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In Vivo Fitness Costs of Different Gag CD8 T-Cell Escape Mutant Simian-Human Immunodeficiency Viruses for Macaques[▽]

Liyen Loh,[1] C. Jane Batten,[1] Janka Petravic,[2] Miles P. Davenport,[2] and Stephen J. Kent[1]*

Department of Microbiology and Immunology, University of Melbourne, Victoria 3010, Australia,[1] and Department of Haematology, Prince of Wales Hospital, and Centre for Vascular Research, University of New South Wales, NSW 2052, Australia[2]

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The kinetics of immune escape and reversion depend upon the efficiency of CD8 cytotoxic T lymphocytes (CTL) and the fitness cost of escape mutations. Escape kinetics of three simian immunodeficiency virus Gag CTL epitopes in pigtail macaques were variable; those of KP9 and AF9 were faster than those of KW9. Kinetics of reversion of escape mutant virus to wild type upon passage to naïve major histocompatibility complex-mismatched macaques also varied. Rapid reversion occurred at KP9, gradual biphasic reversion occurred at AF9, and escape mutant KW9 virus failed to revert. The fitness impact of these mutations is KP9 > AF9 > KW9. These data provide insights into the differential utility of CTL in controlling viremia.

A serious limitation of cytotoxic T lymphocyte (CTL)-based vaccines for controlling human immunodeficiency virus (HIV) type 1 infection in humans and simian immunodeficiency virus (SIV) infection in macaques is the common generation of CD8 T-cell escape mutant viruses (1, 3). However, fitness costs are likely to occur following most immune escape mutations, as is most clearly demonstrated when escaped viruses revert upon transmission to major histocompatibility complex (MHC)-mismatched hosts (6, 7, 11, 12).

SIV Gag epitopes and their escape mutants. It has been difficult to study whether some HIV or SIV-specific T cells are more efficient than others in clearing virus-infected cells in vivo (13). Based on a detailed study of escape and reversion kinetics at a single *Mane-A*10*-restricted SIV Gag CD8 T-cell epitope ($Gag_{164–172}$ KP9), we previously hypothesized that the effectiveness of particular T cells could be measured by analyzing the rate of clearance of wild-type virus during T-cell escape (6, 10). To investigate this rate at other epitopes, we studied escape kinetics at two additional previously defined dominant SIV_{mac239} Gag $CD8^+$ T-cell epitopes, KW9 ($Gag_{28–36}$) and AF9 ($Gag_{371–379}$), in DNA- and fowlpoxvirus-immunized pigtail macaques (4–6, 9). The MHC restrictions of these two epitopes are *Mane-B*10* and *Mane-A*17*, respectively (16, 17).

The chimeric SHIV challenge stocks and serial macaque plasma samples were sequenced from PCR-purified cDNA amplicons as previously described (6). KW9 was amplified with primer pairs 9/5 and 9/82, and AF9 was amplified with primer pair 72/3 and 8/3 (see supplementary Table 1 at www.microbiol.unimelb.edu.au/research/groups/kent); KP9 was amplified as previously described (6). All KW9 and AF9 clones sequenced were wild type in both $SHIV_{mn229}$ and $SHIV_{SF162P3}$ stocks (see supplementary Table 2 at www.microbiol.unimelb.edu.au/research/groups/kent).

To identify escape mutant viruses with mutations at KW9 and AF9, we selected animals responding strongly to these epitopes that had sustained detection of plasma virus (animal 4296 for KW9 and animal 4253 for AF9) (4, 9). The dominant escape motif at KW9 resulted from a single nucleotide change in the first amino acid of the epitope; the lysine residue was replaced by arginine (K28R). The dominant escape motif at AF9 was a novel 6-nucleotide in-frame deletion within the epitope (see supplementary Table 2 at www.microbiol.unimelb.edu.au/research/groups/kent). Several AF9 variants were observed up to week 4, but were all replaced by the 6-nucleotide deletion at week 6 to 8.

To confirm that the single amino acid change K28R indeed resulted in immune escape, serial dilutions of the wild-type (KW9) or escape mutant (RW9) peptides were incubated with whole blood from a *Mane-B*10*-positive animal with KW9-responding T cells. Gamma interferon expression on $CD3^+$ $CD8^+$ T cells was analyzed by intracellular cytokine staining as previously described (4). A significant partial reduction in T-cell recognition was observed from the mutant KW9 epitope peptide RW9 (Fig. 1A).

Kinetics of CD8 T-cell escape varies between epitopes. Variable rates of CD8 T-cell escape were observed (Fig. 1B). KP9 and AF9 both escape rapidly over a 14-day period, although KP9 begins to escape during the acute phase of SIV infection at 2 weeks postinfection (2, 6, 14). The dominant 6-bp AF9 deletion escape mutant is established at between weeks 4 and 6 post infection (Fig. 1C), after the week 2 peak of viremia and a period of generation of multiple other mutations (4). In contrast, KW9 exhibits a more gradual and fluctuating decrease in wild-type virus over 8 weeks.

We then derived the relative growth disadvantage of the wild-type virus in the presence of the relevant CTLs by calculating the rate of loss of wild-type virus as previously described (Table 1). Once escape begins, KP9-specific T-cell responses eliminate wild-type virus more efficiently than AF9- or KW9-specific T cells.

Impact of immune escape mutations on viral fitness. Transmission of escape mutant virus may result in reversion to wild-type virus due to lack of immune pressure from the new host (6, 7, 11). We previously hypothesized that the rate at which

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Melbourne, Parkville 3010, Australia. Phone: 61 3 83449939. Fax: 61 3 83443846. E-mail: skent@unimelb.edu.au.

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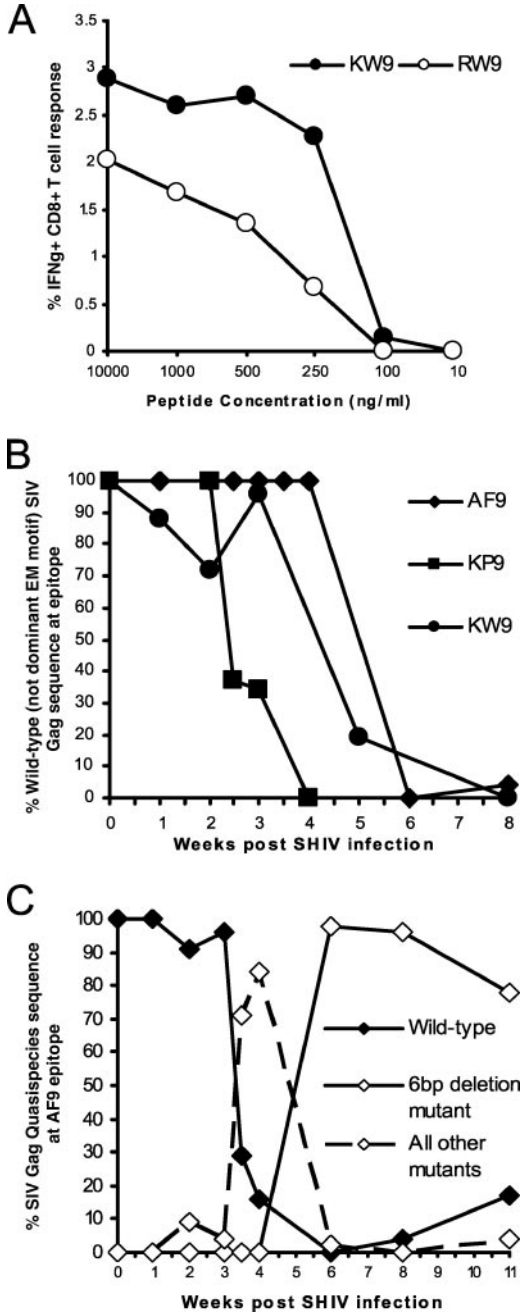

FIG. 1. (A) RW9 is an escape mutant variant of KW9. Recognition of the 9-mer peptide RW9, containing the K28R mutation of the KW9 epitope, was compared to that of the KW9 wild-type peptide. Fresh blood from a pigtail macaque responding to KW9 was stimulated with serial dilutions of each peptide for 6 h, and specific expression of intracellular gamma interferon in $CD3^+$ $CD8^+$ lymphocytes was assessed by flow cytometry. (B) Patterns of escape at the KP9, KW9, and AF9 epitopes. The proportion of viral clones lacking the dominant SIV Gag escape motif (K165R for KP9, the 6-bp deletion for AF9, and K28R for KW9) in pigtail macaques was studied by cloning and sequencing plasma viral cDNA at multiple time points. (C) Growth dynamics of wild-type AF9 and escape variants. Wild-type AF9 is first replaced by a series of minor mutants containing primarily single amino acid changes (see supplementary Table 2 at www.microbiol.unimelb.edu.au/research/groups/kent), which are in turn replaced by the 6-bp mutation.

reversion occurs should be proportional to the in vivo fitness cost of the escape mutant (10). KP9 mutant $SHIV_{mn229}$ reverts rapidly over the first 2 weeks of infection, suggesting a large fitness cost of the K165R KP9 escape mutation (6).

To assess fitness impacts of KW9 and AF9 escape mutations, we analyzed the kinetics of reversion of these immune escape viruses in naïve pigtail macaques not expressing the relevant restricting MHC class I allele. Two macaques negative for the *Mane-B*10* allele were infected with escape mutant KW9 (K28R) virus, and two *Mane-A*17* negative macaques were infected with escape mutant AF9 (6-bp deletion). Pigtail macaque MHC class I typing was performed as previously described (16, 17). We inoculated the animals intravenously with stored plasma (1 ml) and cells (3×10^6 peripheral blood mononuclear cells [PBMC]) from the animals with known complete AF9 and KW9 immune escape virus (animal 4253 at week 6 postinfection and animal 4296 at week 8 postinfection, respectively).

All inoculations resulted in sustained plasma SHIV viremia as measured by quantitative real-time PCR (4, 5). Infection with the escape mutant KW9 virus (derived from parent virus X4-tropic $SHIV_{mn229}$) resulted in a typically rapid reduction of CD4 T cells within 3 weeks of infection (4, 5) (Fig. 2A). Infection with escape mutant AF9 virus (derived from parent virus R5-tropic $SHIV_{SF162P3}$) resulted in a more gradual decline of CD4 T cells in both animals, as expected (9) (Fig. 2C).

Replacement of the 6-bp escape mutant AF9 virus occurred in a biphasic manner. Reversion rates of AF9 mutants were similar to those of KP9 mutants over the first week of infection. However, over the second week of infection the rate of reversion of KP9 remained similar to that during week 1, whereas AF9 reversion was slowed considerably and was not completed for 9 to 11 weeks (Fig. 3A; Table 1).

In contrast to the reversion observed at the KP9 and AF9 epitopes, no reversion occurred in either macaque infected with escape mutant KW9 virus despite high-level viremia (Fig. 3A). It is possible that virus that was wild type at KW9 never occurred in these two animals, so there was no competition between wild-type and escape mutant virus. However, given the high viral loads, this seems unlikely. The lack of reversion at the KW9 epitope suggests minimal in vivo fitness impacts of K28R KW9 mutation. Thus, the comparative growth disadvantage of the various escape mutant viruses in the absence of CTL pressure, at least in the small number of animals studied here, is KP9 > AF9 >KW9 (Table 1). Larger animal studies assessing a wider variety of mutations in both Gag and non-Gag HIV/SIV proteins are warranted, particularly given recent data on the differential utility of Gag and non-Gag T-cell immunity in humans (10a).

Interestingly, viral sequences across the AF9 epitope during the reversion process contained quasispecies in addition to wild-type AF9 virus and the 6-bp deletion escape mutant (see supplementary Table 2 at www.microbiol.unimelb.edu.au/research/groups/kent). Indeed, true wild-type virus was not present in animal 5904 beyond week 9. The most common and persisting variant in both animals was the V375A mutant (Fig. 3B). The V375A mutant was identified as a minor quasispecies in donor animal 4523 at 3.5 to 4 weeks after infection. These results suggest that reversion from immune escape variants may drive enhanced genetic diversity within epitopes.

TABLE 1. Relative growth comparison[a]

Epitope	Escape		Reversion			
			Wk 0–1		Wk 1–2	
	Growth disadvantage of WT[b] (day^{-1})	EM[c] doubling time (days)	Growth advantage of WT (day^{-1})[d]	WT doubling time (days)	Growth advantage of WT (day^{-1})	WT doubling time (days)
KP9	0.71	1.0	0.37 ± 0.06	1.9	0.42 ± 0.11	1.7
AF9	0.28	2.5	0.41 ± 0.08	1.7	−0.01 ± 0.10	>11
KW9	0.19	3.6	<0.06[e]	>11		

[a] The formula used to derive growth difference is $g_{WT/EM} = \{\ln[f_i(t_2)/f_j(t_2)] - \ln[f_i(t_1)/f_j(t_1)]\}/\Delta t$, where $\Delta t = t_2 - t_1$ is the time interval and f_i and f_j are fractions of viral clones.

[b] WT, wild-type virus (KKFGAEVVP for KP9, KYMLKHVVW for KW9, and ALAPVPIPF for AF9).

[c] EM, all non-wild-type virus. The dominant EM variants are KRFGAEVVP (K165R) for KP9, RYMLKHVVW (RW9 or K28R) for KW9, and ALA- -PIPF (6-bp mutation) for AF9.

[d] Mean growth rates are shown for the two animals studied. Error rates reflect the difference between the two animals studied.

[e] No reversion detected; maximum undetected growth rate of WT, 0.06/day.

Reversion could be influenced by unknown compensatory mutations outside the epitope, although none were clearly identified within the fragments sequenced (8). Differences in viral strains studied could also influence reversion rates; however, acute infection with high viremia was uniform. Minor or undetectable populations of wild-type virus in the inocula would enhance the likelihood of reversion occurring during acute infection. It is possible that the KW9 escape mutant viruses transferred had few if any wild-type quasispecies compared to KP9 and AF9. This may have slowed the initial generation of revertant viruses. Further studies using more sensitive tools such as real-time PCR would be useful to identify and track minor viral quasispecies (15).

Rates of reversion could also potentially be influenced by the type and dose of the viral inoculum. The transfer of both PBMC and plasma in our in vivo reversion experiment for reversion at the AF9 and KW9 epitopes rather than a cultured stock of virus would potentially more likely contain multiple

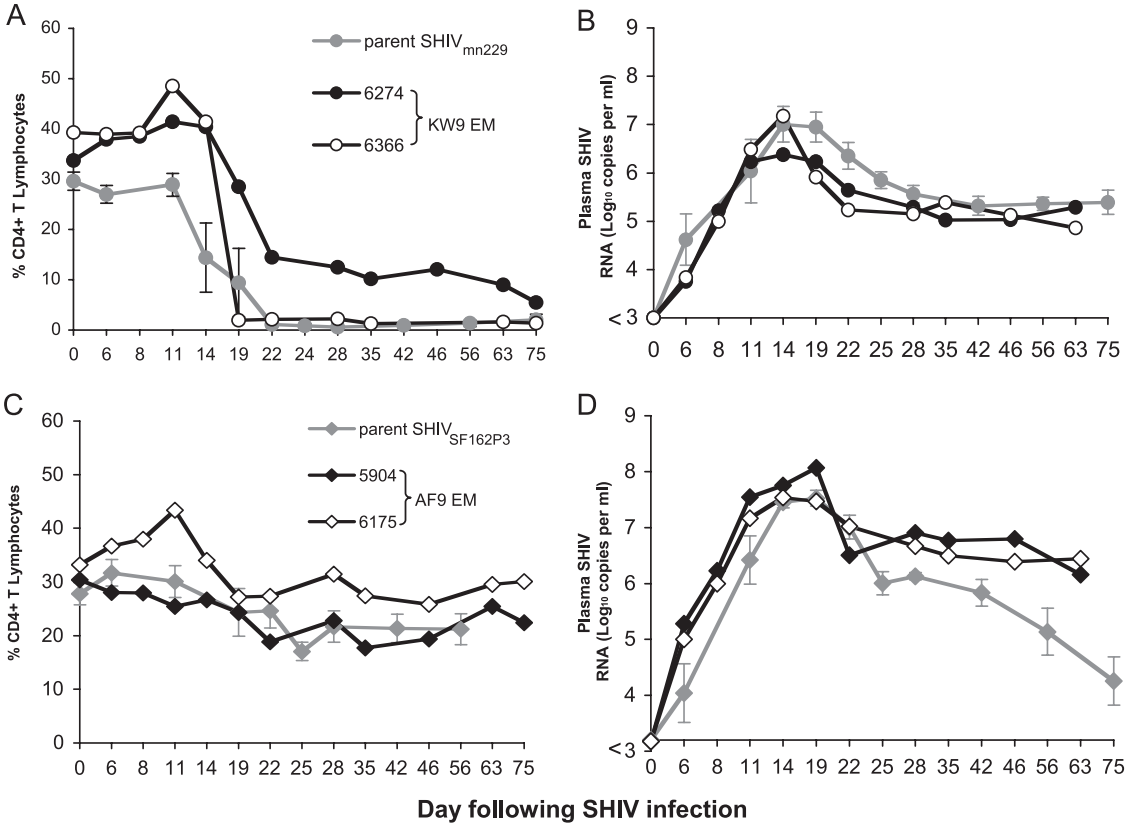

FIG. 2. Successful transmission of KW9 and AF9 escape mutant viruses. (A and B) Pigtail macaques 6274 and 6366 were inoculated with PBMC and plasma from animal 4296 when it had escaped from KW9. CD4 T-cell depletion (A) and SHIV viremia (B) in both animals were analyzed and compared to the means ± standard errors for six animals previously infected with the parent $SHIV_{mn229}$ (4). (C and D) Macaques 5904 and 6175 were inoculated with escape mutant AF9 virus with a 6-bp deletion (AF9 EM), and viral loads were compared to the mean viral load of eight naïve animals previously inoculated with the R5-tropic parent virus $SHIV_{SF162P3}$ as above (9).

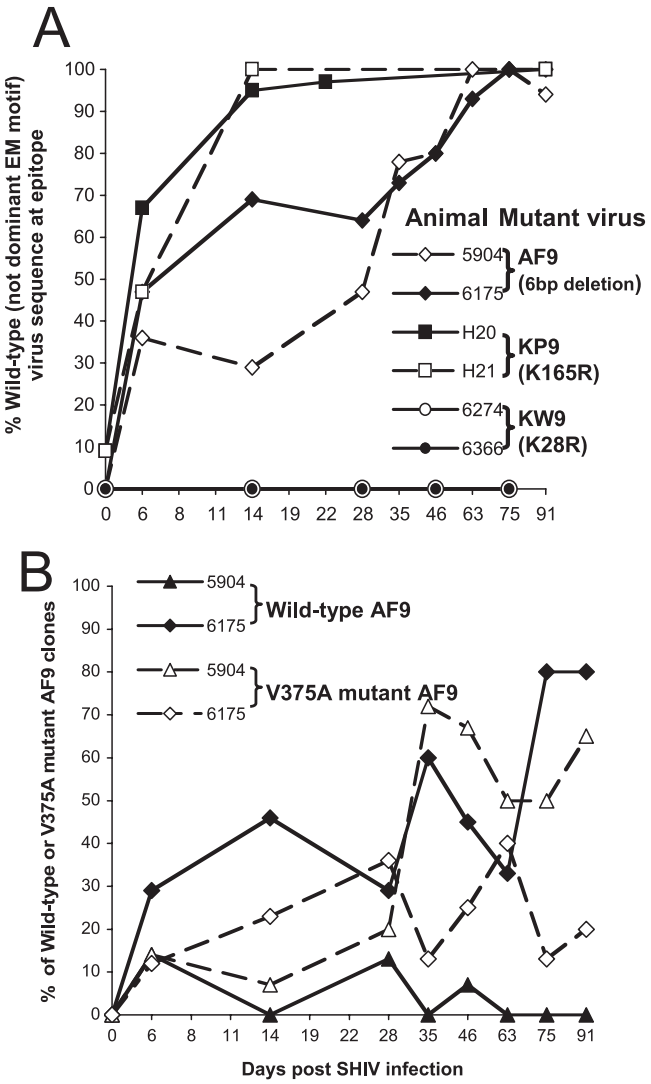

FIG. 3. (A) Reversion of escape mutant viruses AF9 and KW9 compared to KP9. The proportion of viral clones lacking the dominant escape motif for AF9 or KW9 was compared to that for KP9 following inoculation of the escape mutant viruses in two pigtail macaques each. (B) The V375A AF9 mutation persists in both animals inoculated with the 6-bp deletion AF9 mutant virus.

viral quasispecies and promote more rapid reversion. However, the most rapid rates of reversion were detected at KP9 using the cultured $SHIV_{mn229}$ viral stock containing 91% escape mutant virus. Reversion rates are most likely driven primarily by viral fitness effects and by proportions of wild-type and escape mutant virus in the inoculum rather than by the dose or type of inoculum.

In summary, the kinetics of immune escape from SIV Gag-specific CTLs were variable in vivo, suggesting that some CD8 T cells are more efficient at killing virus-infected cells than others. This is consistent with in vitro data on T-cell lines (13). Further, we show that reversion of CTL escape viruses in MHC-mismatched hosts is also variable, suggesting that the in vivo fitness costs of selected mutations differ. This is consistent with emerging data from in vitro competition assays for HIV type 1 Gag escape mutants (18). Targeting CTL epitopes that are the most efficient killers and inflict the biggest fitness cost upon escape should be a rational basis for improving CTL-based HIV vaccines.

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