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Virulence and Reduced Fitness of Simian Immunodeficiency Virus with the M184V Mutation in Reverse Transcriptase

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Drug-resistant mutants with a methionine-to-valine substitution at position 184 of reverse transcriptase (M184V) emerged within 5 weeks of initiation of therapy in four newborn macaques infected with simian immunodeficiency virus (SIVmac251) and treated with lamivudine (3TC) or emtricitabine [(−)-FTC] (two animals per drug). Thus, this animal model mimics the rapid emergence of M184V mutants of HIV-1 during 3TC therapy of human patients. One animal of each treatment group developed fatal immunodeficiency at 12 weeks of age, which is similar to the rapid disease course seen in most untreated SIVmac251-infected infant macaques. To further evaluate the effect of the M184V mutation on viral fitness and virulence, groups of juvenile macaques were inoculated with the molecular clone SIVmac239 with either the wild-type sequence (group A [$n = 5$]) or the M184V sequence (SIVmac239-184V; group B [$n = 5$] and group C [$n = 2$]). The two SIVmac239-184V-infected animals of group C did not receive any drug treatment, and in both animals the virus population reverted to predominantly wild type (184M) by 8 weeks after inoculation. The other five SIVmac239-184V-infected animals (group B) were treated with (−)-FTC to prevent reversion. Although virus levels 1 week after inoculation were lower in the SIVmac239-184V-infected macaques than in the SIVmac239-infected animals, no significant differences were observed from week 2 onwards. Two animals in each group developed AIDS and were euthanized, while all other animals were clinically stable at 46 weeks of infection. These data demonstrate that the M184V mutation in SIV conferred a slightly reduced fitness but did not affect disease outcome.

The emergence of viral mutants with reduced drug susceptibility has been a major barrier to successful drug therapy of human immunodeficiency virus (HIV) infection of humans (49). The main strategy to combat resistance has been the use of drug combinations. The combined use of three or more drugs in highly active antiretroviral therapy has been a major factor in decreasing the number of AIDS-related deaths and the number of new HIV type 1 (HIV-1)-infected individuals in developed countries in the past few years (18). However, the emergence of multidrug-resistant mutants is a growing problem (9), and it is likely that additional strategies to minimize drug resistance will be required to sustain the advances made with highly active antiretroviral therapy.

Drug resistance mutations are expected to reduce the replicative ability of the virus in the absence of drug (8). Indeed, reduced fitness has been demonstrated in vitro for HIV-1 mutants resistant to nucleoside analog inhibitors of reverse transcriptase (RT) or to protease inhibitors (11, 23, 37, 40, 54). A crucial question is whether such reduced fitness will result in attenuated virulence and slower disease progression in HIV-infected patients. If we can identify pathways for resistance that also reduce viral virulence, then it may be possible to take advantage of these in the design of therapeutic strategies. Precedence for attenuation via drug resistance mutations has been provided from work with herpes and influenza viruses. A major reason for the long-term success of the antiherpes drug acyclovir is that most mutants of herpes simplex virus that are resistant to this drug are nonpathogenic and unable to reactivate from nerve tissue (7, 16). Similarly, influenza virus mutants that are resistant to a neuraminidase inhibitor have significantly reduced virulence in mice (55).

The virulence of drug-resistant HIV-1 variants cannot be assessed directly in human patients. Therefore, we use an animal model, simian immunodeficiency virus (SIV) infection of rhesus macaques, to evaluate the virulence of drug-resistant mutants. This model has proven useful for studies of nucleoside analogs, such as 3′-azido-3′-deoxythymidine (AZT; zidovudine) and 9-[2-(phosphonomethoxy)propyl]adenine (PMPA; tenofovir) and for evaluating the emergence, virulence, and clinical implications of drug-resistant viral mutants (59, 60, 62, 67). AZT therapy of SIVmac251-infected newborn macaques resulted in the emergence of SIV mutants highly resistant to AZT; these mutants had a glutamine-to-methionine substitution at position 151 of RT and were fully virulent following inoculation in newborn macaques (62, 67). PMPA treatment of SIVmac251-infected infant macaques resulted in the emergence of mutants with fivefold-reduced susceptibility to PMPA; these mutants had K65R and additional mutations in RT (59). These K65R mutants were as virulent as wild-type

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SIVmac251 following inoculation into newborn macaques (60, 66).

In the work presented here we utilized this model to study the emergence and clinical implications of SIV mutants resistant to the oxathiolane nucleosides (−)-β-L-2′,3′-deoxy-3′-thiacytidine (3TC; lamivudine) and (−)-β-L-2′,3′-dideoxy-5-fluoro-3′-thiacytidine [(−)-FTC; emtricitabine]. (−)-FTC is a 3TC analog with stronger in vitro activity and an improved pharmacokinetic profile (19, 21, 50). HIV-1 variants resistant to these drugs occur predominantly from a methionine-to-valine mutation in position 184 (M184V) of RT, although a mutation to isoleucine (M184I) also occurs transiently (28, 53). Either of these mutations results in high-level (>100-fold) resistance to 3TC or to (−)-FTC (52, 56). These M184V/I mutants have some unique features in vitro. They have impaired replication kinetics in primary T-lymphocyte cultures but not in established T-cell lines (1, 31, 34, 41). The RT enzymes of these mutants have reduced processivity and enhanced fidelity in biochemical assays (1, 3, 69). And the M184V mutation is known to affect the phenotypic expression of other drug resistance mutations (22, 34, 41, 70).

The SIV macaque model was used to further evaluate the in vivo fitness and virulence of M184V viral mutants. We report here the rapid emergence of M184V variants in 3TC- and (−)-FTC-treated SIVmac251-infected macaques. In addition, we investigated the in vivo fitness and virulence of a variant of the molecular clone SIVmac239 with this M184V mutation in RT.

MATERIALS AND METHODS

Animals and sample collection. Newborn and juvenile rhesus macaques (*Macaca mulatta*) were from the type D retrovirus- and SIV-free colony at the California Regional Primate Research Center. Newborn macaques were hand-reared in a primate nursery in accordance with American Association for Accreditation of Laboratory Animal Care Standards. We strictly adhered to the *Guide for the Care and Use of Laboratory Animals* prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (44). When necessary, animals were immobilized with ketamine-HCl (Parke-Davis, Morris Plains, N.J.), 10 mg/kg of body weight, injected intramuscularly. EDTA- and heparin-anticoagulated blood samples were collected regularly to measure viral and immunologic parameters. Complete blood counts were performed on EDTA-anticoagulated blood samples from all animals. Samples were analyzed by using an automated electronic cell counter (Baker 9000; Serono Baker Diagnostics), and differential counts were determined manually. To monitor the immune response to nonviral, nonreplicating antigens, the newborn rhesus macaques were immunized subcutaneously with 0.1 mg of cholera toxin B subunit at 2 weeks of age (i.e., at peak viremia), and a booster immunization was given at 10 weeks of age. The cholera toxin-specific immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) has been described elsewhere (68).

Viruses. Strains of SIV used in this study were uncloned SIVmac251 and virus derived from the molecular clone SIVmac239. The SIVmac251 virus stock used in this study was propagated on rhesus peripheral blood mononuclear cells (PBMC) and had a titer of 10^5 50% tissue culture infectious doses ($TCID_{50}$) per ml. SIVmac239 containing the M184V mutation in RT (SIVmac239-184V) was generated by site-directed mutagenesis, as previously described (5). The 3′ half of SIVmac239 that was used had the premature stop codon at position 93 of *nef* repaired (p239SpE3′/nef-open) (51). The SIVmac239 and SIVmac239-184V virus stocks were propagated in CEMx174 cells and had titers of 3.2×10^5 and 1.8×10^5 $TCID_{50}$ per ml, respectively. Virus titers were determined by limiting-dilution tissue culture methods as previously described (64). Experiments demonstrated that short-term in vitro propagation of M184V virus in the absence of drug does not result in detectable reversion to the wild-type sequence (unpublished data).

Virus inoculation. In initial studies to monitor drug efficacy and emergence of drug-resistant variants, we utilized newborn macaques infected with SIVmac251. Within 3 days of age, the newborn macaques were inoculated orally under ketamine anesthesia with 1 ml of the uncloned SIVmac251 virus stock, as described previously (65). One of the animals (30579) had received two doses of tenofovir near the time of virus inoculation but became infected with an initial viremia that was indistinguishable from that of untreated control animals and was therefore added to this study (65). For experiments to compare the virulence of SIVmac239 and SIVmac239-184V, we utilized juvenile macaques that were 6 to 8 months of age (~1.2 to 2.1 kg) at the time of virus inoculation. Animals were inoculated intravenously with 0.5 ml of virus dilution containing 10^3 $TCID_{50}$ of either SIVmac239 or SIVmac239-184V.

Administration of drugs. Stock solutions of 3TC or (−)-FTC (16 mg/ml) were prepared in phosphate-buffered saline (pH 7.4; Sigma-Aldrich, St. Louis, Mo.), filter sterilized (pore size, 0.2 μm; Nalgene, Rochester, N.Y.), and stored at 4°C. Drugs were administered subcutaneously into the backs of animals at a regimen of 8 mg per kg of body weight once daily. The dosage regimen of 3TC was selected because it is expected to give areas under the concentration-time curve for adult macaques similar to those of the daily dose of 3TC used in human pediatric patients (4 mg/kg twice daily) (36, 42) or adult patients (150 mg orally twice daily) (24, 57), except that for our macaques, it was administered in a single daily dose. Drug dosages were adjusted weekly according to body weight. The untreated control animals did not receive daily sham inoculations.

Quantitative virus isolation (cell-associated and cell-free). Levels of infectious virus in cells and plasma of peripheral blood were determined regularly by a limiting-dilution assay (four replicates per dilution) of PBMC and plasma, respectively, in cultures with CEMx174 cells in 24-well plates and subsequent p27 core antigen measurement, according to methods previously described (64). Virus levels in fresh lymphoid tissues (lymph nodes, spleen, and thymus) collected from the animals at the time of necropsy were determined by aseptically teasing tissues into single-cell suspensions of mononuclear cells and by a limiting-dilution culture assay similar to the one described above for PBMC.

Plasma viral RNA levels. Quantitative assays for the measurement of SIVmac251 RNA were performed by using a branched-DNA signal amplification assay specific for SIV (P. J. Dailey, M. Zamroud, R. Kelso, J. Kolberg, and M. Urdea, Abstr. 13th Annu. Symp. Nonhuman Primate Models AIDS, abstr. 99, 1995). The lower quantification limit of this assay was 1,500 copies of SIV RNA per ml of sample. A real-time TaqMan PCR assay was used to quantitate SIVmac239 RNA as previously described (35). This assay has a sensitivity of 50 copies of viral RNA per ml of plasma (35).

Anti-SIV class-specific antibody determination. The whole-virus anti-SIV IgG antibody ELISA was described previously (63).

Lymphocyte phenotyping by three-color flow cytometry. T-lymphocyte antigens were detected by direct labeling of whole blood with peridinin chlorophyll protein-conjugated anti-human CD8 (clone SK1; Becton Dickinson Immunocytometry Inc., San Jose, Calif.), phycoerythrin-conjugated anti-human CD4 (clone M–T477; Pharmingen), and fluorescein-conjugated anti-human CD3 (clone SP34; Pharmingen). A separate aliquot of blood was labeled with fluorescein-conjugated anti-human CD3 and peridinin chlorophyll protein-conjugated anti-human CD20 (clone L27; Becton Dickinson). Red blood cells were lysed, and the samples were fixed in paraformaldehyde with the Coulter Q-prep system (Coulter Corporation, Hialeah, Fla.). Lymphocytes were gated by forward and side light scatter and were then analyzed with a FACScan flow cytometer (Becton Dickinson). $CD4^+$ and $CD8^+$ T lymphocytes were defined as $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ lymphocyte populations, respectively. B lymphocytes were defined as $CD3^-$ $CD20^+$ lymphocytes.

Drug susceptibility assays. Phenotypic drug susceptibilities of SIVmac isolates were characterized by a previously described assay based on a dose-dependent reduction of viral infectivity. This assay is able to detect SIV mutants with decreased susceptibility to several antiviral drugs (59, 67).

Sequence analysis of the SIV RT-encoding region. DNA sequence analyses were performed on proviral DNA obtained from 5.0×10^5 CEMx174 cells infected with virus isolated from the SIV-infected animals. Infected cells were harvested as soon as culture supernatants were positive by antigen capture ELISA. Genomic DNA was extracted by a silica-guanidine thiocyanate extraction protocol (6) in a 50-μl elution volume (10 μM Tris, pH 8.5). A 2-μl aliquot of each DNA preparation was amplified by nested PCR. The primers used for each round are described in Table 1. Each round of the nested PCR was carried out under the following conditions: incubation at 94°C for 45 s, 30 cycles of 94°C for 45 s, 57°C for 40 s, and 72°C 120 s, and incubation at 72°C for 5 min. All PCRs used RedTaq polymerase (Sigma-Aldrich) under the manufacturer's recommended conditions. Amplicons were sequenced by Davis Sequencing (Davis, Calif.) with the primers listed in Table 1, and data were compared to published sequences of SIVmac251 and SIVmac239 (accession numbers M19499 and M33262, respectively.)

TABLE 1. Primer sequences for PCR and sequencing of SIV RT

Primer	3′ base	Sequence (5′–3′)
PCR primers		
First round		
239–2459	2459	ACC CAG CTG TGG ATC TG
239–4751 (R)	4751	TCC ACT AGC TAC ATG TAC TGC AAC
Second round		
239–2675	2675	GTA ACA GGA ATA GAG TTA GGT CCA C
239–4615 (R)	4615	TCT GTC TGG CCA CTA TTC TG
Sequencing primers		
239–2786	2786	ATT AAA GGG ACA ATC ATG ACA G
SIV-RT3	3153	GAA TAC CAC ACC CTG CAG GAC TAG C
SIV-RT5	3658	ATT GGG CAG CTC AAA TTT ATC CAG G
SIV-RT9	3838	GGC AAG CCA TTA GAA GCC ACG GTA

Necropsy and collection and preparation of tissue samples. Animals were euthanized when it became apparent that their condition was terminal, according to criteria described previously (68). Tissues collected at necropsy were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 6 μm, stained with hematoxylin and eosin, and examined by light microscopy.

Statistical analysis. Statistical analysis was used to compare treated and untreated SIV-infected animals with regard to survival and virus levels. Survival was compared with the generalized Wilcoxon test (12). Virus levels in peripheral blood were compared by calculating the area under the curve for each animal, followed by analysis according to the Wilcoxon rank-sum test (12). It was previously shown that these analyses can distinguish biologically relevant differences (39, 60, 67).

RESULTS

The major goal of this project was to evaluate the effect of the M184V mutation in RT on the virulence and fitness of SIV in vivo. In order to do this we had to first establish whether the M184V mutation emerges following 3TC or (−)-FTC therapy of SIV-infected macaques in a manner similar to the emergence of M184V mutants of HIV-1 in humans. Therefore, we utilized the model of SIVmac251 infection of newborn macaques. Next, the effect of M184V on fitness and virulence was studied by comparing juvenile macaques inoculated with the wild-type molecular clone of SIVmac239 or with SIVmac239 containing the M184V mutation.

Emergence of M184V variants of SIVmac251 in newborn macaques. Within 3 days after birth, four newborn macaques were inoculated orally with virulent, wild-type (i.e., drug-susceptible) uncloned SIVmac251 as described previously (65). All four animals became persistently infected following oral SIVmac251 inoculation and had peak viremia levels at 2 weeks (Fig. 1). Three weeks after virus inoculation, two animals each were started on 3TC and on (−)-FTC (8 mg/kg of body weight, subcutaneously once daily) (Table 2).

Treatment of the four SIVmac251-infected infant macaques with 3TC or (−)-FTC had no significant effect on viral RNA levels in plasma (Fig. 1) or PBMC-associated infectious-virus titers, determined by comparing these data to those of a large number of historical untreated newborn macaques that had previously been inoculated orally with the same virus stock (58, 61, 63). Virus isolates obtained from PBMC and plasma of these animals were cultured in CEMx174 cells and used for phenotypic and genotypic analyses. Population sequence analysis of PCR products of the RT region revealed that after 3 weeks of treatment, all virus isolates still had wild-type sequence but that in the next sample, collected 5 weeks after the start of treatment (i.e., 8 weeks after virus inoculation), the M184V mutation (ATG to GTG) was present in all four animals and the wild-type sequence was no longer detected. No M184I mutation was detected, although we cannot exclude the possibility that such mutants may have been present transiently between 3 and 5 weeks of treatment but were replaced rapidly by M184V mutants due to the high viral turnover rates in these animals. Phenotypic analyses also revealed that the M184V virus isolates from each of these four animals were >100-fold resistant to 3TC. To detect any other mutations in RT that may have emerged during drug treatment, the whole RT-encoding region was sequenced from virus isolated from PBMC of these four animals at the time of death or at 50 weeks of age (for animal 30852). Mutations additional to M184V that were found were R35K (animal 30575), V90I (animal 30852), I341 M (animal 30579), R394K (animals 30579 and 30583), P420L (animal 30852), and E434K (animal 30852). The significance of these mutations is unclear, but they are unlikely to contribute to 3TC resistance; except for 341M and 420L (which were found only in a single animal), all of the amino acid substitutions are found in other wild-type SIV or HIV-2 isolates (43).

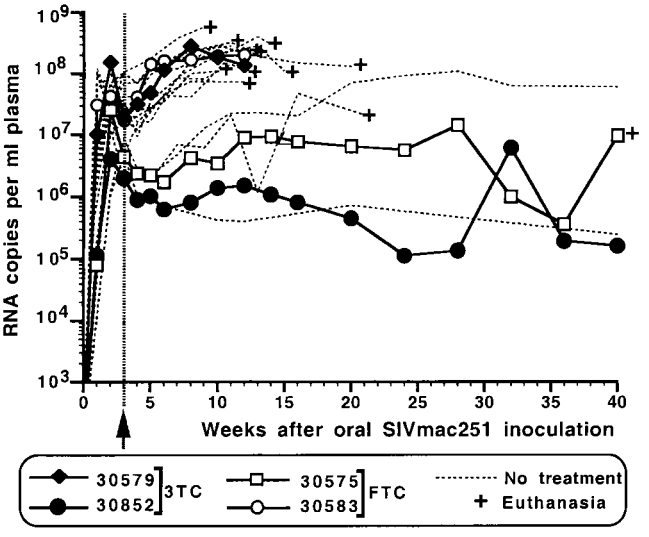

FIG. 1. Virus levels in SIVmac251-infected infant macaques receiving 3TC or (−)-FTC treatment. All newborn macaques were inoculated at birth orally with SIVmac251. The dashed lines represent 14 untreated SIVmac251-infected infant macaques from a number of previous studies. Four animals were started on 3TC or FTC treatment at 3 weeks of age (arrow). Plasma RNA levels were measured by branched-DNA assay.

Two animals, one in each treatment group (30579 and 30583), had persistently high viral RNA levels ($>10^7$ RNA copies/ml) (Fig. 1). These animals failed to make strong and persistent SIV-specific IgG antibody responses (peak titers of 1:400 and 1:25,600 for animals 30853 and 30579, respectively) (65), and their antibody responses following immunizations with cholera toxin B subunit were reduced compared to those in uninfected age-matched control animals (data not shown). These two infants developed fatal immunodeficiency at 12

TABLE 2. Treatment of SIVmac251-infected infant macaques with 3TC or FTC: experimental design and summary of outcome[a]

Animal (sex)	Treatment	Clinical outcome	Histopathology
30579 (F)	3TC	AIDS at 12 wk	Lymphoid depletion, chronic fibrosing pancreatitis with adenovirus infection, enterocolitis, hypoproteinemia (plasma protein, 4 gm/dl), bronchitis
30852 (M)	3TC	Healthy at 22 mo[b]	Lymphoid hyperplasia, cryptosporidium-positive cholecystitis, and pancreatitis with fibrosis and acinar atrophy; pneumonia
30583 (M)	(−)-FTC	AIDS at 12 wk	Enterocolitis, thymic atrophy, cheilitis, hypoproteinemia (plasma protein, 3.1 gm/dl)
30575 (F)	(−)-FTC	Disease at 40 wk	Chronic fibrosing pancreatitis; serositis; mucinous degeneration and fibrosis of intima of pulmonary blood vessels with thrombosis; thymic atrophy; lymphoid depletion

[a] Animals were inoculated at birth orally with SIVmac251; 3 weeks later, they were started on 3TC or (−)-FTC treatment (8 mg/kg, administered subcutaneously once daily).
[b] Animal 30852 was euthanized while still clinically stable at 22 months of age.

weeks of age, with widespread virus dissemination in lymphoid tissues (including thymus, spleen, and lymph nodes), reduced percentages of $CD4^+$ T lymphocytes over total lymphocytes, and reduced $CD4^+/CD8^+$ T-lymphocyte ratios. Histopathological findings in these animals were consistent with terminal SIV disease (Table 2). Sequence analysis was performed on virus isolated from different lymphoid compartments (PBMC, plasma, spleen, and axillary, mesenteric, and inguinal lymph nodes) collected from these animals at time of euthanasia. For all compartments, only M184V sequence was detected; the only exception was the thymus of animal 30583, for which the virus isolate had only wild-type sequence. This fulminant disease course in animals 30579 and 30583, characterized by rapid immunosuppression and widespread virus dissemination, is similar to that seen in the majority of untreated SIVmac251-infected infant macaques, which have persistently high viremia and poor antiviral immune responses due to rapid immunosuppression and develop disease in 2 to 4 months (58, 59, 61, 63, 67).

The other two animals (30575 and 30852) had a delayed and lower primary viremia prior to week 3, and in comparison to untreated SIV-infected animals, drug treatment did not induce a significant reduction in the virus levels in these animals; these animals were able to make a strong and persistent antiviral IgG antibody response (titers > 1:102,400), and their time course resembled that of a minority of untreated SIVmac251-infected infant macaques which have a delayed disease course (survival for 1 to 2 years) (Fig. 1). Animal 30575, which was treated with (−)-FTC, had to be euthanized at 40 weeks of age due to rapidly deteriorating health; the most striking gross lesion found during autopsy was a severely enlarged (~5-cm diameter) and inflamed pancreas and pulmonary thrombosis. Histopathological examination revealed chronic pancreatitis with severe fibrosis and degeneration of the wall of pulmonary blood vessels (Table 2). Pancreatitis has also been considered a serious side effect of 3TC treatment in HIV-infected children with advanced disease (36; product information for 3TC [Epivir]; GlaxoSmithKline). Thus, it is possible that SIV-induced immunosuppression may have predisposed this animal to a similar pancreatic toxicity.

The fitness of a drug-resistant virus can be determined by the rate of reversion following drug withdrawal. Because virus levels in the 3TC-treated animal 30852 were relatively low after the initial viremia (10^5 to 10^6 copies of SIV RNA per ml of plasma) (Fig. 1), treatment was stopped at 50 weeks of age. In virus isolated from PBMC of this animal up to 59 weeks of age (9 weeks after 3TC withdrawal), only mutant sequence (M184V) was detected. During these first 9 weeks after 3TC withdrawal, virus levels in this animal remained relatively constant (between 0.6×10^6 and 0.8×10^6 copies of viral RNA/ml, at all six times when blood samples were collected). However, virus isolated from PBMC at 66 weeks of age (16 weeks after 3TC withdrawal) was wild-type (M) at position 184. Viral RNA levels in plasma at 66 and 76 weeks of age were 1.7×10^6 and 2.0×10^6 copies per ml, respectively, which indicated an approximately threefold increase compared to those at week 59, when M184V mutant was still dominant. Animal 30852 was clinically stable at 22 months of age but, when euthanized at that time, showed evidence of immune dysfunction (Table 2).

In vivo fitness and virulence of SIVmac239-184V. We used juvenile macaques to compare the fitness and virulence of wild-type SIVmac239 to those of SIVmac239-184V. For these studies, juvenile macaques were inoculated intravenously with 1,000 $TCID_{50}$ of SIVmac239 (group A [$n = 5$]) or SIVmac239-184V (groups B [$n = 5$] and C [$n = 2$]) (Table 3). The SIVmac239-184V-infected animals of group B were treated with (−)-FTC (8 mg/kg of body weight, administered subcutaneously once daily), beginning 1 day prior to virus inoculation, in order to prevent reversion of the mutant virus. All animals became persistently infected. Virus levels reached peak levels by 1 week postinoculation in the five animals infected with SIVmac239 ($>10^6$ RNA copies/ml; average ± standard deviation, log = 6.88 ± 0.74) (Fig. 2). In contrast, all seven SIVmac239-184V-inoculated animals had significantly lower (~2 log) viral RNA levels 1 week after inoculation (log = 4.42 ± 0.90 and 4.70 ± 0.18 for groups B and C, respectively; $P < 0.001$, Wilcoxon rank-sum test). But from week 2 on, there was no difference among the groups (Fig. 2), and animals showed similar individual variation over time. Beyond 20 weeks postinoculation, most surviving animals had virus loads greater than 10^4 copies of viral RNA per ml of plasma; the main exception was one animal in group A (number 31304) for which virus levels remained lower. There were no significant differences in virus loads between the groups regardless of whether data from animal 31304 were included (Fig. 2D).

The two SIVmac239-184V-infected animals of group C did not receive any (−)-FTC treatment and were used to monitor reversion. In both animals, mutant M184V virus was detected until 6 weeks postinfection, but wild-type virus was predominant by 8 weeks postinfection. DNA sequence analyses confirmed that the M184V mutation in the (−)-FTC-treated SIVmac239-184V-infected animals (group B) did not revert to wild

TABLE 3. Inoculation of juvenile macaques with wild-type SIVmac239 or SIVmac239-184V: experimental design and summary of outcome[a]

Group	Inoculum	Drug	Animal	Outcome	Histopathology
A	SIVmac239	None	31547	AIDS at 14 wk	Mixed pattern of lymphoid depletion and hyperplasia; thymic atrophy; interstitial pneumonia; pleuritis; fibrosing pancreatitis with amphophilic intranuclear inclusion bodies (possible papovavirus or adenovirus); cryptosporidium-positive enteritis and cholecystitis
			31629	AIDS at 20 wk	Cryptosporidium-positive cholecystitis, gastroenterocolitis, and pancreatic ductitis; *Pneumocystis carinii*-positive pneumonia
			31304	Alive at 46 wk	NA
			31339	Alive at 46 wk	NA
			31632	Alive at 46 wk	NA
B	SIVmac239-184V	FTC	31694	AIDS at 26 wk	Pneumonia; cryptosporidium-positive gastroenterocolitis, oral and esophageal candidiasis; thymic atrophy; lymphoid hyperplasia
			31502	AIDS at 30 wk	Cryptosporidium-positive cholecystitis, cholangitis, and gastroenteritis; lymphoid hyperplasia; thymic atrophy; fibrosing pancreatitis; oral and esophageal candidiasis; histiocytic alveolar pneumonia (possible *P. carinii*)
			31690	Alive at 46 wk	NA
			31579	Alive at 46 wk	NA
			31585	Alive at 46 wk	NA
C	SIVmac239-184V	None	31714	AIDS at 24 wk	Thymic atrophy; *P. carinii*-positive pneumonia; cryptosporidium-positive gastritis and colitis; cholangitis, lymphoid hyperplasia
			31535	AIDS at 39 wk	Lymphoid depletion; protozoan-positive enteritis (possible *Trichomonas*); amphophilic intranuclear inclusion bodies in kidney (possible papovavirus or adenovirus infection)

[a] Juvenile rhesus macaques (6 to 8 months of age) were inoculated intravenously with 1,000 $TCID_{50}$ of SIVmac239 or SIVmac239-184V. Animals of group B were started on (−)-FTC treatment (8 mg/kg subcutaneously once daily) 1 day prior to the virus inoculation. NA, not applicable.

type. This suggests that the (−)-FTC dosage regimen exerted sufficient selection pressure to maintain the M184V mutation.

Absolute $CD4^+$-T-lymphocyte and $CD8^+$-T-lymphocyte counts in peripheral blood were quite variable over time. At the time of virus inoculation, there were no significant differences in these lymphocyte subsets between the different groups ($P > 0.9$). At 2 weeks after virus inoculation, however, four of the five SIVmac239-infected animals had a decrease, while six of the seven SIVmac239-184V-inoculated animals demonstrated an increase in absolute $CD4^+$-T-lymphocyte counts; accordingly, the SIVmac239-infected animals had lower absolute $CD4^+$ T lymphocyte counts than the SIVmac239-184V-infected animals at 2 and 4 weeks after virus inoculation ($P \leq 0.05$, two-tailed t test) (Fig. 3). This difference in absolute $CD4^+$-T-lymphocyte counts disappeared from 6 weeks on. No differences were observed in absolute $CD8^+$-T-lymphocyte counts. The CD4/CD8 T-lymphocyte ratio was significantly lower in the SIVmac239-infected animals than in the SIVmac239-184V-infected animals only 2 weeks after virus inoculation ($P = 0.002$; two-tailed t test) (Fig. 3). After the primary viremia stage there were no significant differences in any of these parameters between the groups of animals. Individual animals in each group showed a decline of $CD4^+$-T-lymphocyte counts to $<500/\mu l$ and CD4/CD8 T-lymphocyte ratios of <1 during disease progression.

Two animals in group A and two in group B developed fatal immunodeficiency within 30 weeks of infection (Table 3), while the remaining animals in these groups remained clinically stable throughout the observation period of 46 weeks. The two animals of group C succumbed to disease at 24 and 39 weeks of age. Accordingly, there were no statistically significant differences in survival between groups ($P > 0.1$, generalized Wilcoxon test). Limiting-dilution culture assays performed on plasma, PBMC, and mononuclear cells from several tissues collected during necropsy (spleen, thymus, and mesenteric lymph node) revealed that all groups had similarly extensive virus dissemination. Histopathological findings in all animals were consistent with terminal SIV disease and immunodeficiency (Table 3).

DISCUSSION

It is well established that the M184V mutation in RT of HIV-1 results in alteration of several in vitro properties. In particular, the RTs of these mutants have reduced processivity, and M184V viral mutants have impaired replication kinetics in primary T-lymphocyte cultures (1, 3, 31, 34, 41). Although the M184V mutation does not prevent patients from developing HIV-associated illnesses, evidence suggests that the M184V mutation may offer clinical benefits, especially during combination therapy (4, 14, 17, 27, 28, 32, 34, 36, 53). However, many unanswered questions remain regarding the effects of these mutations on viral fitness, virulence, and efficacy of drug therapy in HIV-infected patients. Therefore, in the studies presented here, the SIV macaque model was used to further evaluate the in vivo emergence, fitness, and virulence of M184V mutants.

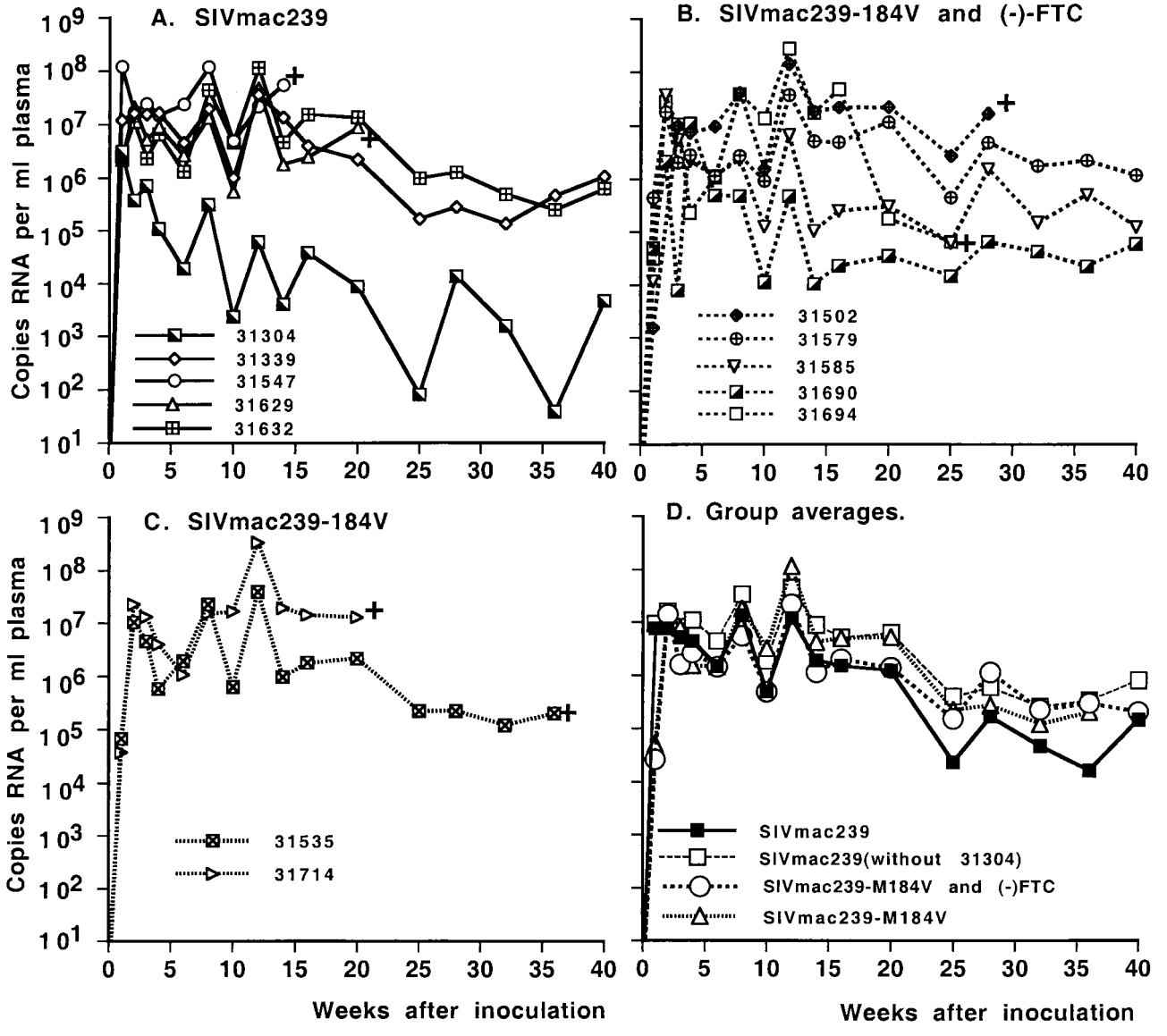

FIG. 2. Virus levels in juvenile macaques infected with SIVmac239 or SIVmac239-184V. Plasma RNA levels, measured by real-time PCR (TaqMan), are presented for animals inoculated intravenously with SIVmac239 (A) or SIVmac239-184V (B and C). Some animals (B) also received (−)-FTC treatment to prevent reversion. +, euthanasia because of simian AIDS. (D) Averages for the different groups, including the average for group A when animal 31304 is excluded. There were no statistically significant differences in virus loads between the groups regardless of whether data from animal 31304 were included.

We have demonstrated that the M184V mutation emerged rapidly in four SIVmac251-infected infant macaques that were started on 3TC or (−)-FTC treatment 3 weeks after virus infection. This emergence of M184V mutants within 5 weeks of drug treatment suggests that drug levels were sufficient to select for the outgrowth of M184V mutants. However, these drug regimens had no significant effect on viral RNA levels in plasma or PBMC-associated infectious virus titers. This finding was unexpected, because in human clinical trials with 3TC or (−)-FTC monotherapy, a 1- to 2-log reduction in viral RNA was observed within 1 to 2 weeks (17, 28, 32, 47, 50, 53). This observation may be the result of a combination of factors. It is unlikely that a higher drug dosage would have been more effective, as oral 3TC dosing of SIV-infected adult macaques at doses up to 20 mg/kg twice daily also did not result in any reduction in viral RNA levels (E. Delwart, personal communication). The high viremia with a highly virulent isolate (SIVmac251) in infants (which do not have fully developed immune systems) may have been overwhelming for these antiviral compounds as monotherapy. In this context, 3TC monotherapy during advanced HIV disease was less effective in suppressing viral RNA for children than for adults (28, 36). Finally, in vitro studies demonstrated that the concentration of 3TC required to inhibit SIV in vitro is slightly higher (about fivefold) than that for HIV-1 (2, 5; A. Giuffre and T. North, unpublished data). It is not known whether this is due to differences in the

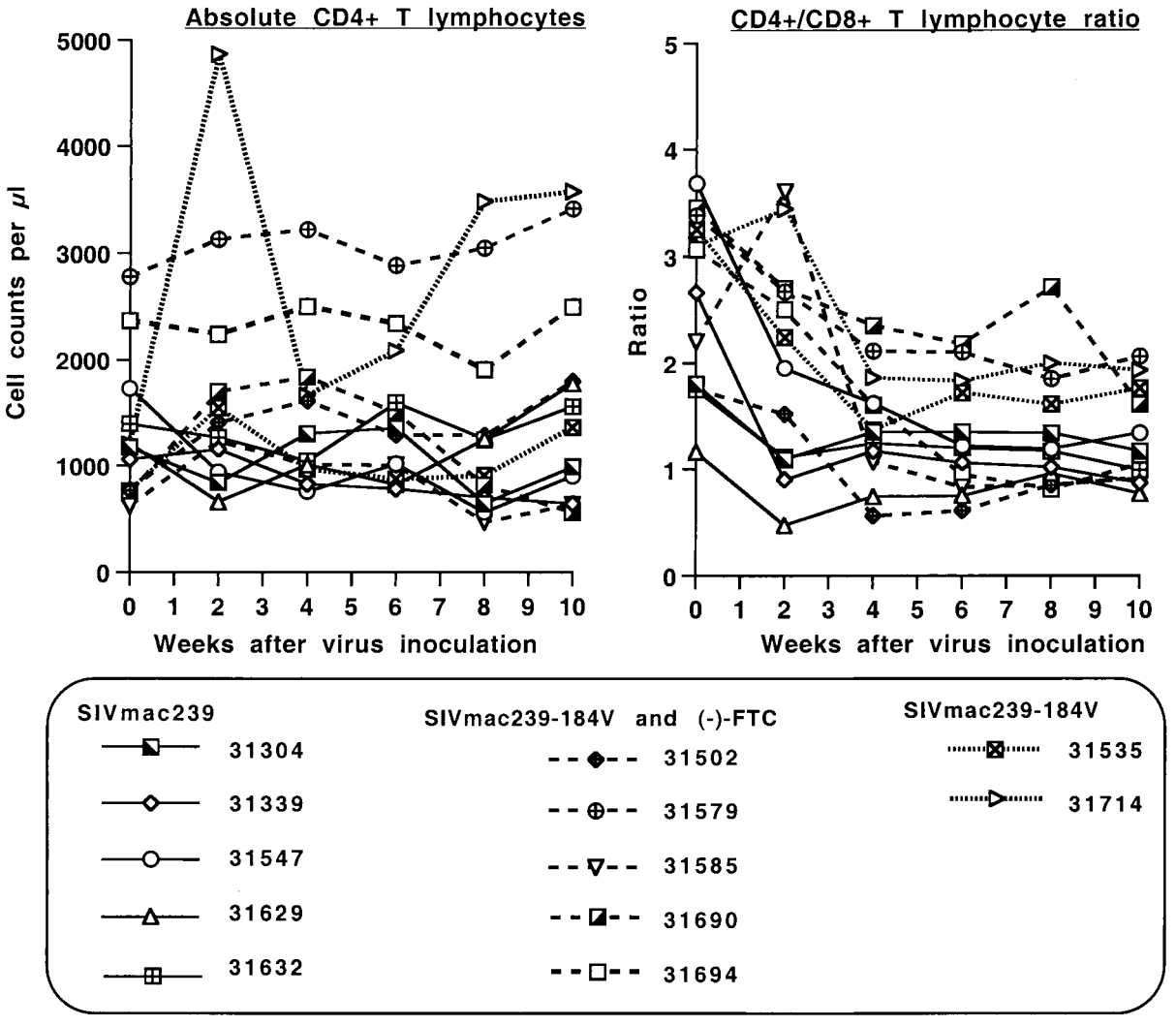

FIG. 3. Changes in absolute $CD4^+$-T-lymphocyte counts and CD4/CD8 T-lymphocyte ratios. Animals were inoculated intravenously with SIVmac239 or SIVmac239-184V. Five of the SIVmac239-184V-infected animals also received (−)-FTC treatment. Absolute $CD4^+$ $CD3^+$ T lymphocytes and the ratio of $CD4^+$ $CD3^+$ to $CD8^+$ $CD3^+$ T lymphocytes are presented for the first 10 weeks of infection.

two RTs, but wild-type SIV isolates have amino acids at two positions in RT (44D and 118I) that differ from those of HIV-1, and these mutations in HIV-1 confer approximately threefold resistance to 3TC in vitro (26). Most importantly for the studies we report here, despite the absence of a transient reduction in virus levels, the drug regimens exerted sufficient selection pressure for the outgrowth of M184V mutants in the SIVmac251-infected macaques.

The rapid disease course in two of the SIVmac251-infected infant macaques that were treated with 3TC or (−)-FTC (animals 30579 and 30853, respectively), characterized by persistently high virus levels, widespread systemic virus dissemination, poor antiviral immune responses, and rapidly fatal immunodeficiency within 12 weeks, was indistinguishable from that of the majority of newborn macaques infected with wild-type SIVmac251. Thus, a shift to a predominant M184V population did not prevent disease in these animals.

To directly evaluate the effect of the M184V mutation on fitness and virulence, we used two molecular clones that differ only at codon 184 of RT, namely, SIVmac239 and SIVmac239-184V. In vitro experiments found that SIVmac239-184V has a slightly reduced replication rate in CEMx174 cells and rhesus macaque PBMC relative to SIVmac239 (J. B. Whitney, M. Oliviera, M. Detorio, Y. Guan, and M. A Wainberg; submitted for publication). To study the effect of the M184V mutation on in vivo fitness and virulence, groups of juvenile macaques were inoculated with SIVmac239 without drug treatment or with SIVmac239-184V in the presence of (−)-FTC treatment (to maintain selection pressure) or absence of drug treatment (to monitor reversion in the absence of selective pressure). Reversion of SIVmac251-184V in animal 30852 occurred between 9 and 16 weeks after 3TC withdrawal. For the two SIVmac239-184V-infected animals which did not receive any (−)-FTC treatment, reversion was detected 8 weeks after virus inoculation. These findings of a reversion from M184V to wild-type sequence confirm that in the absence of drug treatment, SIV

isolates with the M184V mutation have reduced fitness relative to wild-type virus, but the rate of reversion suggests that this reduction in fitness is of moderate magnitude. Other mutations (such as a stop codon in *nef*) that affect the in vivo replication rates of SIV more severely reverted to the wild-type sequence within 2 weeks after inoculation (30, 33).

It was recently reported that the M184V mutation did not revert when macaques were infected with SIVmac239 containing both the M184V and the E89G mutations, whereas the E89G mutation did revert (45). However, the M184V mutation in that study was engineered with two base changes in codon 184, whereas our mutant was constructed to have the single base change observed in drug-resistant SIVmac251 in vivo or SIVmac239 in vitro. Thus, our mutant construct is expected to revert more rapidly than the construct with two base changes. Those authors also reported the appearance of a P272S mutation in virus from animals that had the M184V mutants (45). We did not detect this mutation in virus from any of the animals infected with SIVmac239-184V, including late samples that were collected 40 weeks after infection.

The reduced fitness of M184V mutants may explain why virus levels in the SIVmac239-184V-infected animals 1 week after inoculation were lower than those in animals inoculated with wild-type SIVmac239. Virus levels at 1 week were similarly low in the SIVmac239-184V-infected animals whether they received (−)-FTC treatment or not. Thus, this effect is not due to (−)-FTC inhibition of the M184V virus in vivo, which was predicted to be unlikely based on the >100-fold level of in vitro resistance induced by this mutation. Therefore, the lower virus levels observed in the SIVmac239-184V-infected animals 1 week after virus inoculation are best explained by a reduced fitness of the mutant versus wild-type virus. However, from week 2 on, no difference in virus levels could be observed, as virus levels in the SIVmac239-184V-infected animals reached levels similar to those observed for the wild-type virus. Observations of primary infection of humans with populations of HIV-1 containing M184V mutants also suggest that this mutation does not affect the ability to reach high levels of viremia. In these patients, primary infection with HIV-1 M184V mutant populations led to symptoms of acute infection and peak virus levels similar to those of wild-type HIV-1 (10, 13, 25), although it is unknown whether the time to reach this peak was longer than that for wild-type virus.

An important finding was that, although M184V mutants of SIV were initially less fit (as evident from slower replication during the first week of infection and from reversion in the absence of drug), they were virulent and capable of inducing disease. There was no statistically significant difference in disease-free survival between animals infected with M184V and those with wild-type SIV isolates. The histopathological lesions in the animals with SIVmac239-184V were consistent with SIV-induced fatal immunodeficiency.

The observation that a decreased replicative fitness of the M184V SIV mutants did not cause a significant change in virulence (i.e., their ability to cause disease) or in viremia from week 2 on deserves further attention. Virulence and replicative fitness usually correlate well, as large differences in replicative fitness translate into significant differences in virulence and disease course (15, 38, 39, 48). However, this correlation is not stringent, especially when virus isolates with relatively smaller differences in replicative fitness are compared. Virulence is a broad issue of viral pathogenesis that depends not only on replicative fitness but also on other factors. These factors include the ability of the virus to weaken the immune system and to evade the different antiviral immune responses that the host is mounting in an attempt to control virus replication. Although in vitro studies often provide useful information on replicative fitness, they are currently not able to completely model these complex interactions with the immune system and predict in vivo virulence (30). Animal models are the best tools for providing further insights in fitness and virulence. Our data suggest that the reduction in replicative fitness of SIV caused by the M184V mutation was too small to alter its virulence or was compensated for by other mechanisms. In this context, Frost et al. observed that the drop in viral fitness associated with the M184V mutation in HIV-1 resulted in a drop in virus levels only in individuals with high $CD4^+$-T-cell counts, from which the relative fitness of M184V mutants was estimated to be approximately 10% of that of the wild-type virus prior to therapy. In contrast, in individuals with low $CD4^+$-T-cell counts, this decrease in viral fitness had no effect on the viral load, which suggests that the functional status of the immune system further affects the complex interactions between viral fitness, virus levels, and virulence (20).

There are other mechanisms through which the M184V mutation can alter the pathogenesis for HIV-infected patients which were not addressed in the present macaque studies. As mentioned earlier, the M184V mutation is known to affect the phenotypic expression of some drug resistance mutations. In this regard, addition of M184V to a virus with AZT resistance mutations in RT restores phenotypic susceptibility to AZT by decreasing pyrophosphorolysis (22, 34), and other mutations are required to make the virus coresistant to both drugs (29, 46, 71). The M184V mutation also renders virus more susceptible to inhibition by the nucleotide analogs adefovir [9-(2-phosphonylmethoxyethyl)adenine] and tenofovir (PMPA) (41, 70). Thus, the demonstrated benefits of 3TC in combination therapy, including salvage therapies, even after M184V mutants arise (4) can perhaps be attributed more to its effect on the phenotypic expression of resistance to other drugs than to a direct effect on viral fitness and virulence. Studies in animal models using 3TC or (−)-FTC as part of combination regimens can be performed to further elucidate the role of the M184V mutation on these aspects of viral diversification and pathogenesis.

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