The Intracytoplasmic Domain of the Env Transmembrane Protein
Is a Locus for Attenuation of Simian Immunodeficiency Virus SIVmac in Rhesus Macaques

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The human and simian immunodeficiency viruses (HIV-1 and SIVmac) transmembrane proteins contain unusually long intracytoplasmic domains (ICD-TM). These domains are suggested to play a role in envelope fusogenicity, interaction with the viral matrix protein during assembly, viral infectivity, binding of intracellular calmodulin, disruption of membranes, and induction of apoptosis. Here we describe a novel mutant virus, SIVmac-M4, containing multiple mutations in the coding region for the ICD-TM of pathogenic molecular clone SIVmac239. Parental SIVmac239-Nef+ produces high-level persistent viremia and simian AIDS in both juvenile and newborn rhesus macaques. The ICD-TM region of SIVmac-M4 contains three stop codons, a +1 frameshift, and mutation of three highly conserved, charged residues in the conserved C-terminal alpha-helix referred to as lentivirus lytic peptide 1 (LLP-1). Overlapping reading frames for tat, rev, and nef are not affected by these changes. In this study, four juvenile macaques received SIVmac-M4 by intravenous injection. Plasma viremia, as measured by branched-DNA (bDNA) assay, reached a peak at 2 weeks postinoculation but dropped to below detectable levels by 12 weeks. At over 1.5 years postinoculation, all four juvenile macaques remain healthy and asymptomatic. In a subsequent experiment, four neonatal rhesus macaques were given SIVmac-M4 intravenously. These animals exhibited high levels of viremia in the acute phase (2 weeks postinoculation) but are showing a relatively low viral load in the chronic phase of infection, with no clinical signs of disease for 1 year. These findings demonstrate that the intracytoplasmic domain of the transmembrane Env (Env-TM) is a locus for attenuation in rhesus macaques.
with SIVmac-M4 developed transient viremia, which dropped below detectable levels by 8 to 12 weeks postinoculation, and these animals have remained asymptomatic for 1.5 years. Neonatal macaques that received SIVmac-M4 survived the acute phase of infection and are showing relatively low viral loads at 1 year postinoculation, with no disease signs. Taken together, these results demonstrate that the ICD-TM can be considered a locus for attenuation of SIVmac in rhesus macaques.

**MATERIALS AND METHODS**

**Construction of mutants.** Mutants SIVmac-M1 and SIVmac-M4 were described in an earlier report as SIVmac239WT- stop and SIVmac239-M3 stop, respectively (50). The GenBank accession number for SIVmac239 is M33262 (42). Both mutants were created by oligonucleotide-directed mutagenesis of SIVmac239-Nef+. The oligonucleotide primers used are described in reference 50. SIVmac-M4 contains clusters of mutations at four sites: (i) a single stop codon at nucleotides (nt) 9056 to 9058 of env; (ii) two stop codons at nt 9197 to 9199 and 9203 to 9205; (iii) a single-base-pair deletion, nt 9318, creating a +1 frameshift mutation in the ICD-TM; and (iv) three conserved Arg codons changed to Gly codons in the C-terminal amphipathic alpha-helices of ICD-TM, encompassing nt 9459 to 9470. Taken individually, mutations ii, iii, and iv each reduce viral infectivity, as previously reported (50). SIVmac-M1 contains only the stop codon at nt 9056 to 9058 of env. Cassettes containing individual mutations were recombined into a plasmid encoding the 3′ half of the SIVmac239 genome (p239-3′-psp72, obtained from D. Regier, New England Regional Primate Center). All mutations were verified by extensive restriction analysis followed by DNA sequencing of the entire mutated region. Nucleotide numbering in molecular clone SIVmac239 corresponds to that of Regier and Desrosiers (42).

**Preparation of virus stocks.** To recover infectious virus, plasmids encoding the 5′ and 3′ halves of SIVmac239 (wild type or mutants) were linearized in equimolar amounts by digestion with SmaI, ligated with bacteriophage T4 DNA ligase, precipitated in isopropanol-sodium acetate, and resuspended in sterile distilled water (50). COS-7 cells, grown in Dulbecco’s minimal essential medium (DMEM) with 8% fetal bovine serum, were infected with the baculovirus plasmid. All experiments were performed in the absence of antibiotics (R-10). Then 1 × 10⁶ to 2 × 10⁷ CEMx174 cells were added to each plate in 5 ml of R-10 medium. Coculture was continued overnight. The following day, CEMx174 cells were removed from virus-producing COS-7 cells and placed in individual T-25 flasks. These cultures were maintained for 10 to 15 days, and virus-containing supernatant was harvested on days 7, 10, and 14. Supernatants were clarified by centrifugation for 10 minutes at 15,000 rpm, and virus-containing supernatants were used as a source of virus for the inoculation of each animal.

**In vitro growth kinetics.** Peripheral blood mononuclear cells (PBMC) were obtained from whole blood of uninfected rhesus macaques by Ficoll-Hypaque density centrifugation (Pharmacia). Cells were stimulated in R10 medium, supplemented with 0.5 μg of Staphylococcus enterotoxin A (Toxin Technology, Inc., Sarasota, Fla.) per ml, for 3 days before being infected. For each virus, duplicate infections were set up using 10⁵ cells at a multiplicity of infection (MOI) of 0.1. Cells were harvested in RPMI, placed in duplicate wells of a 24-well culture plate, and maintained for up to 3 weeks. Supernatants were withdrawn for p27 antigen testing every 3 to 5 days and replaced with fresh R10 medium containing 50 U of recombinant interleukin-2 per ml.

**Inoculation of rhesus macaques and collection of samples.** The animals used in this study were colony-bred juvenile or newborn rhesus macaques (Macaca mulatta) housed at the California Regional Primate Research Center and determined to be free of simian type D retroviruses, SIV, and simian T-lymphotropic virus. These animals were maintained in accordance with the standards of the American Association for Accreditation of Laboratory Animal Care. Physical examinations were performed at regular intervals to detect lymphadenopathy, splenomegaly, and opportunistic infections. Clinical criteria for euthanasia consisted of three or more of the following: (i) greater than 10% weight loss within 2 weeks or greater than 20% within 2 months; (ii) chronic diarrhea unresponsive to treatment; (iii) infections unresponsive to antibiotic treatment; (iv) inability to maintain body heat or fluids without supplementation; and (v) persistent, marked hematological abnormalities or persistent, marked splenomegaly or hepato-omegaly.

Juvenile rhesus macaques were inoculated with 10⁶ or 10⁷ TCID₅₀ of cell-free virus by the intravenous route. From each juvenile macaque, 8 to 15 ml of blood was collected by venipuncture immediately prior to inoculation, at 1, 2, 3, 4, 6, and 8 weeks postinoculation, and at regular intervals thereafter. Plasma was stored for virus load and antibody determinations. Complete blood count and T-lymphocyte subset analysis were performed at each time point. Lymph node biopsies were performed at 2, 8, 16, 32, and 80 weeks postinoculation. Neonatal rhesus macaques were inoculated intravenously at 2 days of age with 2 × 10⁶ or 1 × 10⁷ TCID₅₀ of cell-free virus. Prior to inoculation, every 2 weeks throughout week 8 and at regular intervals thereafter, 1.5 ml of blood was collected from each macaque.

**Virus load in plasma and mononuclear cells.** Viral p27ₐg antigen in longitudinal plasma samples was measured with an SIVmac p27ₐg ELISA kit (Coulter Immunology). Viral RNA in plasma samples was determined by the branched DNA (bDNA) assay (J. Booth and F. Bailey, Bayer Diagnostics, Emeryville, Calif.) (42).

**Cell-associated viremia was measured in peripheral blood and lymph node mononuclear cells (LNMC) by a limiting-dilution assay as previously described (34). Briefly, serial 10-fold dilutions of rhesus PBMC or LNMC were co-cultivated in the SIV-susceptible cell line CEMx174 in replicate wells of a 24-well tissue culture plate. Cultures were maintained for 4 weeks. The presence of SIV in culture supernatants was assessed by a p27 antigen ELISA as described above. The cell-associated virus load was calculated using the method of Reed and Muench (34).**

**PCR amplification and sequencing of viral DNA.** Genomic DNA was isolated from either 10⁷ PBMC or 10⁵ CEMx174 cells cocultured with PBMC from infected macaques (Qiagen tissue kit). TM sequences were amplified in a nested PCR using first-round primers SIV-340 (nt 8901 to 9290) and SIV-341 (nt 9918 to 9940) and second-round primers SIV-342 (nt 8907 to 9019) and SIV-343 (nt 9816 to 9834) and sequenced on an ABI automated sequence analyzer (Applied Biosystems, Foster City, Calif.).

**Antibody titers.** Antibodies to SIV were assessed in longitudinal samples from experimentally inoculated macaques and control macaques by using an HIV-1/HIV-2 peptide ELISA (Genetic Systems Corp., Redmond, Wash.).

**RESULTS**

**Rationale for ICD-TM mutations.** A single stop codon in the ICD-TM would be expected to revert rapidly in vivo in rhesus macaques (19, 25, 29). However, in previous studies we identified several point mutations in the ICD-TM that, taken individually, each reduced viral infectivity and/or delayed virus replication in vitro (50). The presence of stops 2 and 3 (Fig. 1) reduced the stability of Env, reduced Env incorporation into virions, and delayed viral replication by 2 to 3 weeks (50). The +1 frameshift dramatically reduced Env incorporation into virions.
virions and abrogated viral replication (50). The RRIR-to-
GGIG mutation delayed viral replication by 2 to 3 weeks (50).
We reasoned that the presence of all of these mutations down-
stream of a stop codon would minimize reversion to a full-
length TM reading frame in vivo (Fig. 1). None of the muta-
tions in SIVmac-M1 or SIVmac-M4 affected the translation 
frames for tat, rev, or nef.

In vitro replication kinetics of SIV mutants. It has been 
reported that SIVmac encoding a truncated ICD-TM replic-
ates more efficiently than wild-type SIVmac in established 
human T-cell lines such as HUT-78 (7, 19, 24, 27, 50). In an 
earlier study, we observed that both SIVmac-M1 and SIVmac-M4 
showed more rapid replication than did wild-type SIVmac239-
Nef + in HUT-78 cells (50). In CEMx174 cells, SIVmac239-
Nef +, SIVmac-M1, and SIVmac-M4 replicated with identical 
kinetics (50). It was unclear from these earlier studies whether 
the presence of ICD-TM mutations would impede the repli-
cation of SIVmac in primary mononuclear cells from rhesus 
macaques. To address this question directly, we prepared cell-
free virus stocks of SIVmac-M1 and SIVmac-M4 on CEMx174 
cells and used these to infect primary PBMC obtained prior to 
initiation of the study from each of the eight juvenile macaques 
that were later inoculated with infectious virus. The viral an-
tigen content (p27) of the stocks was determined by ELISA, 
and the TCID50 was measured by serial dilution on CEMx174 
cells. To rule out the possibility that mutations had reverted 
during expansion of the viruses in vitro, we performed reverse 
transcription-PCR, followed by sequence analysis, of the viral 
nucleic acid isolated from each stock (data not shown). Results 
of representative experiments are shown in Fig. 2; each exper-
iment was performed in duplicate. For each infection, 10⁶ 
PBMC were infected with 10⁴ TCID50 of virus and superna-
tants were harvested every 3 to 5 days for SIV p27 antigen 
quantitation. Intriguingly, in primary PBMC from all eight rhesus 
macaques, SIVmac-M1 and SIVmac-M4 replicated less effi-
ciently than wild-type SIVmac in the human T-cell line HUT-78; 
(ii) with a profile identical to wild-type SIVmac in human T/B 
hybrid cell line CEMx174, and (iii) less efficiently than wild-type SIVmac in primary rhesus PBMC.

Infection of juvenile macaques with SIVmac-M4. The goal 
of this study was to determine whether the SIVmac ICD-TM 
region is essential for high-level virus replication and patho-
genesis in rhesus macaques. During the acute phase of infec-
tion, plasma viremia in all four juvenile macaques peaked 2 
weeks after infection at 1.2 × 10⁸ to 1.5 × 10⁹ copies/ml 
(bDNA assay, Fig. 3C) and declined to undetectable levels 
(<1,500 copies/ml) by 8 to 12 weeks. Plasma viremia has 
remained below detectable limits in all four juveniles up to 80 
weeks after infection.

The viral load in PBMC exhibited a similar pattern, with a 
peak in cell-associated viremia at 2 weeks; subsequently, the 
PBMC-associated viral load declined to below detectable lev-
els at 8 to 16 weeks and remained at or below the detection 
threshold thereafter (Fig. 3A). In LNMC, the viral load peaked 
at 2 weeks and dropped steadily during the first 6 months, 
reaching borderline-detectable levels (less than 10 TCID₅₀ per 
10⁶ cells) at 32 weeks (Fig. 3B). At over 80 weeks postinocu-
lation, all four SIVmac-M4 infected juveniles currently remain 
clinically healthy and asymptomatic, with CD4⁺ T-cell counts 
above 500 (Fig. 4B; Table 1).

To determine whether the ICD-TM stop codons and other 
mutations of SIVmac-M4 had reverted to the wild-type coding 
sequence, DNA was isolated from PBMC or from PBMC-
CEMx174 cocultures for all four juvenile macaques at time 
points ranging from 25 to 66 weeks. A portion of the SIV Env 
transmembrane protein (Env-TM) coding region was ampli-
fied by PCR, and the amplified DNA fragment was subcloned 
into a plasmid vector for DNA sequence analysis. Assessment 
of a total of 28 clones obtained from all four juvenile macaques 
revealed no reversion of any of the ICD-TM mutations (Table 2).

Infection of juvenile macaques with SIVmac-M1. Earlier 
studies revealed rapid in vivo reversion of single stop codons in 
the nef gene and in Env-TM of SIVmac (15, 19, 25, 27). Based 
on the results of these studies, we chose to use SIVmac-M1, which contains a single stop codon at position 733 of Env, as 
a control in our experiments. Juvenile rhesus macaques inocu-
lated with SIVmac-M1 developed persistent plasma- and cell-
associated viremia, as shown in Fig. 3. Plasma viremia, as 
measured by the bDNA assay, attained peak values at 2 to 4 
weeks postinoculation (1 × 10⁸ to 4 × 10⁷ copies/ml) and 
remained above 0.9 × 10⁴ copies/ml in all animals throughout 
the first year of infection (Fig. 3C). Virus was consistently 
rescued from PBMC and LNMC of these four animals at all 
time points from 2 to 52 weeks (Fig. 3A and B). One animal, 
Mmu 28853, had persistent viremia of over 10⁹ viral RNA 
copies per ml of plasma; this macaque developed lymphade-
nopathy, splenomegaly, weight loss, and CD4⁺ T-cell decline 
and was euthanized at week 57 of the study (Table 1; Fig. 4).

Two other animals in this group were euthanized due to dete-
riorating health: Mmu 28864 developed severe hemorrhagic enteritis and peritonitis and was euthanized at week 82, and Mmu 28682 developed an abdominal abscess and was euthanized at week 86. The fourth animal, Mmu 28746, succumbed to pneumonia at week 88; this animal had a history of lymphadenopathy and opportunistic infections prior to developing pneumonia.

The high viral loads measured in the four SIVmac-M1-infected animals suggested rapid reversion of the single ICD-TM stop codon in vivo. To evaluate potential reversions, genomic DNA was isolated from PBMC or from PBMC-CEMx174 co-cultures for all four animals at 8 weeks postinoculation. TM sequences were amplified by PCR and cloned into a plasmid vector as described above. As shown in Table 2, all clones obtained from three of four animals (Mmu 28682, Mmu 28746, and Mmu 28853) inoculated with SIVmac-M1 showed reversion of the stop codon to the original CAG (Leu) codon. Sequences from the fourth animal (Mmu 28864) showed mutation of the stop codon to TGG (Trp) in all clones examined (Table 2).

Juvenile macaques infected with SIVmac239-Nef+ and SIVmac239Δ152Nef. We previously described virologic and clinical data for two juvenile macaques intravenously inoculated with 10^3 TCID_{50} of SIVmac239-Nef+ (animals Mmu 26084 and Mmu 27098), and two juveniles (Mmu 26873 and Mmu 26939) inoculated in the same manner with SIVmac239Δ152Nef (26, 49). These data are briefly summarized here (Table 1; Fig. 5) to provide a basis for comparison with animals in the present study. The plasma viral load in animals Mmu 26084 and Mmu 27098 reached a peak at 1.6 × 10^8 to 2.3 × 10^8 copies/ml, 1 to 2 orders of magnitude higher than observed in the SIVmac-M1-inoculated animals during the acute phase, and remained above 3 × 10^6 copies/ml in both animals throughout the first year of infection (Fig. 5) (26). Both animals developed simian AIDS (Table 1). In contrast, plasma viremia in Mmu 26873 and Mmu 26939, which received SIVmacΔ152Nef, reached a peak at 4 × 10^5 to 6 × 10^5 copies/ml at 2 weeks postinoculation and dropped below detection limits by 12 weeks, similar to the pattern observed in SIVmac-M4-infected animals (Fig. 5B) (49). Mmu 26873 remained healthy and asymptomatic for 2 years, at which time it was euthanized. Mmu 26939 exhibited an increase in viral load after 70 weeks, concurrent with viral genetic changes leading to the expression of a novel truncated form of Nef, designated t-Nef (Fig. 5B) (49).

FIG. 3. Virus load in juvenile rhesus macaques infected with SIVmac ICD-TM mutants. (A and B) The cell-associated virus load was measured in PBMC (A) and LNMC (B) by the limiting-dilution assay (34). Briefly, serial 10-fold dilutions of rhesus PBMC or LNMC were cocultured with CEMx174 cells in replicate wells of a 24-well tissue culture plate. Cultures were maintained for 4 weeks. The presence of SIV in individual wells was assessed by a p27 antigen ELISA. The cell-associated virus load was calculated using the method of Reed and Muench (34). The sensitivity of this method is 1 TCID_{50} per 10^6 cells. (C) Viral RNA in plasma samples was determined by the bDNA assay; the sensitivity of the assay used in these experiments was 1,500 copy equivalents/ml of plasma. In all panels, animals inoculated with SIVmac-M1 are indicated by black symbols and animals inoculated with SIVmac-M4 are indicated by white symbols.

FIG. 4. CD4+ T-cell counts in peripheral blood of juvenile macaques. Lymphocyte subset analysis was performed by flow cytometry in a FACScan apparatus (Becton Dickinson, Mountain View, Calif.) after staining fresh whole blood with monoclonal antibodies recognizing CD4+ T cells (OKT4), CD8+ T cells (Leu2A), CD2+ T cells (Leu5b), and CD19+ B cells (Leu16). The figure shows CD4+ T-cell counts in macaques inoculated with SIVmac ICD-TM mutants SIVmac-M1 (A) or SIVmac-M4 (B).
Antibody titers in SIV-infected macaques. Antibodies to SIVmac were assessed in longitudinal samples from experimentally inoculated macaques and controls in an ELISA utilizing HIV-1 and HIV-2 peptides (Fig. 6). All animals exhibited detectable antibody responses by 8 weeks postinoculation. Peak reciprocal titers during the first 52 weeks ranged from $8 \times 10^4$ to $3 \times 10^5$ in the four animals that received SIVmac-M1 (Fig. 6A). Peak reciprocal titers were 2 to 3 orders of magnitude lower in animals that received SIVmac-M4, ranging from $6 \times 10^2$ to $1 \times 10^4$ (Fig. 6A). Of two macaques that received SIVmac239-Nef, one had antibody titers comparable to those of animals that received SIVmac-M1; the titers in the other macaque were lower (Fig. 6B) (26). Antibody titers in juvenile macaques that received SIVmac-M4 were similar to those observed in animals that received SIVmac239-D152Nef (Fig. 6B) (49).

Infection of neonatal macaques with SIVmac-M4. Recent reports suggested that multiply deleted viruses derived from SIVmac239, which may have an attenuated phenotype in adults, are pathogenic when administered orally or intravenously to newborn macaques (3, 4, 35, 49). It was therefore critical to assess the pathogenicity of SIVmac-M4 in rhesus neonates. Four neonatal macaques were inoculated intravenously with a cell-free preparation containing infectious SIVmac-M4,

| Age          | Virus | Macaque | Inoculation dose (TCID$_{50}$) | Clinical status                                                                 |
|--------------|-------|---------|--------------------------------|--------------------------------------------------------------------------------|
| Juvenile     | M1    | Mmu 28682 | $1 \times 10^4$                | Abdominal abscess; euthanized at 86 wk                                        |
| Juvenile     | M1    | Mmu 28746 | $1 \times 10^3$                | Lymphadenopathy, opportunistic infections; died of pneumonia at 88 wk         |
| Juvenile     | M1    | Mmu 28853 | $1 \times 10^4$                | Lymphadenopathy, splenomegaly, weight loss, CD4$^+$ decline; euthanized at 57 wk |
| Juvenile     | M1    | Mmu 28864 | $1 \times 10^3$                | Severe hemorrhagic enteritis, peritonitis; euthanized at 82 wk                |
| Juvenile     | M4    | Mmu 28300 | $1 \times 10^4$                | Healthy, asymptomatic through 80 wk                                          |
| Juvenile     | M4    | Mmu 28323 | $1 \times 10^3$                | Healthy, asymptomatic through 80 wk                                          |
| Juvenile     | M4    | Mmu 28388 | $1 \times 10^3$                | Healthy, asymptomatic through 80 wk                                          |
| Juvenile     | M4    | Mmu 28543 | $1 \times 10^4$                | Healthy, asymptomatic through 80 wk                                          |
| Juvenile     | 239-Nef+ | Mmu 26084$^a$ | $1 \times 10^3$                | Simian AIDS; euthanized at 51 wk                                              |
| Juvenile     | 239-Nef+ | Mmu 27098$^a$ | $1 \times 10^3$                | Simian AIDS, euthanized at 87 wk                                              |
| Juvenile     | 239-D152Nef | Mmu 26873$^a$ | $1 \times 10^3$                | Healthy through 105 wk                                                        |
| Juvenile     | 239-D152Nef | Mmu 26939$^a$ | $1 \times 10^3$                | CD4$^+$ T cells declined at 70 wk; euthanized at 105 wk                      |
| Neonate      | M4    | Mmu 31342 | $2 \times 10^3$                | Unremarkable (acute phase), healthy (chronic phase)$^b$                       |
| Neonate      | M4    | Mmu 31345 | $2 \times 10^3$                | Treated for mild gastrointestinal disorders (acute phase), healthy (chronic phase) |
| Neonate      | M4    | Mmu 31346 | $1 \times 10^4$                | Treated for gastrointestinal disorders; shigella (acute phase), healthy (chronic phase) |
| Neonate      | M4    | Mmu 31348 | $1 \times 10^4$                | Unremarkable (acute phase), healthy (chronic phase)                          |

$^a$ These animals were described in references 26 and 49.

$^b$ The acute phase consisted of weeks 0 to 12; the chronic phase consisted of weeks 12 to 52.

| Age          | Virus | Macaque | Time (wk) postinoculation | No. of clones showing reversion or no reversion |
|--------------|-------|---------|---------------------------|-----------------------------------------------|
| Juvenile     | M1    | Mmu 28682 | 8                         | 7 of 7 TAG $\rightarrow$ CAG                  |
| Juvenile     | M1    | Mmu 28746 | 8                         | 8 of 8 TAG $\rightarrow$ CAG                  |
| Juvenile     | M1    | Mmu 28853 | 8                         | 7 of 7 TAG $\rightarrow$ CAG                  |
| Juvenile     | M1    | Mmu 28864 | 8                         | 12 of 12 TAG $\rightarrow$ TGG                |
| Juvenile     | M4    | Mmu 28300 | 32                        | 6 of 6 no reversion of TM mutations           |
| Juvenile     | M4    | Mmu 28323 | 66                        | 4 of 4 no reversion of TM mutations           |
| Juvenile     | M4    | Mmu 28543 | 25                        | 6 of 6 no reversion of TM mutations           |
| Juvenile     | M4    | Mmu 28388 | 66                        | 4 of 4 no reversion of TM mutations           |
| Neonate      | M4    | Mmu 31342 | 24                        | 5 of 5 no reversion of TM mutations           |
| Neonate      | M4    | Mmu 31345 | 24                        | 10 of 10 no reversion of TM mutations         |
| Neonate      | M4    | Mmu 31346 | 24                        | 5 of 5 no reversion of TM mutations           |
| Neonate      | M4    | Mmu 31348 | 24                        | 6 of 6 no reversion of TM mutations           |

$^a$ Genomic DNA was isolated from either $10^7$ PBMC or $10^7$ CEMx174 cells cocultured with PBMC from infected macaques (Qiagen tissue kit). TM sequences were amplified in a nested PCR as describe in Materials and Methods. PCR products were sequenced on an ABI automated sequencer analyzer. Both strands were sequenced.
either $2 \times 10^3$ TCID$_{50}$ (Mmu 31342 and Mmu 31345) or $1 \times 10^4$ TCID$_{50}$ (Mmu 31346 and Mmu 31348) (Fig. 7). All four animals survived the acute phase of infection. Two of the four neonates (Mmu 31345 and Mmu 31346) were treated with antibiotics for gastrointestinal symptoms during the first 2 weeks; however, these animals responded to therapy. Plasma viral loads, as measured by bDNA, peaked at $1 \times 10^6$ to $5 \times 10^7$ copies/ml at 2 weeks and declined steadily thereafter, falling below the detection threshold in three of four animals (Fig. 7A). The PBMC-associated viral load also declined steadily during the first 16 weeks of infection (Fig. 7B). All four neonates had detectable antibodies to SIVmac (Fig. 7C). Currently, at 52 weeks postinoculation, all four neonates remain clinically healthy, with CD4$^+$-T-cell counts of >1,500/μl (Fig. 7D), and will continue to be closely monitored.

To assess the stability of the ICD-TM mutations in newborn macaques, genomic DNA was isolated from PBMC or from cocultures of PBMC and CEMx174 cells for all four animals at 24 weeks and from one animal (Mmu 31345) at 32 weeks. PCR amplification followed by DNA sequence analysis of the TM region from a total of 31 clones from all four neonates revealed no reversion of any of the M4 mutations (Table 2).

**DISCUSSION**

ICD-TM and viral attenuation. The major finding of this study, based on in vivo analysis of SIV clones with specific mutations, is that the ICD-TM domain of SIVmac is necessary for the development of persistent viremia and pathogenesis in rhesus macaques. The precise virologic functions of lentivirus ICD remain unresolved; however, in vitro studies have pointed to a role for these domains in viral replication kinetics, infectivity, host cell tropism, virion assembly, cytopathicity, intracellular trafficking, endocytosis, and signal transduction (5, 7, 10, 14, 17, 18, 24, 28, 44, 51, 61). The length of ICD-TM has been shown to influence envelope incorporation into virions, probably through interaction with the viral matrix protein (10, 17).

The density of the Env glycoprotein is believed to be greater on viruses with a short ICD-TM (e.g., SIVmac239-M1 and SIVmac239-M4) than on those with a full-length TM (e.g., SIVmac239Nef) (24). Envelope proteins with a short ICD-TM are also more fusogenic than those with a full-length TM (43, 51, 61). Although higher Env density and/or greater fusogenicity might be required for efficient viral entry in certain cell types (e.g., HUT-78), lower Env density and fusogenic capacity might permit more favorable virus-host cell interactions in other cases (e.g., rhesus PBMC) (24, 50). Another hypothesis can be drawn from studies that implicate conserved regions near the C terminus of ICD-TM in induction of cytopathic effects (lentivirus lytic peptides), calmodulin binding, and enhanced apoptosis (37, 38, 40, 58). According to this view, a full-length ICD-TM would be essential for gp160-enhanced apoptosis, mediated via calmodulin binding (37, 40). Calmodulin binding by ICD-TM might also be relevant to T-cell signaling events that play a role in viral replication (54). Further studies are required to identify the ICD-TM functions that are relevant to pathogenesis and to understand the virologic basis for attenuation of ICD-TM mutants.

In light of a recent report suggesting that genetic changes in the ICD-TM may compensate in vivo for deletion of nef sequences, it is intriguing to speculate on potential functional implications of these findings.
similarities between these proteins (L. Alexander, P. Ilyinski, X. Alvarez, R. Veazey, A. A. Lackner, and R. C. Desrosiers, Abstr. 7th Conf. Retroviruses Opportunistic Infect. abstr. 150, 2000). The SIVmac nef gene is dispensable for virus replication in cultured T-cell lines but enhances replication in unstimulated PBMC (1, 39, 53). Like ICD-TM, Nef interacts with cellular adaptor proteins, is membrane associated, and has been implicated in modulation of signal transduction in T cells (reviewed in references 16, 46, and 48). Because of these similarities, it will be important to monitor viral sequences isolated from macaques infected with SIVmac-M4 for potential compensatory changes in the nef coding region.

A hierarchy for live-attenuated SIVmac239 mutants. A hierarchy for live-attenuated SIVmac239 mutants has been proposed, based on parameters such as disease induction, cell-associated virus load, plasma viremia, and level of antiviral antibody responses in rhesus macaques (13). The proposed ranking, from least attenuated to most highly attenuated, was as follows: SIVmac239ΔVpxΔVprΔNef > Δ3 > Δ2 > Δ1 > ΔΔ1 > ΔΔΔ1 > ΔΔΔΔ1 (13). It is difficult to precisely situate SIVmac-M4 in this hierarchy because the methods for evaluating virus load and antibody titers differ among laboratories and because the Nef-deleted virus (SIVmac239Δ152Nef) constructed in our laboratory is not identical to the SIVmac239ΔNef mutant described elsewhere (25, 49). However, SIVmac-M4, which contains multiple mutations in ICD-TM, is clearly more strongly attenuated than SIVmac-M1, which contains a single stop codon in ICD-TM. Animals infected with SIVmac-M4 developed transient viremia in the range of \(1 \times 10^5\) to \(1.5 \times 10^6\) viral RNA equivalents/ml of plasma (mean, \(7.5 \times 10^5\)), 2 to 3 orders of magnitude below that detected in animals infected with SIVmac239-Nef+ but comparable to that observed in one juvenile macaque, Mmu 26873, infected with SIVmac239Δ152Nef (26, 49). In our laboratory, four juvenile macaques that received SIVmac-M4 remain healthy and asymptomatic with low viral loads at 80 weeks postinoculation whereas one of two macaques inoculated with SIVmac239Δ152Nef developed a high viral load and progressed to simian AIDS (49).

In a study of three highly attenuated mutants (SIVmacΔ3, SIVmacΔ3x and SIVmacΔ4) in juvenile macaques, peak plasma viral loads for SIVmacΔ3 were comparable to those we detected in animals infected with SIVmac-M4 (13). Peak levels of PBMC-associated virus for SIVmacΔ3 showed a 13.5-fold reduction relative to those of wild-type SIVmac239-Nef+ compared to a 16.8-fold reduction for SIVmac-M4 relative to SIVmac239-Nef+ in the present study (Fig. 3) (13). Taken together, these results suggest that the attenuation level of SIVmac-M4 is similar to or slightly greater than that of SIVmac239Δ152Nef or SIVmacΔ3.

Correlation of acute-phase virus load with clinical outcome. Several recent reports suggested that events occurring during the first 6 weeks of infection determine the eventual disease course (20, 30, 36, 56, 59). In the present study, sequence analysis of viral nucleic acid obtained from SIVmac-M1-inoculated animals at week 8 postinoculation indicated that the single ICD-TM stop codon had mutated to a coding triplet in all clones obtained from all four macaques. In contrast, viral
Genomes obtained from SIVmac-M4-inoculated animals as late as 66 weeks showed no reversion of any of the ICD-TM mutations (Table 2). This result strongly suggests that early restoration of the wild-type ICD-TM in the SIVmac-M1 animals correlated with a high viral load during the acute phase. High acute-phase viremia, in turn, was predictive of persistent viremia and progression to disease, as previously reported (20, 30, 36, 56, 59).

Attenuation threshold. The “threshold hypothesis” was proposed to account for the in vivo attenuation of SIVmac clones containing mutations in accessory genes (45). According to this view, mutations in individual viral genes contribute to attenuation not by abrogating essential virologic functions but by modulating virus replication efficiency in vivo. The reduced replication observed for SIVmac-M1 and SIVmac-M4 in primary macaque PBMC (Fig. 2) supports the notion that a full-length ICD-TM is required for optimal replication in primary macaque mononuclear cells. Therefore, decreased replication efficiency alone may be sufficient to account for the observed in vivo attenuation of SIVmac-M4. Additional studies are required to establish whether the ICD-TM domain also performs an essential virologic function (i.e., cellular activation, modulation of protein trafficking, or facilitation of viral assembly) in primary cells.

Implications for vaccine development. Live-attenuated viruses derived from SIVmac molecular clones have shown promising levels of protection in macaques (2, 9, 11, 60). However, the ability of an attenuated viral vaccine to induce protective immunity is dependent on the ability of the virus to replicate in the host (12, 22, 31) and the length of time between vaccination and pathogenic challenge (8, 9). The most highly attenuated viral strains, which replicate poorly in vivo, are unlikely to induce protection; additionally, an inverse relationship was demonstrated between the level of attenuation and the degree of protection from challenge with virulent viruses (13, 22, 31). Enthusiasm for this approach has also been somewhat moderated by the observation that live, attenuated mutants can induce disease in newborn (3, 4) and juvenile (4, 49) macaques. Additional investigations are required to determine to what extent the macaques that received SIVmac-M4 can be protected from challenge with pathogenic viruses. However, used in combination with mutations in other viral genes, site-directed mutations in the ICD-TM may serve to expand the repertoire of attenuated SIVmac molecular clones that will be useful in vaccine development.

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