of CTL EM and wild-type (WT) viruses, if accurately defined, could be used to model the impact of CTL killing and fitness costs.[4,10–12]

Monitoring escape mutations by the traditional method of cloning and sequencing is, however, very insensitive to viral quasispecies of low (<10%) frequency. More accurate and high-throughput assays are required to quantitate minor viral quasispecies, which may influence the timing and kinetics of CTL EM viremia. Advances in real-time polymerase chain reaction (PCR) technology for sequence-specific detection have resulted in quantitative real-time PCR (qRT-PCR) assays utilizing DNA probes to measure the evolution of common drug-resistant HIV-1 strains that arise after antiretroviral treatment.[13] A molecular beacon–based approach has also been used to measure SIV Gag CM9 CTL EMs restricted by the *Mamu-A*01* allele[14]; however, that assay was insensitive to EM variants <5% of total virus populations. This may be problematic for quantifying low-frequency quasispecies arising during the peak of viremia where the total copy number of virus can be in excess of 10^6 copies/ml of plasma.

In this study, we developed a sensitive qRT-PCRs to concurrently measure EM and WT viremia at the SIV Gag AF9 *Mane-A*17*-restricted allele in the simian-HIV (SHIV)-infected pigtail macaque model. We studied escape at the AF9 CTL epitope; this epitope undergoes a six-base-pair deletion that should enable the development of sensitive and specific assays in comparison to single nucleotide change escape mutations previously studied.[14,15] Our qRT-PCR assays, which utilize minor-groove binding (MGB)-DNA probes and locked nucleic acid (LNA)-modified primers, have the potential to advance our understanding of EM viremia kinetics in both scenarios of CTL escape and reversion in MHC-mismatched hosts.

Materials and Methods

Animals and viruses

Experiments on outbred pigtail macaques (*Macaca nemestrina*) were approved by the University of Melbourne and Commonwealth Scientific Industrial Research Organization (CSIRO) livestock industries Animal Ethics Committees. Pigtail macaques were MHC typed by reference strand-mediated conformational analysis for *Mane-A*17* allele, which presents SIV $Gag_{371–379}$ AF9 epitope.[16,19] If this is a reference, please add to references and cite here by number. CTL escape was stud-

Department of Microbiology and Immunology, University of Melbourne, 3010, Australia.

ied in *Mane-A*17*-positive pigtail macaque 4253, previously reported in a DNA prime/recombinant fowlpoxvirus (rFPV) boost vaccine study involving a viral challenge with R5-tropic $SHIV_{SF162P3}$.[4,17] Reversion of AF9 was studied in two naive *Mane-A*17*-negative macaques infected with 1 ml of plasma and 3×10^6 peripheral blood mononuclear cells (PBMC) from *in vivo* passaged $SHIV_{SF162P3}$ with known six-nucleotide AF9 EM.[10] SIV Gag AF9 region was amplified from viral cDNA and cloned/sequenced as previously described.[4,10]

RNA standards for qRT-PCR

To quantify and validate the SIV Gag AF9 qRT-PCRs, RNA standards were constructed from WT and EM virus sequence. In short, pDNA clones previously sequenced at the SIV Gag AF9 were selected for EM and WT RNA standards, after confirmation from resequencing of the specified region. Prior to transcription of *in vitro* RNA, pDNA was linearized with the restriction endonuclease *Spe*I (Promega, Madison, WI). *In vitro* RNA was transcribed using the ribomax T7 promoter kit (Promega) according to manufacturer's instructions. After transcription, RNA was purified with the QIA RNA MinElute Kit according to manufacturer's instructions (QIAGEN, Valencia, CA). Purified RNA was electrophoresed on a 1.5% agarose gel to check for the presence of correct transcript. Purity and concentration of RNA was derived to calculate RNA total copy number from UV spectrophotometry absorbance readings at 260 nm using Ultrospec 3100Pro UV/Vis spectrophotometer (Amersham Biosciences, Piscataway, NJ). Total RNA copy number quantification was also confirmed using Quant-iT™ RiboGreen RNA Assay Kit (Invitrogen, Carlsbad, CA).

qRT-PCR

To quantify WT and EM viral loads, standard curves were produced by reverse transcribing 10^8 copies of RNA standard and diluting cDNA 10-fold (10^7–10^1 copies) in nuclease-free water to produce WT and EM cDNA standards for analysis. RNA was extracted from serial plasma samples isolated from infected pigtail macaques using a QIAmp Viral RNA minikit (QIAGEN). For each time-point 10 μl of RNA was subjected to two-step qRT-PCR. The first step involved reverse transcription of RNA in a 30-μl reaction carried out as follows: 10 μl of RNA was added to a PCR mix containing: 10× PCR buffer II (ABI), 10× PCR buffer A, 5 mM of $MgCl_2$ (Promega), 10 μM of random hexamers, 0.5 mM of each dNTP (Promega), 20 U of RNasin (Promega), and 20 U of SuperScript III RNase H- reverse transcriptase (Invitrogen). The cycling conditions were: 25°C for 15 min, 42°C for 40 min, and 75°C for 5 min, using GeneAmp 9700 PCR thermal cycler (ABI) or Reaplex^4 (Eppendorf). qRT-PCR reactions (50 μl) were achieved by the addition of 30 μl of cDNA to 20 μl of qRT-PCR mix containing 17 μl of 2× TaqMan universal mastermix (ABI), 100 nM of MGB-Gag-AF9 DNA probe: 5′ 6FAM-TTGCAGCAGCCCAAC- MGBNFQ 3′ where NFQ = non-fluorescent quencher (ABI), 400 nM of reverse primer No. 117: 5′ CCCTCTTTCCCACAATTCCAACAC 3′, 400 nM of WT forward primer or EM forward primer was also added to each PCR reaction. The WT and EM primers were No. 128 5′ G+CCCTCGCACCAGT+G 3′, and No. 129 5′G+CCCTCGCACCAA+ATC 3′ respectively (+ precedes LNA-modified bases; mutated codons are denoted by underlined bases). The cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 45 cycles of: 95°C for 15 s, 63°C for 60 s, using an ABI Prism 7700 sequence detection system PCR thermal cycler or an Eppendorf Realplex^4 cycler. Analysis was performed using Sequence Detection Systems applications version 1.9 (ABI) or Eppendorf Realplex^4 software. Baselines were set two cycles earlier than real reported fluorescence, and threshold value was determined by setting threshold bar within the linear data phase. Samples amplifying after 40 cycles was regarded as negative and corresponds to <1.5-Log_{10} SHIV RNA copies/ml of plasma (threshold value of quantification).

Results

We have previously suggested that the kinetics of CTL escape and reversion (demonstrated upon transmission to MHC-mismatched hosts) are determined by the effectiveness of CTL and the fitness cost of the escape mutations.[4] Furthermore, we identified a novel six-nucleotide in-frame deletion within the immunodominant $CD8^+$ T cell epitope, $Gag_{371–379}$ AF9.[10] This six-nucleotide deletion lends itself to the development of qRT-PCR assays because primers across this region have the potential to be highly discriminatory. We sought to develop sensitive qRT-PCR assays to detect minor WT or EM AF9 virus that may arise during the peak of viremia or when escape appears to be "complete" by cloning and sequencing plasma virus RNA. The qRT-PCR assays were developed with LNA-modified forward DNA primers that discriminate WT AF9 virus from the six-nucelotide deletion mutant; a 5′ FAM-labeled DNA probe with a 3′ MGB modification was added to each reaction to quantify both targets (Fig. 1).

The SIV Gag AF9 qRT-PCR assays were validated on WT and EM RNA standards created from *in vitro* transcription of plasmid DNA. Primers were validated for specificity by adding the WT forward primer to six serial 10-fold dilutions of EM RNA standards (2×10^7–2×10^1 copies); the inverse was performed with the EM primer. There was no significant cross-reactivity of SIV Gag AF9 EM primer, reflective of the six-nucleotide deletion (Table 1). Minimal cross-reactivity was observed when the WT forward primer was added to excessive amounts of EM target ($\geq 10^6$ copies; Table 1). To rigorously evaluate the specificity and sensitivity of qRT-PCR a series of mixed dilutions of WT and EM, RNA standards were prepared to mimic the mixed target ratios that may occur during SHIV infection. The cycle threshold (Ct) value (cycle number at which amplification cross above background fluorescence) was then compared with amplification of WT target without competing target (EM) and showed <1 cycle difference at all concentrations tested (Table 1). The AF9 qRT-PCR assay detected up to 10 copies of WT target in an excess of 10^6 EM copies. Similar experiments were performed with the EM forward primers that were equally as specific (data not shown).

We previously studied SIV Gag AF9 CTL escape kinetics in a *Mane-A*17+* pigtail macaque (4253), which was immunized with DNA and rFPV vaccines and infected with chimeric $SHIV_{SF162P3}$.[4,10,17] The kinetics of escape were defined by cloning and sequencing a limited number of cDNA amplicons from plasma virus RNA at multiple time points across the Gag AF9 region. We observed that the emergence

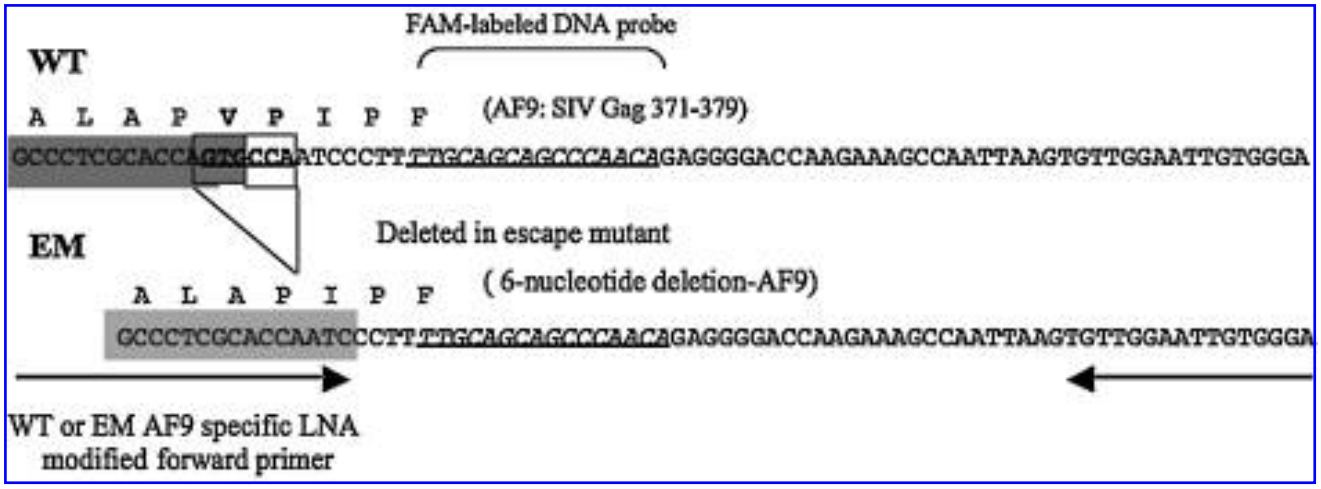


FIG. 1. SIV Gag quantitative real-time PCR (qRT-PCR) assay outline for SIV $Gag_{371–379}$ AF9. Wild-type (WT)-specific locked nucleic acid (LNA) primer sequence highlighted in dark gray, escape mutant–specific sequence highlighted in light gray. Bold underlined nucleotides represent mutations within epitopes. DNA reverse primer and FAM-labeled minor groove binding DNA probe allow quantification of both WT and EM target.

of 6-bp EM AF9 virus was only detected by sequencing viral clones from week 6 postinfection onward, well after the peak of viremia at week 2.[10] It is possible that minor populations of EM viruses may be established during or early after the peak of viremia that may not be detectable until much later time points by cloning and sequencing. To address this hypothesis, we analyzed CTL escape at Gag AF9 in animal 4253 by qRT-PCR of archive plasma RNA samples. The slow emergence of the EM AF9 virus could be consistently detected from as early as week 3 postinfection with this qRT-PCR assay (open triangles, Fig. 2). Note that emergence of the EM virus was consistently earlier, and slower, than detected by cloning and sequencing.[10]

Fitness costs are incurred by CTL escape mutations; this is most clearly demonstrated when EM virus "reverts" to (or outgrown by) the fitter WT upon transmission to MHC-mismatched hosts.[4,7–9] To more accurately track rates of reversion, we utilized the AF9 EM/WT qRT-PCR to track the evolution of EM AF9 virus used to infect two naïve *Mane-A**17*-negative pigtail macaques. The virus used for the passage was taken from animal 4253 when all sequenced clones contained the six-nucleotide deletion escape mutation. We were able to accurately define the rate of outgrowth of the SIV Gag AF9 mutant using the qRT-PCR assay. Immediate growth of WT and EM virus was observed from qRT-PCR in pigtail macaques 5904 and 6175, with similar rates of growth of both viruses during the first 8–11 days (Fig. 3). The immediate outgrowth of both viral forms is consistent with the presence of significant amounts of WT virus in the inoculum, as suggested by our qRT-PCR (Fig. 2), even though sequencing of viral clones suggested only EM virus was present.

However, from beyond 19 days after infection, the WT virus more efficiently outgrows the EM virus in both animals, and by day 133 the EM virus is below detectable levels in both animals. This biphasic reversion suggests more rapid reversion of EM virus to WT during acute infection and slower during chronic infection. Comparing the

TABLE 1. VALIDATION OF SIV $GAG_{371–379}$ AF9 QUANTITATIVE REAL-TIME PCR

Specificity of AF9 qRT-PCR			*Sensitivity of AF9 qRT-PCR*				
EM copy no.	*EM Ct value*	*Ct value[a] WT primer amplification of EM standards*	*WT copy no.*	*WT Ct value (without EM)*	*Amount of target WT:EM*	*WT Ct value (with EM)*	ΔCf[c]
2×10^7	16.8	37.5	10^7	17.6	$10^7:10^6$	18	0.4
2×10^6	19.9	40.1	10^6	20.4	$10^6:10^6$	20.8	0.4
2×10^5	23.8	—[b]	10^5	24.9	$10^5:10^6$	24.2	0.7
2×10^4	28.0	—	10^4	28	$10^4:10^6$	27.6	0.4
2×10^3	32.0	—	10^3	31.7	$10^3:10^6$	31.2	0.5
2×10^2	36.6	—	10^2	34.7	$10^2:10^6$	34.7	0.0
2×10^1	39.1	—	10^1	38.2	$10^1:10^6$	38.3	0.1

SIV = simian immunodeficiency virus; qRT-PCR = quantitative real-time polymerase chain reaction; EM = escape mutant; Ct = cycle threshold; WT = wild-type.

[a]Cycle threshold (Ct) value = Cycle no. of target DNA amplification above background fluorescence.

[b]No amplification or amplification after 40 cycles.

[c]ΔCt = WT Ct value (without EM) − WT Ct value (with EM).

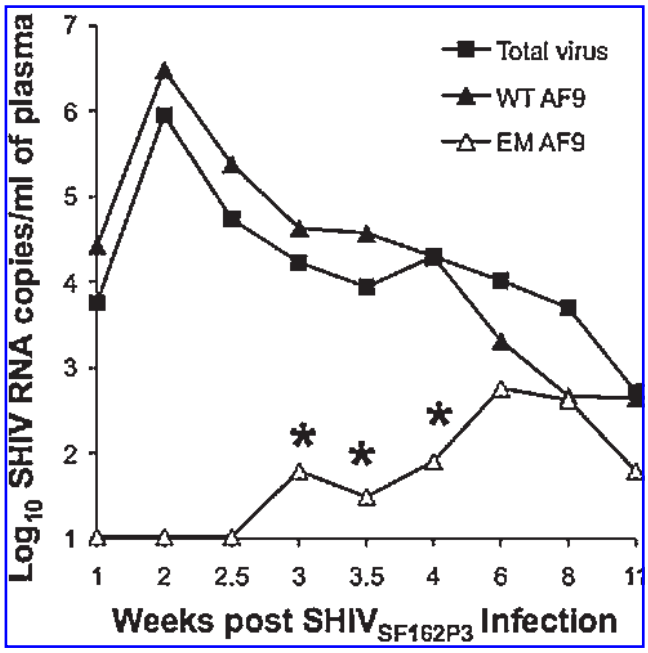


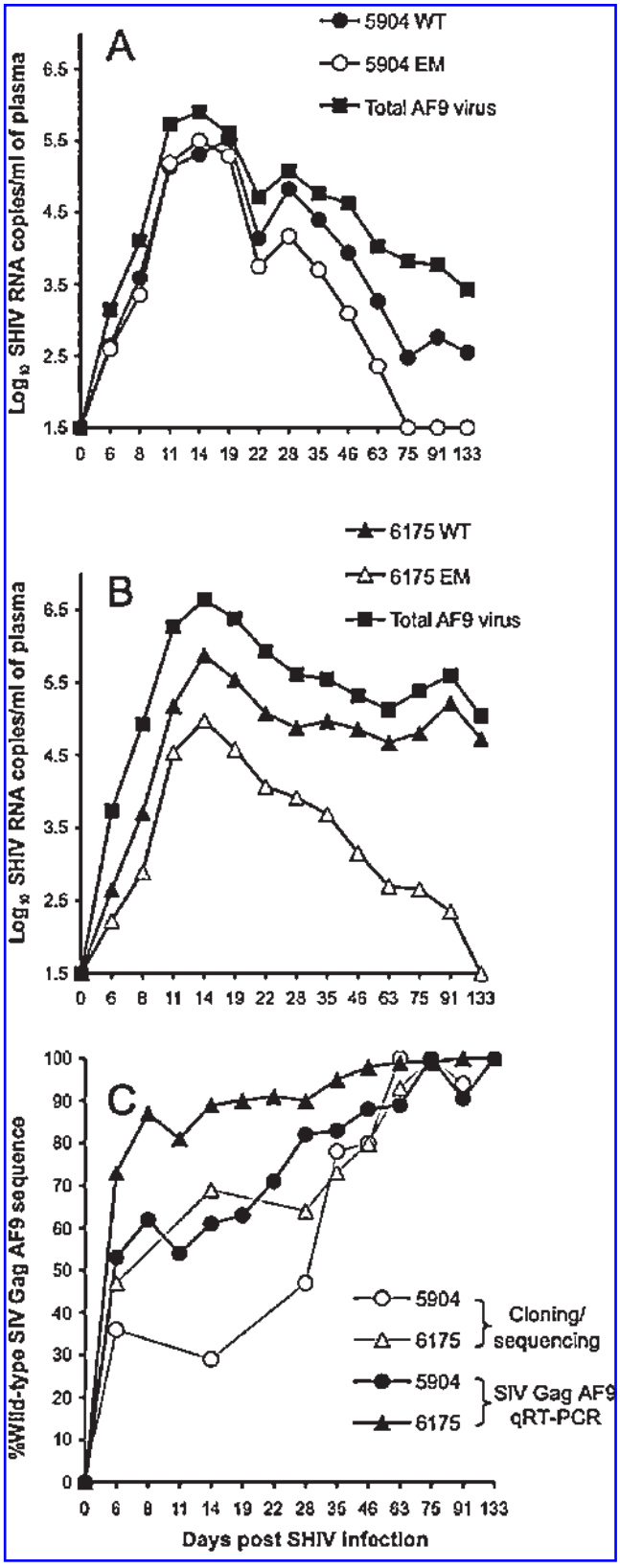
FIG. 2. Minor populations of six-nucleotide deletion escape mutant (EM) AF9 virus can be detected by qRT-PCR early after acute infection. SIV Gag AF9 qRT-PCR was performed on serial archived RNA samples extracted from plasma from *Mane-A*17* pigtail macaque 4253 infected with $SHIV_{SF162P3}$. EM AF9 viral load is represented by open triangles, wild-type virus at AF9 by closed triangles, and the total AF9 virus quantified is represented by solid squares. The 6-bp EM AF9 was only detected beyond week 6 by cloning/sequencing[10] but was detected at weeks 3, 3.5, and 4 by the qRT-PCR (asterisks).

cloning/sequencing to qRT-PCR at day 6, the percentage of WT AF9 virus detected in animal 5904 was 36% and 53%, respectively. Similarly, in animal 6175, the percentages of WT AF9 were higher during acute infection (day 6) than cloning and sequencing: 47% and 73%, respectively. A remarkably slow and gradual decay of EM virus was observed after the peak of infection, slower than predicted by cloning and sequencing (Fig. 3C). Minor amounts of EM AF9 were detected in animal 6175 after day 63, and no EM virus was observed in plasma RNA after day 75 from animal 5904, which also controlled viremia.

FIG. 3. *In vivo* reversion rates of six-nucleotide deletion escape mutant (EM) AF9 viruses assessed by qRT-PCR (**A, B**) and compared with cloning/sequencing (**C**). Wild-type (WT) and EM AF9 virus was quantified concurrently by qRT-PCR from serial RNA samples extracted from plasma in two naïve MHC-mismatched pigtail macaques, 5904 (**A**) and 6175 (**B**) and compared with total viral load (black squares). The naïve macaques received inoculum consisting of 3×10^6 peripheral blood mononuclear cells and 1 ml of plasma, derived from donor pigtail macaques with 100% EM virus (cloning/sequencing). The percentage of WT AF9 sequence was then compared from qRT-PCR in animals 5904 and 6175 (open circles and triangles, respectively) to cloning/sequencing (14–20 clones/time point[10] in solid circles and triangles, respectively) (**C**).

Discussion

In this study, we show that qRT-PCR assays can be developed to monitor and quantify a six-nucleotide deletion CTL escape mutation with high levels of sensitivity in comparison with cloning and sequencing methods, other RT-



PCR based assays,[14] and similar assays detecting single nucleotide changes.[15] The SIV Gag AF9 qRT-PCR assay readily detected 10 copies of WT virus in the presence of 10^6 copies of six-nucleotide deletion EM virus. This large dynamic range allows the detection of minor populations of AF9 quasispecies that are not detectable from cloning/sequencing. We were able to track the slow emergence of minor six-nucleotide deletion EM variant generated early after the peak of virus infection that was not observed by cloning and sequencing. Further, following passage of AF9 EM virus into naïve macaques, we were able to track the simultaneous expansion of both WT and EM virus at the AF9 epitope (consistent with significant WT virus in the inoculum as detected by the qRT-PCR not observed by cloning and sequencing) and the very slow and gradual decay of EM virus and outgrowth of WT virus occurring during the chronic phase of infection. Our results are consistent with the emergence of WT virus at the KP9 CTL epitope when a mix of EM and WT are used to infect naïve pigtail macaques unable to respond at that epitope.[15] Taken together, our results suggest that the emergence and decay of CTL EM viruses may be slower than originally anticipated, particularly after the peak of viremia. The maximum rates of escape and reversion are expected to be seen during the exponential phase of virus growth in early infection, and by contrast, mutants should be selected more slowly in chronic infection.[4] This may reflect the "steady-state" virus growth during post–acute infection where selection pressures may be more muted compared with the exponential virus growth during acute infection. Further studies are suggested to analyze the rates of selection of AF9 WT and EM viruses arising at different times during infection. A more rigorous understanding of the pressures impacting upon the evolution of EM viruses is obtained using sensitive quantitative assays. Ultimately, this should facilitate the design of vaccines to generate specific immune responses that can maximally suppress viremia, or, where escape occurs, to inflict a maximally decrease in viral fitness. This general approach is applicable to all immune responses where the dominant EM variant has been identified, including escape and reversion from neutralizing antibody responses.

Although we describe in this report novel sensitive qRT-PCR assays to detect minor viral quasispecies, several limitations are apparent. Our assay specifically discriminates WT AF9 from the dominant six-nucleotide deletion EM. Most CTL EM variants are, however, single nucleotide changes, and it is common for multiple EM variants to emerge prior to selecting a dominant variant. It is, however, feasible to design similar assays for detection of single nucleotide escape mutations to CTL escape motifs or drug-resistant escape motifs.[13,14] We recently developed and evaluated a similar qRT-PCR assay for the common K165R EM virus at the *Mane-A*10*-restricted SIV $Gag_{164–172}$ KP9 epitope in pigtail macaques.[15] The KP9 assay detected a single nucleotide EM change, compared with the six-nucleotide deletion studied herein. As expected given the larger deletion being studied, this AF9 EM assay is somewhat more sensitive than the KP9 EM assay, detecting no cross-reactivity until $\sim 10^6$ copies compared with $\sim 10^4$ copies, respectively, and having ΔCT values of <1 cycle compared with frequently >1 cycle when studying EM/WT combinations (Table 1[15]). Additional qRT-PCRs could be designed to quantify rarer or transient escape mutations that may arise during the peak of viremia in addition to the dominant EM. With regard to the sensitivity of this assay, although the greater dynamic range allows for detection of minor quasispecies, the integrity of the plasma/RNA sample is critical for quantification of the sample—it is essential that the plasma/RNA be stored appropriately with minimal freeze/thaws to avoid RNA degradation. The lower limit of detection of this and other viral load assays can be further improved using ultracentrifugation of larger plasma samples.[18] This or similar quantitative PCR assays could also be adapted to the quantification of total or integrated SIV or HIV DNA in PBMC or other cell subsets. Sensitive assays for CTL EM viruses may detect "archived" population in nonreplicating reservoirs, similar to drug resistant HIV strains that which reemerge when selection pressure is reapplied.

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Address reprint requests to:
Stephen J. Kent
Department of Microbiology and Immunology
University of Melbourne
Parkville, Victoria, 3010, Australia

E-mail: skent@unimelb.edu.au