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Persistent Infection of Rhesus Macaques by the Rev-Independent Nef(−) Simian Immunodeficiency Virus SIVmac239: Replication Kinetics and Genomic Stability

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We generated previously a Nef(−), replication-competent clone of SIVmac239 in which the Rev protein and the Rev-responsive element were replaced by the constitutive transport element (CTE) of simian retrovirus type 1 (A. S. von Gegerfelt and B. K. Felber, Virology 232:291–299, 1997). In the present report, we show that this virus was able to infect and replicate in rhesus macaques. The Rev-independent Nef(−) simian immunodeficiency virus induced a persistent humoral immune response in all monkeys, although viral loads were very low. Upon propagation in the monkeys, the genotype remained stable and the virus retained its in vitro growth characteristics. The infected monkeys showed normal hematological values and no signs of disease at more than 18 months post-virus exposure. Therefore, replacement of the essential Rev regulation by the CTE generated a virus variant that retained its replicative capacity both in vitro and in vivo, albeit at low levels.

All lentiviruses depend on the regulatory protein Rev for expression. Rev functions by binding to the Rev-responsive element (RRE), which is present on a subset of viral mRNAs encoding structural proteins. This regulatory mechanism is most conserved among lentiviruses and may play an important role in viral pathogenesis. Binding of Rev to RRE leads to stabilization, nucleocytoplasmic export, and efficient expression of Gag/Pol- and Env-encoding mRNAs. Rev is essential, since in its absence no virus is produced. We and others have demonstrated that Rev regulation of human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV) can be replaced by the constitutive transport element (CTE) of simian type D retroviruses (SRV-D) (3, 20, 24, 26, 30). For these studies, we have generated Rev-independent clones of HIV and SIV by introducing multiple point mutations into both *rev* and RRE that do not affect the overlapping *tat* and *env* reading frames and have demonstrated that these viruses can be propagated in primary lymphocytes (26, 30). Rev exits the nucleus through its interaction with CRM1, a protein responsible for export of many cellular proteins (for a recent review, see reference 10). We recently showed that the CTE mediates its function via the cellular TAP protein (9), which is present ubiquitously in mammalian cells and *Xenopus laevis* oocytes (16, 21). Therefore, although both Rev/RRE and TAP/CTE promote export and expression of *gag*/*pol* and *env* mRNAs, they utilize distinct nuclear export pathways (16, 21, 29).

The Rev-independent clones of HIV and SIV are the first replication-competent mutant viruses to target the *rev* gene. Rev regulation is essential for virus expression, but it can be replaced by an alternative transport mechanism such as the CTE (3). Replacement of the Rev/RRE system by the CTE has generated HIV and SIV variants that show reduced replication in primary cultured cells and lower infectivity than wild-type virus (25, 26, 30). Upon long-term propagation in vitro, these viruses maintain stable genotypes and have also stable in vitro growth properties.

Using SCID-hu mice as a model system, we tested the replication and pathogenic potential of Rev-independent HIV-1 clones. We found that infection by these viruses resulted in reduced viral load and did not cause depletion of CD4-bearing lymphocytes within human lymphoid tissues implanted in mice (25). Importantly, this observation was made after infection with either the Nef(+) or the Nef(−) variant of the Rev-independent clones. These data suggested that the Rev-independent HIV-1 clones have reduced replication capacity and cytotoxicity, independently of the presence of Nef. In addition, this study showed that in the SCID-hu mouse, Rev-independent viruses had lower virus loads than HIV-1 mutant viruses lacking either *nef* alone or any of the other accessory genes. This finding suggested that replacement of the Rev/RRE regulatory axis is mostly responsible for the observed phenotype.

To test the effect of Rev replacement in a primate model, a Rev(−)RRE(−)Nef(−)CTE(+) SIVmac239 clone was generated (26). As previously described (26), this clone contains the CTE inserted into the *Nco*I site at nucleotide (nt) 9181, downstream of the *env* terminator. The N-terminal Nef peptide produced by this virus variant spans 70 amino acids, whereas that of SIVΔNef (11) spans 58 amino acids. Like its HIV-1 counterpart, the Rev-independent SIV showed reduced replicative capacity in primary monkey lymphocytes in vitro and had lower infectivity (26). As a proof-of-concept study, we examined whether the Rev-independent SIV can replicate in rhesus macaques and whether its genome is stable. Here, we report that all three rhesus macaques inoculated with this virus became persistently infected and we provide a follow-up study of these animals over a period of 18 months. Our study shows that replacement of the Rev/RRE regulatory axis did not eliminate viability and persistence of the virus variant in vivo, although virus loads were persistently low. We show further that

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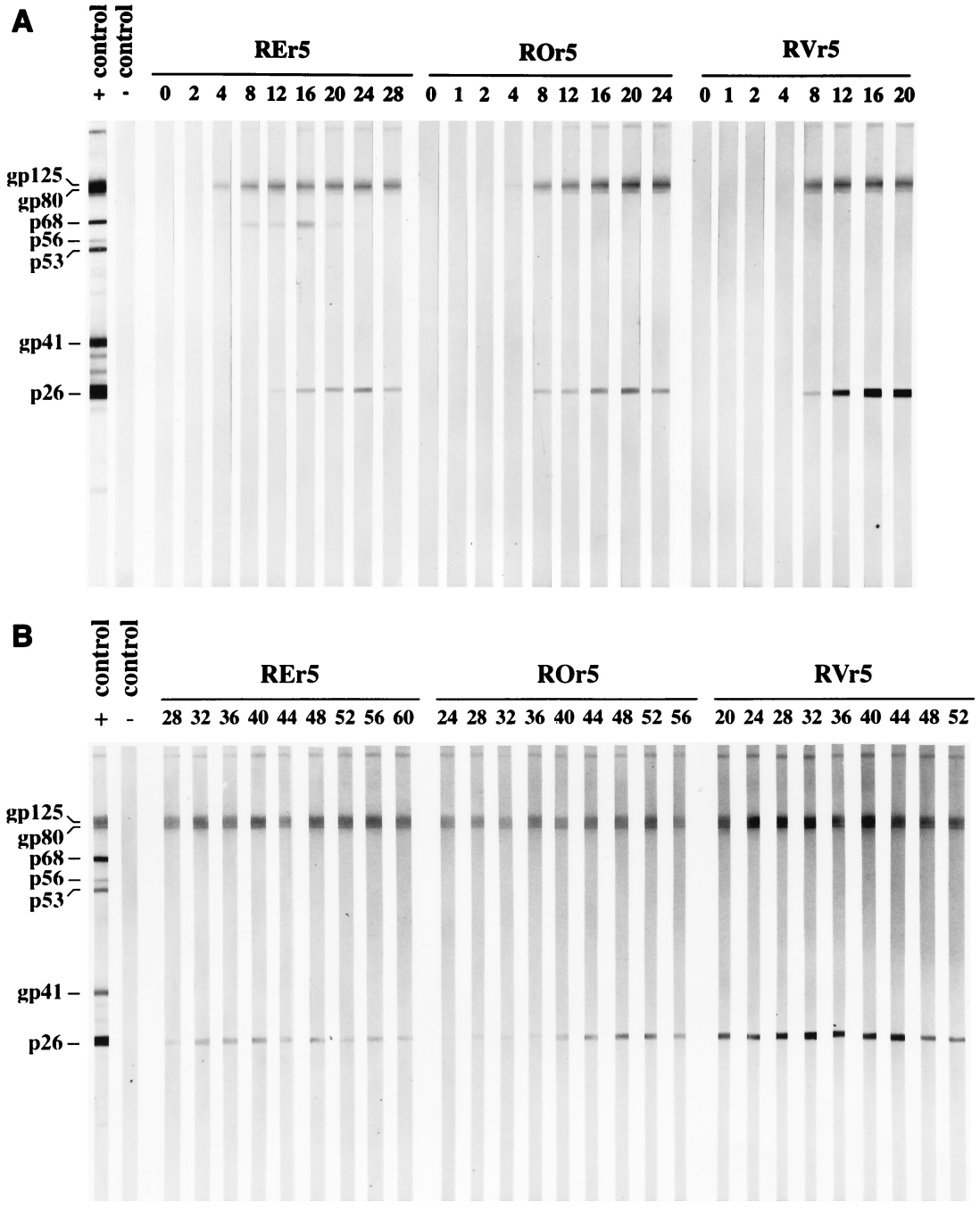

FIG. 1. Western immunoblot analysis of rhesus macaques infected by Rev-independent Nef(−) SIVmac239. Plasma samples from the three monkeys obtained at the indicated time points (in weeks) were subjected to Western blot analysis with a commercial assay. Panels A and 1B show samples collected over different time spans. The numbers above the individual lanes indicate weeks postinoculation. Note that different HIV-2 kits (Cambridge Biotech) were used in the two panels and that the last time point of panel 1A is repeated in panel B.

the genome of the Rev-independent SIV is stable upon propagation in rhesus macaques. Interestingly, we found that replacement of Rev/RRE results in lower virulence, which suggests a novel approach for lowering of virulence of a pathogenic lentivirus.

We generated a virus stock of the Rev-independent Nef(−) SIV in rhesus macaque peripheral blood mononuclear cells (PBMC) collected from healthy, pathogen-free animals. The stock was titrated in CEMx174 cells, as described previously (26). Three macaques were inoculated intravenously with 10,000 (REr5), 1,000 (ROr5), and 100 (RVr5) 50% tissue culture infective doses. Three different virus doses were administered to characterize our virus stocks in vivo. It has been previously shown that disease development by pathogenic SIV is independent of the infecting dose (6); therefore, no effect of the different doses was anticipated in the infected animals. The monkeys were prescreened for the presence of SRV-D and simian T-lymphotropic virus type 1 sequences by PCR (12, 13)

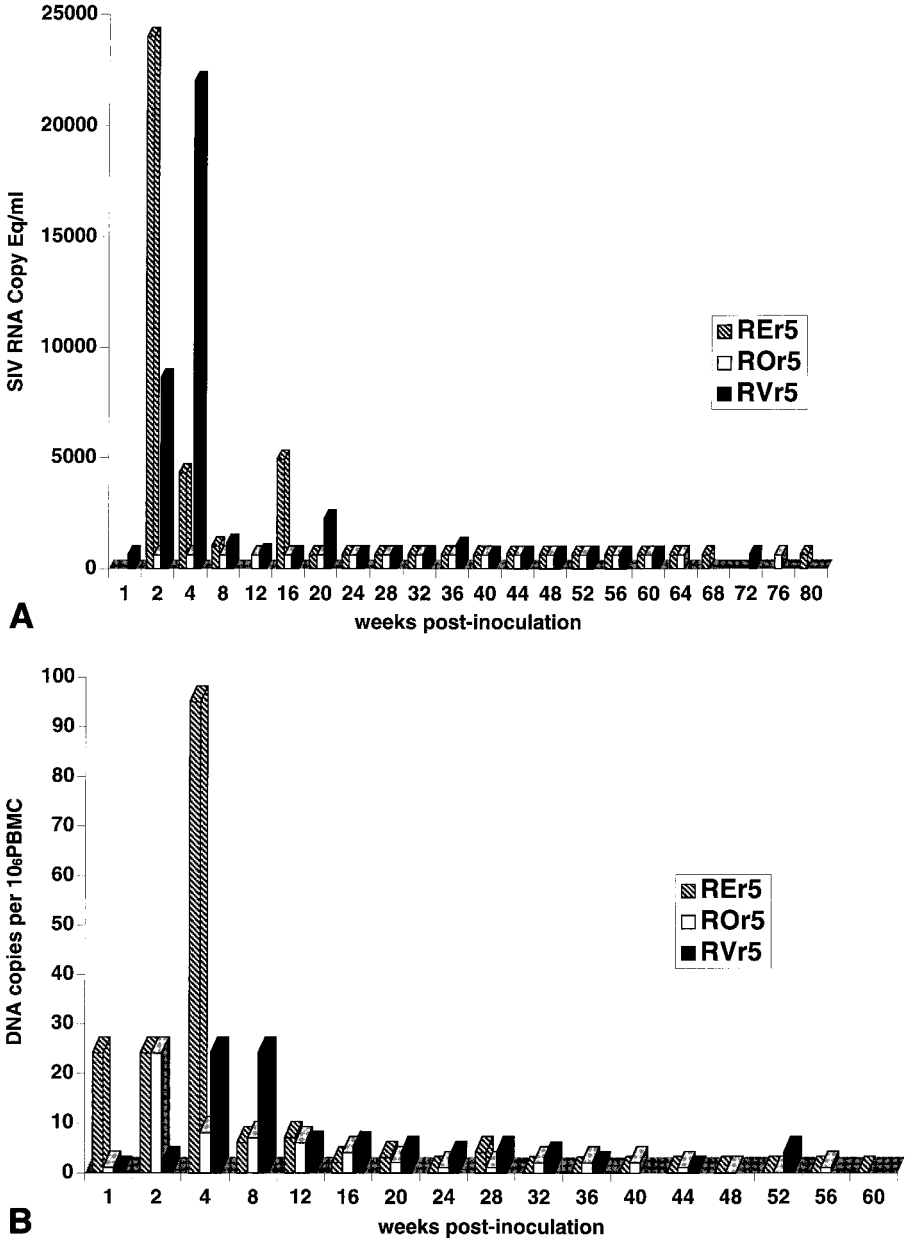

FIG. 2. Low levels of viral RNA and DNA. Rhesus macaques REr5, ROr5, and RVr5 were inoculated with the Rev-independent Nef(−) SIV. (A) Plasma RNA levels were determined at the indicated time points by using real-time RT-PCR (23) and are shown as RNA copy equivalents per ml. After the initial peak, the values are below the threshold of detection of the assay (<600 RNA copy equivalents per ml). (B) DNAs isolated at the indicated time points from PBMC were subjected to PCR analysis with a nested primer spanning a region in *gag* from nt 1406 to 1928 (+1 represents the first nucleotide of U3 [18]).

and found to be negative. The ages of the monkeys ranged from 9 to 11 months at the time of inoculation. Following inoculation, blood samples were collected at 1, 2, and 4 weeks and monthly thereafter. The samples were analyzed for the presence of anti-SIV antibodies, for levels of plasma viral RNA and cell-associated viral DNA, and for the ability to isolate virus. Complete blood cell counts and T-cell subsets were monitored at regular intervals.

Plasma samples were subjected to Western immunoblot analysis to detect anti-SIV humoral responses. As shown in Fig. 1, all three monkeys reacted with Env and Gag antigens starting at 4 to 8 weeks post-virus exposure (Fig. 1A) and remained positive for about 1 year of follow-up (Fig. 1B). Therefore, all three monkeys generated a persistent antibody response against SIV, indicating persistent infection with the Rev-independent Nef(−) SIV. Notably, none of the three monkeys showed, over the course of the study, a significant selective loss of anti-Gag antibody response, which is an indicator of disease progression in HIV-1-infected persons (2, 8) as well as in SIV-infected macaques (17, 28).

Despite chronic infection, all monkeys had low levels of viremia. First, plasma samples were analyzed for viral RNA levels by using a real-time quantitative reverse transcriptase (RT) PCR assay (23). Figure 2A shows that two of the three monkeys (REr5 and RVr5) had positive values at several time points postinoculation, whereas the RNA levels for ROr5 were

TABLE 1. Comparison of plasma RNA levels in juvenile macaques infected by different SIV mutant viruses

Virus	Genotype					RNA copy equivalents at peak[a]
	rev	*vpr*	*vpx*	*nef*	U3	
Wild type	+	+	+	+	+	$1–5 \times 10^7$
Δ3	+	−	+	−	−	$10^4–2 \times 10^6$
Δ3X	+	+	−	−	−	$10^4–2 \times 10^6$
Δ4	+	−	−	−	−	$<600–1 \times 10^6$
Rev/RRE(−)CTE(+)	−	+	+	−	+	$<600–2 \times 10^4$

[a] Data for all viruses except Rev/RRE(−)CTE(+) are from reference 7.

always below the threshold of the assay. The values for REr5 and RVr5 reached a peak of about 2×10^4 RNA copy equivalents per ml of plasma within 2 to 4 weeks post-virus exposure. These RNA levels were 3 to 4 logs lower than those obtained after infection with wild-type SIVmac239 (7) (Table 1). Interestingly, we observed a rapid decline in plasma RNA to levels below the threshold of the assay. The same method was used to determine plasma RNA levels of mutant SIV strains that lack, in addition to *nef* and a portion of U3, other viral genes, such as *vpr* and *vpx*, as in SIVmac239 Δ3 (*nef*, *vpr*, U3), Δ3X (*nef*, *vpx*, U3), and Δ4 (*nef*, *vpr*, *vpx*, U3) (7). Comparison of mean plasma RNA equivalents at peak showed that the values for Rev-independent Nef(−) SIV and SIV Δ4 are similar (Table 1). These data indicate that replacement of Rev/RRE, in the presence of all other viral genes, is sufficient to decrease virus loads of a Nef(−) SIV variant significantly. Note also that we found reduced levels of virus replication both in rhesus PBMC in vitro (26) and in infected animals in vivo, indicating that the in vitro growth properties were a predictor for reduced propagation of this virus variant in the monkeys.

We also determined cell-associated viral DNA levels, by using a quantitative PCR protocol amplifying a portion of *gag*, as described elsewhere (13a). All three monkeys were SIV negative at the day of inoculation but became SIV DNA positive within 1 to 2 weeks postinoculation, showing a peak of 24 to 95 copies per 10^6 PBMC at 2 to 8 weeks postinoculation (Fig. 2B). Overall, the DNA copy levels were low and declined over time to either few copies or undetectable levels in the circulating blood cells. We also measured viral DNA levels in lymph node biopsies of RVr5 (at 12 weeks), ROr5 (at 16 weeks), and REr5 (at 20 weeks). All three monkeys showed about a 10-fold-higher level of proviral DNA in lymph nodes than in matching PBMC, as expected. Despite this, the viral DNA levels were low (24 [REr5] and 95 [ROr5 and RVr5] copies per 10^6 cells) compared to the levels found in infection by wild-type virus (5, 19, 22, 27).

Virus isolations were performed with 10^6 PBMC and fourfold serial dilutions thereof in a standard cocultivation assay with CEMx174 cells (1). We were able to isolate virus from the PBMC of ROr5 but only at 1, 2, and 4 weeks postinoculation (Table 2). Note that although virus could be isolated, we were unable to detect viral RNA in the plasma of this monkey at all time points analyzed (Fig. 2A). From the other two monkeys, we were unable to isolate virus from the PBMC throughout the follow-up period of the study (more than 1 year). The frequencies of virus isolation were similar to those obtained from SIV mutants Δ3X and Δ4 (7). Virus isolations were also performed from lymph node biopsies at two time points, as indicated in Table 1. In addition to ROr5 (at week 60), we were also able to isolate infectious virus from RVr5 (at week 12). Since at least 10^6 PBMC were necessary to isolate virus, these findings showed that the virus levels were also low in the lymph nodes. In contrast, we were never successful in isolating virus from REr5, although plasma viral RNA and cell-associated proviral DNA were readily detectable in this animal. Since variation exists among outbred monkeys, it is expected that different virus levels can be obtained after infection by the same virus stock. In summary, all monkeys infected by the Rev-independent Nef(−) SIV showed low levels of persistent viremia.

This observation was further corroborated by determinations of changes that occurred in the region spanning V1 and V2 (amino acids 90 to 200 of Env) (4, 15) of the Rev-independent Nef(−) virus. Genomic DNA isolated from the lymph node of ROr5 at 60 weeks postinoculation was PCR amplified, cloned, and sequenced. In the 20 clones analyzed, we did not detect any amino acid changes within V1 or V2, although a few changes occurred in the surrounding region. We cannot exclude the possibility that some of the changes were present in the input virus stock and/or occurred upon propagation in CEMx174 cells. Clearly, preservation of V1 and V2 in the Rev-independent Nef(−) SIV is in contrast with the changes reported for an actively replicating SIVmac239 (4). Taken together, these data are consistent with the findings above and support the observation that the Rev-independent Nef(−) virus shows reduced replication in vivo.

Hematological values were analyzed at regular intervals. Hemoglobin levels and platelet counts remained normal for all three monkeys (data not shown). Figure 3 shows absolute $CD4^+$ T cells (A), $CD4^+/CD8^+$ T-cell ratios (B), and $CD4^+$ $CD29^+$ T cells (C). The latter T-cell subset measures memory T cells, which are affected early on during disease progression. Persistent drops to levels of <10% indicate the early stages of immune dysfunction and are associated with a poor prognosis (14). As expected, the hematological parameters we evaluated prospectively fluctuated in individual animals. We noted that REr5 had a decline in $CD4^+$ T cells to 482 at week 76, which was followed by a subsequent increase to normal levels at week 80. However, since no correlation with other hematological parameters (see also Fig. 3B and C) or changes in levels of viremia (Fig. 2A) were noted, the significance of this change in $CD4^+$ T cells is not known. The $CD4^+/CD8^+$ T-cell ratios remained normal in one animal (REr5 [Fig. 3B]), whereas they were slightly low in the other two animals. Note that these values fluctuated minimally during the experiment and are

TABLE 2. Virus isolation upon coculture of rhesus PBMC with the CEMx174 cell line

Wk	Monkey[a]					
	REr5		ROr5		RVr5	
	PBMC	LN	PBMC	LN	PBMC	LN
1	−		+		−	
2	−		+		−	
4	−		+		−	
8	−		−		−	
12	−		−		−	+
16	−		−	−	−	
20	−	−	−		−	
24–54	−		−		−	
56	−		−		−	−
60	−		−	+	−	
64	−	−	−		−	
68	−		−		−	
72	−		−		ND	
76	−		ND		ND	

[a] LN, lymph nodes; ND, not done.

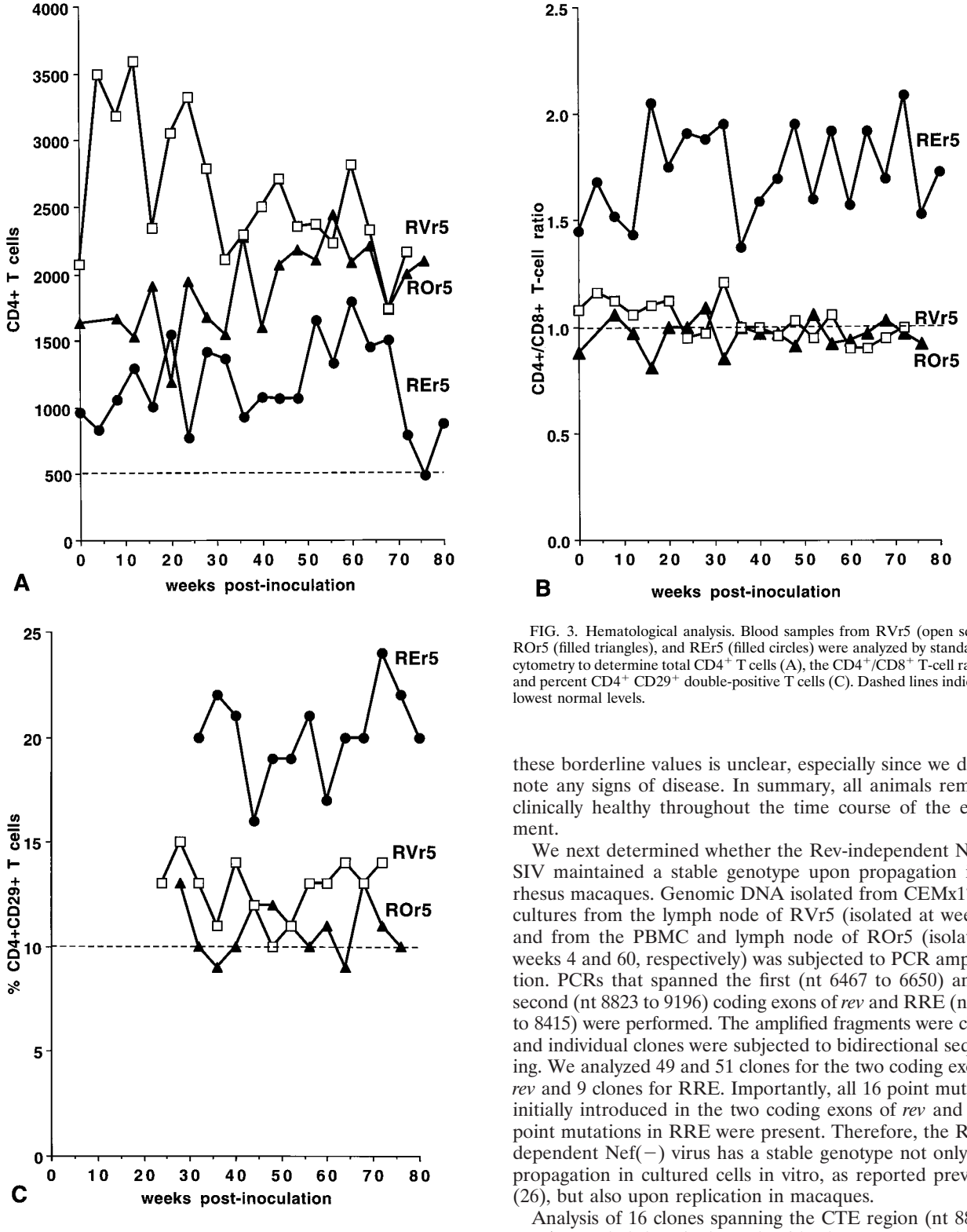

FIG. 3. Hematological analysis. Blood samples from RVr5 (open squares), ROr5 (filled triangles), and REr5 (filled circles) were analyzed by standard flow cytometry to determine total $CD4^+$ T cells (A), the $CD4^+/CD8^+$ T-cell ratio (B), and percent $CD4^+$ $CD29^+$ double-positive T cells (C). Dashed lines indicate the lowest normal levels.

close to the values obtained preinoculation. In two of the three infected macaques, the $CD4^+$ $CD29^+$ T-cell subsets stayed within normal limits (>10%). In the third animal, ROr5, borderline or slightly low levels were observed occasionally. Given the undetectable levels of viral RNA and the difficulty of isolating virus from these animals, the clinical significance of these borderline values is unclear, especially since we did not note any signs of disease. In summary, all animals remained clinically healthy throughout the time course of the experiment.

We next determined whether the Rev-independent Nef(−) SIV maintained a stable genotype upon propagation in the rhesus macaques. Genomic DNA isolated from CEMx174 cocultures from the lymph node of RVr5 (isolated at week 12) and from the PBMC and lymph node of ROr5 (isolated at weeks 4 and 60, respectively) was subjected to PCR amplification. PCRs that spanned the first (nt 6467 to 6650) and the second (nt 8823 to 9196) coding exons of *rev* and RRE (nt 8072 to 8415) were performed. The amplified fragments were cloned, and individual clones were subjected to bidirectional sequencing. We analyzed 49 and 51 clones for the two coding exons of *rev* and 9 clones for RRE. Importantly, all 16 point mutations initially introduced in the two coding exons of *rev* and all 14 point mutations in RRE were present. Therefore, the Rev-independent Nef(−) virus has a stable genotype not only upon propagation in cultured cells in vitro, as reported previously (26), but also upon replication in macaques.

Analysis of 16 clones spanning the CTE region (nt 8823 to 9400) revealed that single nucleotide changes occurred within 40% of the CTE elements analyzed. These changes are predicted to be neutral, since they do not affect the binding site of TAP or the secondary structure of the element, which are both essential for function (9, 24). In our detailed study on the CTE sequence after long-term culture in vitro (24), we reported that the primary sequence of the internal loop regions and the

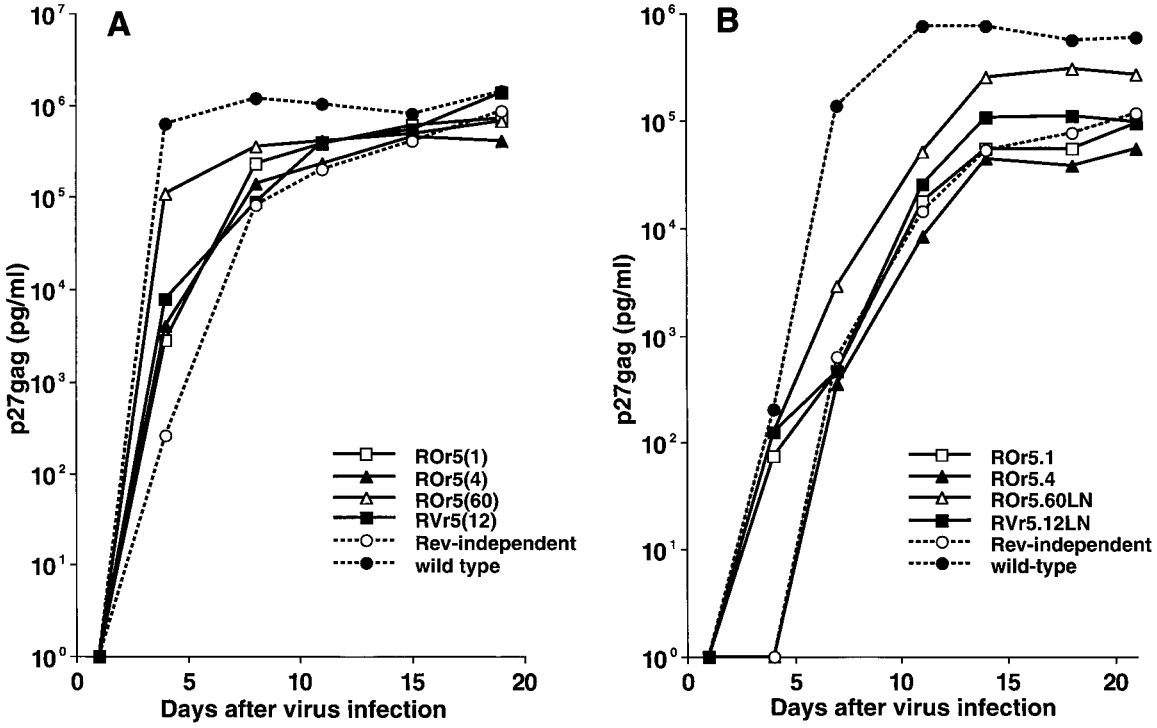

FIG. 4. Rev-independent Nef(−) SIV maintains its in vitro growth characteristics. Virus was isolated from ROr5 PBMC at 1 week [open square; ROr5(1)] and 4 weeks postinoculation [filled triangle; ROr5(4)] and from the lymph node at 60 weeks postinoculation [open triangle; ROr5(60)]. Virus isolated from the lymph node of RVr5 at 12 weeks is also indicated [filled square; RVr5(12)]. These virus isolates were passaged once through rhesus PBMC and then used for cell-free infection of CEMx174 cells (A) and rhesus PBMC (B). As controls, parallel cultures were infected with rhesus PBMC-derived viruses which are wild-type SIVmac239 (filled circles) and Rev-independent Nef(−) SIV (open circles). Virus production was monitored with the Cellular Products antigen-capture assay.

secondary structure of the element are maintained, since they are essential for CTE function. Therefore, the findings in vivo and in vitro are in agreement.

Although the introduced point mutations in *rev*, RRE, and the CTE are maintained stably within the genome of the Rev-independent SIV, sequence analysis revealed nucleotide changes in some of the cloned fragments. Some of these changes affect the coding potential of the overlapping *tat* and/or *env* open reading frames, although the significance of these changes is not known. In addition, sequence analysis of 31 clones containing the coding exons of *rev* of the input virus not only confirmed the presence of the introduced point mutations but also showed some changes affecting either the *tat* or *env* coding potential. Since the input virus is a mixture of several genotypes, we cannot exclude the possibility that some of the observed changes originated in the virus stock and/or were the result of virus propagation in the monkey or in the CEMx174 cell line. Clearly, nucleotide changes occur within the analyzed regions upon virus replication; importantly, none of the changes affected the introduced point mutations.

We also studied the growth properties of the Rev-independent Nef(−) virus isolated after propagation in monkeys. We previously reported that the Rev-independent Nef(−) SIV has a reduced replicative capacity in some cell types, such as rhesus macaque PBMC in vitro, but not in the CEMx174 cell line (26). Therefore, CEMx174 cells and rhesus PBMC were infected with viruses isolated from ROr5 and RVr5. As shown in Fig. 4A, there are no significant differences in the growth properties of these viruses in the CEMx174 cell line. In contrast, propagation in rhesus PBMC (Fig. 4B) shows that all of the Rev-independent Nef(−) SIV isolates grow at lower levels than wild-type SIVmac239. We noted smaller differences in the plateau levels reached by virus isolated from the lymph node of ROr5 at week 60 postinoculation. We cannot exclude the possibility that changes within *env* or within other segments of the viral genome occurred, which could have enabled this virus to propagate more efficiently. On the other hand, if this were the case, we would expect increased viremia, which was not observed within the 4 subsequent months of follow-up (see Fig. 2 and Fig. 4, week 76). Alternatively, this isolate has a slightly increased in vitro replicative capacity in rhesus PBMC. Longer follow-up of this monkey is necessary to understand this phenomenon. Thus far, the Rev-independent Nef(−) SIV has maintained its in vitro reduced growth properties upon propagation in macaques.

In conclusion, we have shown that deletion of *nef* and replacement of the essential Rev/RRE regulatory mechanism by the SRV-D CTE element generates a virus variant that is infectious in vivo. Importantly, the anti-Gag and anti-Env immune responses were readily detectable and persistent, demonstrating that the virus is able to replicate in rhesus macaques. Although the Rev-independent Nef(−) virus caused an active infection, all parameters analyzed revealed low levels of replication and viremia. It is interesting to note that the change in Rev/RRE regulation generated a virus that is expressed at a lower level in cultured, activated primary monkey and human cells, which is not the case for the *nef*(−) mutant viruses. Replacement of Rev regulation generated a virus variant that has reduced growth properties both in rhesus PBMC in vitro (26) and in rhesus macaques. These findings suggest that Rev/RRE is likely to be essential for high virus replication in the host. It is possible that replacing Rev/RRE by the CTE alters the replicative capacity of the virus such that it also affects virus-host interactions. Although we did not observe that this

virus variant caused disease in the juvenile macaques during the 18 months of follow-up, a longer observation time is essential to evaluate the pathogenicity of this virus variant. We have begun to address this question in neonatal macaques, which are a more sensitive host for SIV (1).

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