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Role of Env in Resistance of Feline Immunodeficiency Virus (FIV)-Infected Cats to Superinfection by a Second FIV Strain as Determined by Using a Chimeric Virus[▽]

Simone Giannecchini,[1,2] Mauro Pistello,[1] Patrizia Isola,[1] Donatella Matteucci,[1] Paola Mazzetti,[1] Giulia Freer,[1] and Mauro Bendinelli[1]*

Retrovirus Center and Virology Section, Department of Experimental Pathology, University of Pisa, I-56127 Pisa,[1] and Department of Public Health, University of Florence, I-50134 Firenze,[2] Italy

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A more or less pronounced resistance to superinfection by a second strain of the infecting virus has been observed in many lentivirus-infected hosts. We used a chimeric feline immunodeficiency virus (FIV), designated FIV_{χ}, containing a large part of the *env* gene of a clade B virus (strain M2) and all the rest of the genome of a clade A virus (a p34TF10 molecular clone of the Petaluma strain modified to grow in lymphoid cells), to gain insights into such resistance. FIV_{χ} was infectious and moderately pathogenic for cats and in vitro exhibited the neutralization specificity of the *env* donor. The experiments performed were bidirectional, in that cats preinfected with either parental virus were challenged with FIV_{χ} and vice versa. The preinfected animals were partially or completely protected relative to what was observed in naïve control animals, most likely due, at least in part, to the circumstance that in all the preinfecting/challenge virus combinations examined, the first and the second virus shared significant viral components. Based on the proportions of complete protection observed, the role of a strongly matched viral envelope appeared to be modest and possibly dependent on the time interval between the first and the second infection. Furthermore, complete protection and the presence of measurable neutralizing antibodies capable of blocking the second virus in vitro were not associated.

Recent reportsman immunodeficiency virus type 1 (HIV-1)-infected individuals can become superinfected with a second strain of the virus more frequently than previously estimated (1, 9, 26, 28, 46, 50). On the other hand, there is also convincing evidence that an established HIV-1 infection may confer on the host the ability to ward off acquisition of another HIV-1, especially if it belongs to the same clade as the initial virus and if exposure occurs after a sufficient time interval from the first infection (reviewed in references 8 and 50). The latter evidence is corroborated by studies with several animal lentiviruses, including simian immunodeficiency virus (SIV) in macaques, equine infectious anemia virus in ponies, and feline immunodeficiency virus (FIV) in cats, showing that prior infection with live attenuated and even wild-type viruses can prevent subsequent infection by fully virulent strains of the same viruses or at least afford substantial protection against their pathological effects (10, 11, 13, 18, 38 reviewed in reference 17). The mechanisms mediating such protection are, however, unresolved in both the primate and nonprimate systems (4, 27, 29, 32, 33, 55). The issue is important, since a clear understanding of the nature of these mechanisms might help in identifying immune correlates of protection against lentiviruses. That such correlates have so far remained elusive is considered a major obstacle to development and testing of candidate AIDS vaccines (2, 7, 21, 22, 37).

FIV is both a significant pathogen of domestic cats (42) and a widely used model to investigate HIV pathogenesis and approaches to AIDS vaccination (14, 16, 51, 54). In the present study, we developed a chimeric FIV, designated FIV_{χ}, having most of the *env* gene of a clade B virus and the rest of the genome of a clade A virus and used it in an attempt to gain insight into the variables that may affect the resistance to superinfection by a second strain of virus in this system, including the neutralization specificity of the viral envelope (Env).

MATERIALS AND METHODS

Animals, cells, and viruses. Specific-pathogen-free (SPF) female domestic cats, purchased from Iffa Credo (L'Arbresle, France) when they were 7 months old, were housed individually in our climate-controlled animal facility and had ad libitum access to fresh water and a proprietary brand of cat food in accordance with European Community guidelines. They ranged between 36 and 72 months of age when enrolled in the experiments. MBM cells are an interleukin 2 (IL-2)-dependent line of T lymphocytes originally established from the peripheral blood mononuclear cells (PBMC) of an FIV- and feline leukemia virus-negative cat. They are routinely grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 5 μg of concanavalin A (Sigma-Aldrich, Milan, Italy), and 20 U of human recombinant IL-2 (Roche Diagnostics, Monza, Italy) per ml. Preinfection of the cats was carried out by intravenous (i.v.) administration of 1 ml of the indicated dilution of virus. The stocks of FIV_{PET} and FIV_{M2} used for this purpose were pooled cell-free plasma from acutely infected cats titrated in 50% cat infectious doses (CID_{50}) as previously described (36, 43). The chimeric virus, designated FIV_{χ} (Fig. 1A), was obtained starting from clone p34TF10, which carries the whole genome of FIV_{PET} (GenBank accession no. NC_001482) (53). First, the stop codon in the open reading frame A accessory gene of p34TF10, which impairs the capability of this clone to grow in lymphoid cells, was removed by site-specific mutagenesis (43). Second, in an attempt to increase in vivo fitness,

* Corresponding author. Mailing address: Dipartimento di Patologia Sperimentale, Università di Pisa, Via San Zeno, 37 I-56127 Pisa, Italy. Phone: 39 050 2213 641. Fax: 39 050 2213 639. E-mail: bendinelli@biomed.unipi.it.

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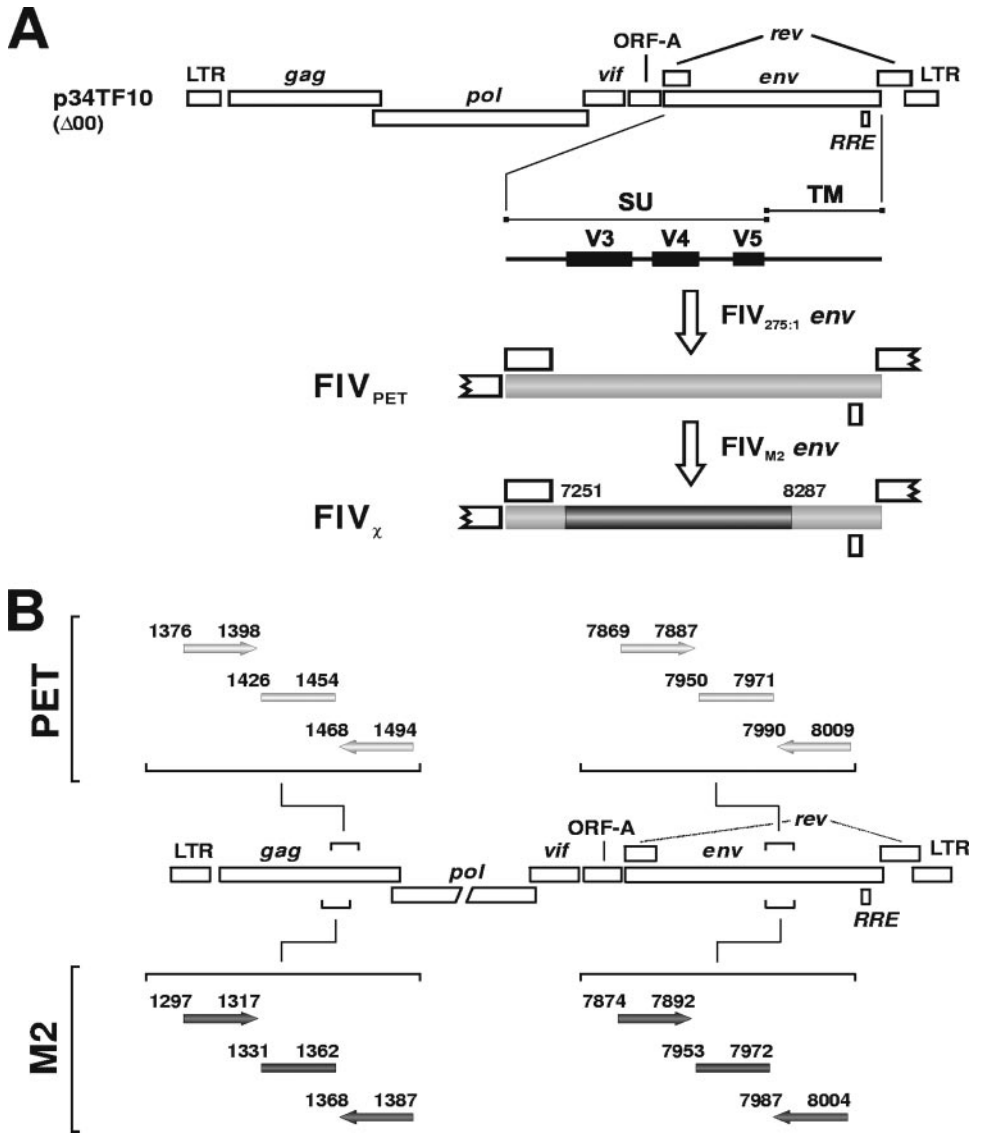

FIG. 1. Schematic representation of the chimeric virus FIV_{χ} and of how it was prepared, starting from the parental viruses FIV_{PET} and FIV_{M2}. The locations of the primers used for the PCR assays in Fig. 2 are also shown.

the entire *env* gene was replaced with that of an FIV_{PET} reisolated from an experimentally infected cat ($FIV_{275:1}$ [3]). Third, most of the *env* of the latter clone (amino acid positions 329 to 673) was replaced with the corresponding sequence amplified from ex vivo FIV_{M2}. The viral stock was finally obtained by transfecting Crandell feline kidney cells and expanding the progeny virus in feline T-lymphoid MBM cells (3). Proper insertion and the absence of unwanted mutations were checked by sequencing the entire *env* gene exactly as described previously (44). Upon characterization, FIV_{χ} proved readily infectious for SPF cats, where it produced a florid acute-phase infection and a clearly evident depletion of circulating $CD4^+$ T lymphocytes (data not shown; see below).

Virus challenges. The viral stocks used for the challenges consisted of the same frozen pooled plasma preparations from acutely infected SPF cats (FIV_{M2} and FIV_{PET}) and supernatant of acutely infected MBM cells (FIV_{χ}) used for the preinfections. The challenges were performed under slight anesthesia. The i.v. challenge was carried out by injecting 1 ml of the virus preparations diluted to contain the indicated dose. The mucosal inoculum was deposited onto the anterior vagina in 100 μl pyrogen-free saline using smooth pipette tips. No discharge from the vagina was observed after the inoculum was injected.

Quantitation and discrimination of the viruses replicating in infected cats. To quantitate and genotype the FIV(s) growing in the study cats, four *gag*- and *env*-based real-time PCR and reverse transcription (RT)-PCR assays were developed, which made use of the primers and probes shown in Fig. 1B. After preliminary experiments using plasmid DNA as standards, the following assay conditions were adopted: reaction mixture, 25-μl final volume containing 5 μl of either genomic DNA (200 to 500 ng) or cDNA, 100 nM probe, and 300 to 900 nM primers. The thermal cycling profile was as follows: 50°C, 2 min; 95°C, 10 min; 95°C, 15 s; and 60°C, 1 min (50 cycles) on the ABI Prism 7700 Sequence Detection System instrument (Applied Biosystems, Monza, Italy). The selectivity of these assays for the specific FIV targeted, albeit not absolute, was considered sufficient for the purposes of the study. Indeed, as shown by Fig. 2, the proviral and viral loads detected by measuring DNA or RNA of the homologous FIV with the *gag*-based assays were at least 2 log units higher than those detected by measuring DNA of the heterologous virus, regardless of the ratio between the two DNAs, and this differential was at least 3 log units with the *env*-based assays, which exploited the greater diversity existing between FIV_{PET} and FIV_{M2} in this gene relative to *gag* (23.8%, with peaks of 36.4% in the variable regions targeted, versus 17.7%). For proviral-load quantitation, genomic DNA was extracted from PBMC or lymphoid tissues using the QIAamp DNA Blood Mini Kit (QIAGEN, Milan, Italy). The extracted DNA was then amplified in parallel with serial 10-fold dilutions (10^1 to 10^7) of the corresponding DNA plasmid standards diluted in 1 μg of genomic DNA. The sensitivity of the PCR assays was 50 copies per μg of genomic DNA, regardless of the target. For plasma viral-load quan-

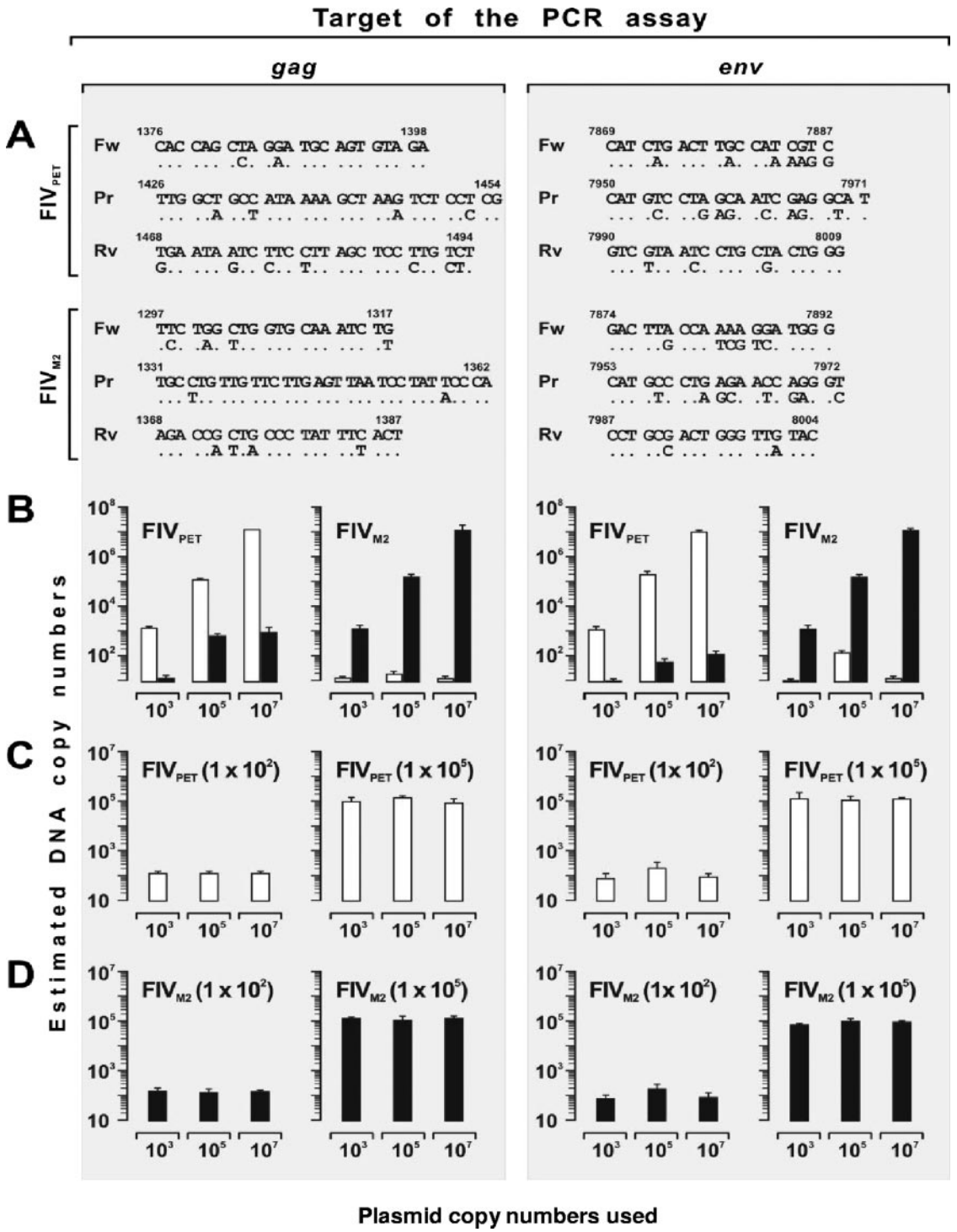

FIG. 2. Features of the PET*gag*-, PET*env*-, M2*gag*-, and M2*env*-specific real-time PCR assays used in the study. (A) Forward primers (Fw), probes (Pr), and reverse primers (Rv) were designed based upon appropriately studied segments of *gag* or *env* of FIV_{PET} or FIV_{M2} using Primer Express software (version 1.5; Applied Biosystems, Monza, Italy) and differed from the heterologous virus, as indicated under each oligonucleotide (nucleotide positions are based on FIV_{PET} clone p34TF10, GenBank accession number NC_001482) (53). Note that in order to improve selectivity and fulfill real-time probe requirements, the M2*gag* primers and probe were designed from the antisense strand. (B to D) Selectivity of the PET-specific (open bars) and M2-specific (closed bars) assays, as determined by quantitating FIV_{PET} and FIV_{M2} plasmids; the DNAs examined were FIV_{PET} plasmids alone or FIV_{M2} plasmids alone (indicated at the tops of the graphs) at the copy numbers shown in the abscissa (B), 1×10^2 or 1×10^5 FIV_{PET} plasmids (indicated at the tops of the graphs) mixed with the numbers of FIV_{M2} plasmids shown in the abscissa (C), and 1×10^2 or 1×10^5 FIV_{M2} plasmids (indicated at the tops of the graphs) mixed with the numbers of FIV_{PET} plasmids shown in the abscissa (D). These degrees of selectivity were conserved in the RT-PCR assays, as determined by performing similar experiments with RNA transcribed on the viral plasmids (data not shown). The error bars represent standard deviations from three independent experiments.

TABLE 1. Parameters of infection in study cats at times of challenge

Preinfecting virus	Cat no.	Age at challenge (mo)	RNA copies/ml of plasma[a]	DNA copies/mg of PBMC DNA[b]	Infectious units in 10^6 PBMC	$CD4^+$ T lymphocytes/ml of blood[c]
FIV_{PET}	2682	72	2,000	3,000	1	690
	3552	72	6,100	4,200	10	200
	3608	72	3,300	7,600	1	300
	3633	72	11,300	1,900	10	440
	3646	72	17,900	10,000	1	398
FIV_{M2}	2646	57	2,100	34,100	10	250
	2647	57	5,000	62,000	100	210
	2658	57	9,000	84,700	100	326
	2664	57	20,000	87,600	100	230
	2668	57	28,000	98,300	100	408
FIV_X[d]	2839	36	6,000	2,600	10	800
	2841	36	700	900	1	910
	2893	36	3,000	800	10	870
	3335	36	3,200	1,000	1	980
	3437	36	900	1,900	1	700
	2731	36	1,400	800	10	700
	2881	36	800	700	1	700
	2884	36	6,500	5,100	1	800
	2897	36	800	2,000	10	500
	3316	36	2,000	3,000	1	760

[a] RNA copies determined with the appropriate RT-PCR-specific assay.
[b] DNA copies determined with the appropriate PCR-specific assay.
[c] Normal range, 1,000 to 3,500.
[d] The first five animals in this group were challenged with FIV_{PET}, the others with FIV_{M2}.

titation, RNA was extracted from EDTA-collected blood with the QIAamp Viral RNA kit (QIAGEN) and reverse transcribed with reverse primer, and the cDNA was amplified in parallel with serial 10-fold dilutions (10^2 to 10^7) of the corresponding RNA transcripts. As evaluated by extracting and amplifying FIV-negative plasma spiked with serial 10-fold dilutions of RNA transcripts, the sensitivity of RT-PCR assays was 200 copies per ml, regardless of the target. Precautions to avoid misquantitation and false-positive and -negative results have been previously described (44).

Virus isolation and infectious-cell enumeration. For FIV reisolation from infected cells, 10^6 Ficoll-Paque gradient-purified PBMC were stimulated with concanavalin A and cocultured with 10^6 MBM cells in RPMI 1640 medium containing 10% fetal bovine serum and 20 U/ml human recombinant IL-2. The culture supernatants were monitored biweekly for p25 production by enzyme-linked immunosorbent assay (ELISA) for up to 5 weeks. Quantitation of circulating infectious units was performed exactly as described above, except that the cocultures were established with 10-fold serial dilutions of the PBMC under scrutiny.

Serology. Plasma was tested for antibodies against whole FIV_{PET} antigen by ELISA as described previously (36). Plasma specimens found to be reactive at dilutions of 1:100 or greater were considered antibody positive. Neutralizing antibodies (NA) were determined against 10 50% tissue culture infective doses ($TCID_{50}$) of the appropriate FIV by using MBM cells as indicator cells and quantitation of reverse transcriptase activity in the supernatant as an end point readout, exactly as described previously (19).

Hematology and lymphocyte subset analyses. Complete blood counts and differential leukocyte counts were performed by standard methods. $CD4^+$ and $CD8^+$ T-cell counts were determined by flow cytometry using a FACScan (BD Biosciences-Life Science Research, Milan, Italy).

RESULTS

FIV_X challenge of cats preinfected with the parental viruses. Two groups of five 72- and 57-month-old cats preinfected with 5 to 10 CID_{50} of FIV_{PET} or FIV_{M2} 34 and 36 months earlier, respectively, were used in this part of the study. Consistent with what is known about long-term steady-state FIV infections (51), the animals had moderate (FIV_{M2}-preinfected) to low (FIV_{PET}-preinfected) viral and proviral loads and markedly reduced circulating $CD4^+$ T-cell counts (Table 1). Of note, the results of prechallenge monitoring corroborated the exquisite selectivity demonstrated by the assays used to differentiate/quantitate the infecting viruses during characterization (see Materials and Methods), since the animals consistently tested positive only if they had been infected with the FIV strain targeted by the assays (Fig. 3B and C).

(i) Systemic challenge. The above-mentioned animals and five uninfected cats were injected i.v. with 20 $TCID_{50}$ of FIV_X and then monitored for 9 months. Postchallenge (PC), all the controls developed sustained infections by all the parameters measured, including a progressive decline in $CD4^+$ T-lymphocyte counts, which by the end of follow-up were approximately halved. Importantly, the kinetics and levels of plasma viremia and PBMC proviral loads of these animals were similar independently of the PET*gag* or M2*env* specificity of the assay used to monitor FIV_X infection, confirming the comparable performance characteristics of these assays (Fig. 3A).

In the preinfected cats (Fig. 3B and C), FIV_X challenge produced no appreciable changes in the preexisting infection set points. Furthermore, only one preinfected animal showed traces of the chimeric virus in the bloodstream. This was cat no. 2647, preinfected with FIV_{M2}, which, although constantly negative for FIV_X in plasma, reacted transiently positive for the chimeric provirus in the PBMC 1 month PC. The other nine preinfected animals presented no evidence of FIV_X in the circulation, as shown by uniformly negative FIV_{M2}- and FIV_{PET}-specific PCR and RT-PCR assays in the FIV_{PET}-preinfected cats and in the FIV_{M2}-preinfected cats, respectively, throughout the follow-up. Also, preexisting anti-FIV ELISA antibody titers, $CD4^+$ T-cell counts (Fig. 3B and C), and infectious-cell loads in the PBMC (data not shown) underwent no appreciable variations PC.

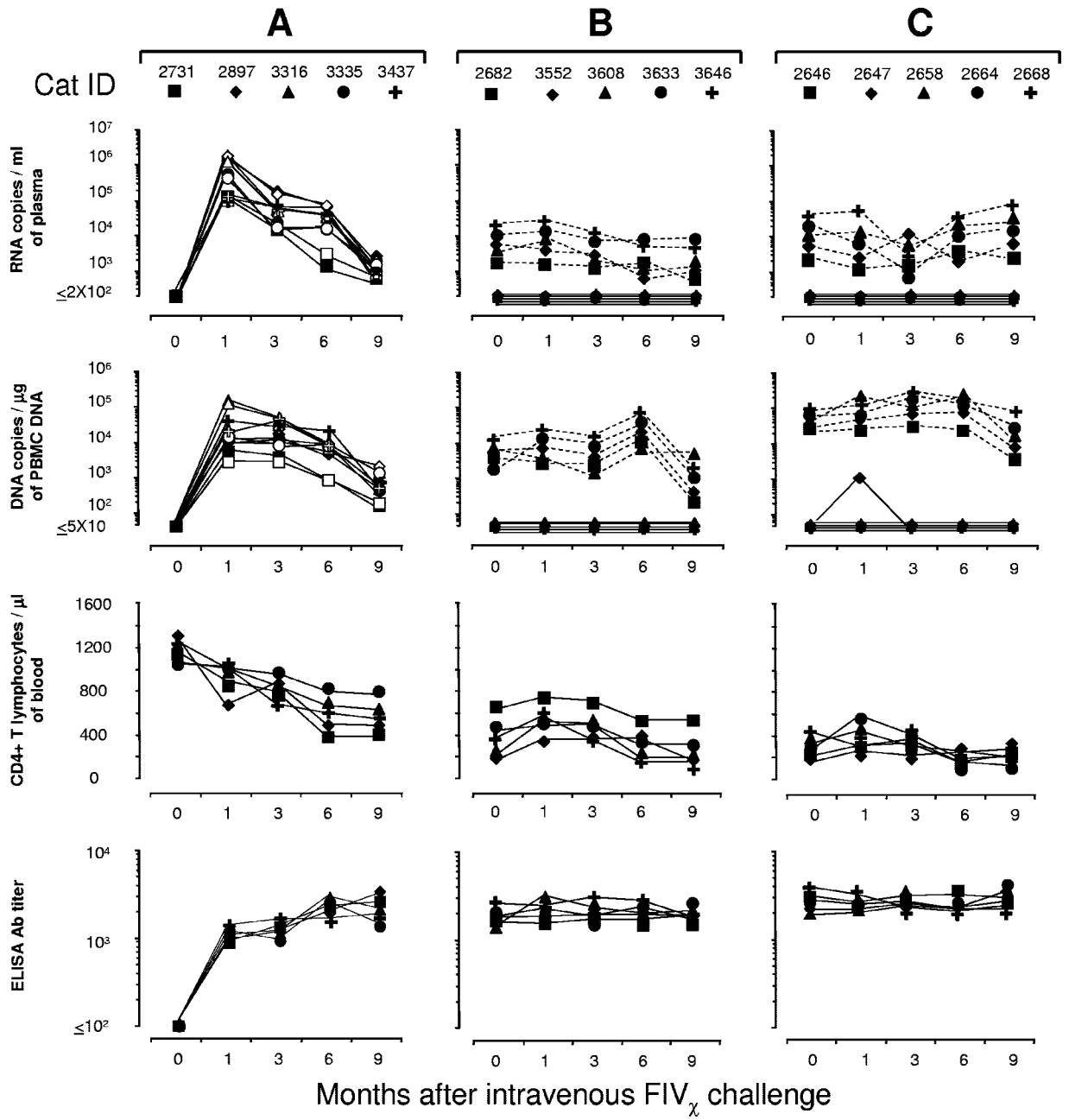

FIG. 3. Set points in FIV_{PET}- or FIV_{M2}-preinfected cats and naïve controls at various times after i.v. challenge with FIV_{χ}. (A) Naïve cats. FIV_{χ} plasma viremia and proviral loads were determined with both the PET*gag*-specific assay (empty symbols) and the M2*env*-specific assay (solid symbols). Note that cats in this age range (27 months at challenge) showed no major spontaneous $CD4^+$ T-lymphocyte count changes. (B) FIV_{PET}-preinfected cats. FIV_{PET} (dashed lines) and FIV_{χ} (continuous lines) plasma viremia and proviral loads determined with the PET*env*- and M2*env*-specific assays, respectively. (C) FIV_{M2}-preinfected cats. FIV_{M2} (dashed lines) and FIV_{χ} (continuous lines) plasma viremia and proviral loads determined with the M2*gag*- and PET*gag*-specific assays, respectively. Anti-FIV antibodies were determined by ELISA using whole FIV antigen.

(ii) Mucosal challenge. Compared to systemic challenge, mucosal FIV infections may be more difficult to control immunologically (5, 6, 39, 40, 44). To assess how the animals preinfected with the parental viruses dealt with mucosal challenge with the chimera, after follow-up of the systemic challenge described above was terminated, all the FIV_{PET}- and FIV_{M2}-preinfected cats were exposed intravaginally to 100 $TCID_{50}$ of FIV_{χ} and monitored for an additional 9 months. While two of three age-matched uninfected cats used as controls became readily FIV_{χ} infected (Fig. 4A), none of the FIV_{PET}-preinfected cats yielded FIV_{χ} RNA or provirus in peripheral blood throughout the follow-up (Fig. 4B). In contrast, among the FIV_{M2}-preinfected cats, two (no. 2658 and 2668) yielded the FIV_{χ} provirus in PBMC at month 1 PC, and

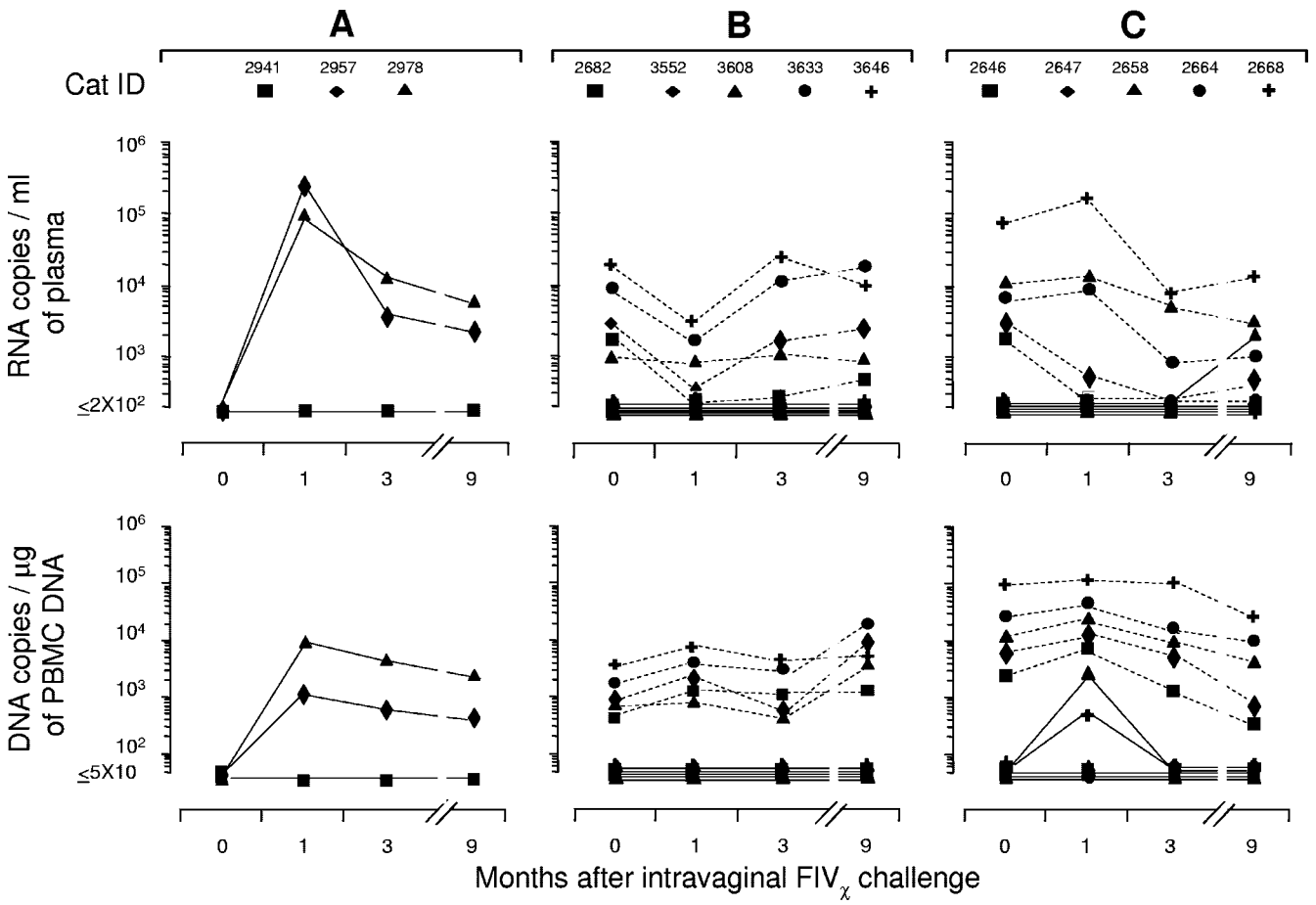

FIG. 4. Viral set points in FIV_{PET}- or FIV_{M2}-preinfected cats and naïve control cats at various times after intravaginal challenge with FIV_{χ}. (A) Naïve cats. FIV_{χ} plasma viremia and proviral loads determined with the M2*env*-specific assay. (B) FIV_{PET}-preinfected cats. FIV_{PET} (dashed lines) and FIV_{χ} (continuous lines) plasma viremia and proviral loads determined with the PET*env*- and M2*env*-specific assays, respectively. (C) FIV_{M2}-preinfected cats. FIV_{M2} (dashed lines) and FIV_{χ} (continuous lines) plasma viremia and proviral loads determined with the M2*gag*- and PET*gag*-specific assays, respectively.

one of these (no. 2658) was also positive for FIV_{χ} RNA in plasma at month 9 PC (Fig. 4C).

(iii) Detection of the challenge virus in the lymphoid organs. At the end of the mucosal challenge experiment described above, four cats in the FIV_{PET}-preinfected group (one animal in this group could not be examined because it was mistakenly given for adoption to a volunteer) and all five FIV_{M2}-preinfected cats were euthanized, and their mesenteric lymph nodes, spleen, and bone marrow were tested with the appropriate PCR assays. As shown by Fig. 5A, the animals preinfected with FIV_{PET} showed generally high levels of the preinfecting virus in all or most tissues examined but no evidence of FIV_{χ} provirus. On the other hand, three animals preinfected with FIV_{M2} yielded both the preinfecting virus and the FIV_{χ} provirus at comparable copy numbers per μg of extracted tissue DNA (Fig. 5B). Of these, one (no. 2647) had tested transiently FIV_{χ} positive in the circulation after the first challenge and two after the second challenge (no. 2658 and 2668).

(iv) NA at the challenges. Sera taken at the time of systemic challenge, from the FIV_{PET}- and FIV_{M2}-preinfected cats were tested for NA to the chimera, as well as the parental viruses, in standard neutralization assays using lymphoid cells as a substrate. FIV_{χ}-neutralizing activity was detected in three FIV_{M2}-preinfected cats, two of which also possessed NA for the homologous virus, and in one FIV_{PET}-preinfected animal, who instead invariably had NA for the homologous virus. Consistent with previous findings (12), essentially no cross-neutralization of FIV_{PET} and FIV_{M2} was observed (Table 2). The tests were repeated at mucosal challenge to assess possible changes relative to the above-mentioned NA status resulting from the prior systemic challenge, but no changes were detected (data not shown).

Challenge of cats preinfected with FIV_{χ} with the parental viruses. The animals used in this part of the study were 36-month old cats that had been infected with FIV_{χ} 10 months earlier. Five were the ones used as controls for systemic FIV_{χ} challenge in the study described above, and five had been infected contemporaneously but had been left untreated. As expected, they were all positive by the PET*gag*- and PET*env*- but not by the M2*gag*- and M2*env*-specific assays and had viral set points typical of a postacute FIV infection of moderate severity (Table 1 and Fig. 6B and 7B). These animals were sorted into two groups of five with comparable viral set points and used as reported below.

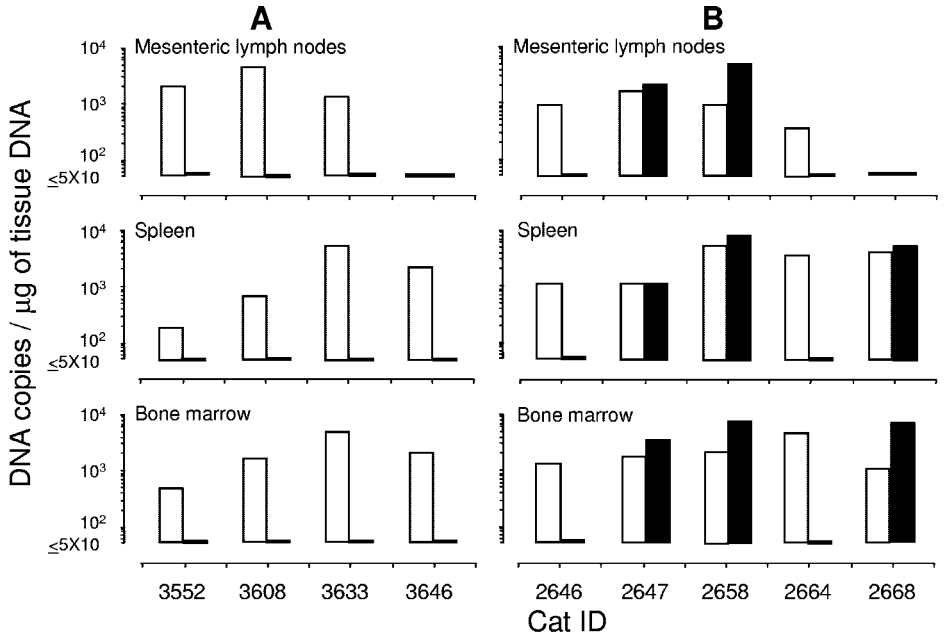

FIG. 5. Proviral loads of preinfecting viruses and challenge FIV_{χ} in selected tissues of cats at the end of the experiment shown in Fig. 4. (A) FIV_{PET}-preinfected cats examined with the M2*env*- and PET*env*-specific assays. (B) FIV_{M2}-preinfected cats examined with the M2*gag*- and PET*gag*-specific assays. Open bars, preinfecting viruses; closed bars, FIV_{χ}.

(i) Challenge with FIV_{PET}. In the first experiment, five FIV_{χ}-preinfected cats and five virus-naïve age-matched controls were challenged i.v. with 10 CID_{50} of FIV_{PET} and monitored for 9 months. PC, all the controls readily developed the expected plasma viremia and PBMC provirus loads (Fig. 6A). In the FIV_{χ}-preinfected cats, the challenge failed to modify the preexisting plasma viremia with regard to both the viral-RNA load and the FIV strain detected throughout the follow-up. The numbers of infectious units found in the PBMC and FIV binding antibody titers also remained unchanged relative to what was observed prechallenge (data not shown); however, when observations were limited to 1 and 3 months PC, these cats underwent moderate to low elevations in the preexisting levels of FIV_{χ} provirus in PBMC and showed clear evidence that the PBMC also harbored FIV_{PET} (Fig. 6B).

(ii) Challenge with FIV_{M2}. The second group of FIV_{χ}-preinfected cats and a group of four age-matched uninfected controls were inoculated i.v. with 10 CID_{50} of FIV_{M2}. For 9 months PC, none of the FIV_{χ}-preinfected cats showed evidence that FIV_{M2} was circulating in the blood, as indicated by consistently negative M2*gag*-specific PCR and RT-PCR (Fig. 7B). However, although a similar dose of virus had been fully effective at infecting younger cats in the previous experiment, the challenge was only partially successful, since two of four controls escaped infection, as revealed by persistently negative virological (Fig. 7A) and serological (data not shown) tests. For this reason, all the FIV_{χ}-preinfected cats, along with the two controls that had escaped the infection, as described above, and three additional naïve cats, were inoculated i.v. with 30 CID_{50} of FIV_{M2}. As shown by Fig. 7C, this second challenge with a higher viral dose readily infected all the controls. Conversely, the FIV_{χ}-preinfected cats showed no substantial changes in preexisting plasma viremia and PBMC provirus loads, even after this robust challenge. However, two cats in the group (no. 2884 and 2897) yielded the challenge virus in plasma from month 3 PC onward, as well as the challenge provirus in PBMC starting from month 6 PC.

(iii) Detection of the challenge viruses in the lymphoid organs. At the end of the follow-up, all of the cats preinfected with the chimera and challenged with FIV_{PET} or FIV_{M2} were euthanized, and the viral contents in their mesenteric lymph nodes, spleens, and bone marrow were characterized. As shown by Fig. 8A, five of five FIV_{PET}-challenged cats were found to carry the challenge virus in some or all the tissues examined, and in one (no. 2839), this was the only virus detected. Among the FIV_{M2}-challenged cats, two of five tested positive for the challenge virus, and these were the same animals whose peripheral blood had tested positive (Fig. 8B). Interestingly, in a few instances, the proviral loads of the challenge viruses exceeded or even replaced those of FIV_{χ}.

(iv) NA at the challenges. Sera obtained at challenge from the FIV_{χ}-preinfected cats were examined for NA. All had moderate to low titers of NA for both FIV_{χ} and FIV_{M2}, but none blocked FIV_{PET} (Table 2).

DISCUSSION

The chimeric virus FIV_{χ} was generated by replacing a large part of the Env of FIV_{PET} (clade A), specifically, the part including the domains known to be involved in antibody-mediated neutralization (3, 35, 48, 49), with the corresponding region of FIV_{M2} (clade B). In previous superinfection studies, preinfection with FIV_{PET} had exerted long-term beneficial effects against fully virulent FIV_{M2} (45) and significantly protected against a heterologous intraclade challenge given systemically or mucosally (44). Importantly, FIV_{χ} was effectively

TABLE 2. Virus NA in day-of-challenge sera of the preinfected cats

Preinfecting virus	Cat no.	NA titer against indicated virus			Challenge virus	Complete protection[a]
		FIV_{PET}	FIV_{M2}	FIV_{χ}		
FIV_{PET}	2682	64	—	—	FIV_{χ}	Yes
	3552	64	—	—		Yes
	3608	128	—	—		Yes
	3633	128	—	—		Yes
	3646	128	—	16		Yes
FIV_{M2}	2646	—	—	16	FIV_{χ}	Yes
	2647	—	—	—		No
	2658	—	32	128		No
	2664	—	—	—		Yes
	2668	—	32	256		No
FIV_{χ}	2839	—	16	128	FIV_{PET}	No
	2841	—	16	32		No
	2893	—	16	16		No
	3335	—	32	64		No
	3437	—	16	16		No
	2731	—	32	32	FIV_{M2}	Yes
	2881	—	16	64		Yes
	2884	—	64	128		No
	2897	—	16	16		No
	3316	—	16	16		Yes

[a] As determined from the overall results obtained pre- and posteuthanasia. The efficiencies of the challenges were as follows: only 1 of 25 total naïve controls escaped infection. This was cat no. 2941 in the FIV_{χ} mucosal challenge study shown in Fig. 4. —, <8.

neutralized by the sera of FIV_{M2}-infected cats but very poorly or not at all by the sera of FIV_{PET}-infected cats and elicited NA that neutralized itself and FIV_{M2} but not FIV_{PET}, showing that in the new setting the transferred Env had conserved a great part, and possibly all, of its neutralization specificity. Furthermore, FIV_{χ} readily infected naïve cats and, as judged by the depletion of circulating $CD4^+$ T cells produced, was also moderately pathogenic.

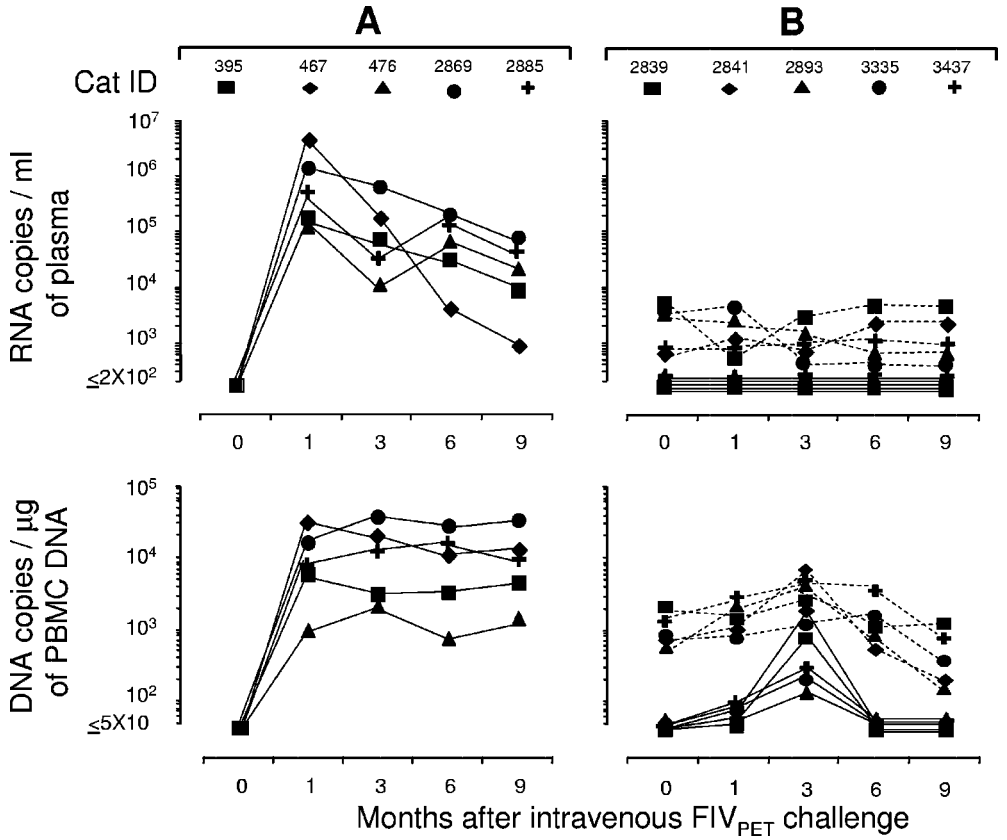

FIG. 6. Viral set points in FIV_{χ}-preinfected cats and naïve control cats after i.v. challenge with FIV_{PET}. (A) Naïve cats. FIV_{PET} plasma viremia and proviral loads determined with the PET*env*-specific assay. (B) FIV_{χ}-preinfected cats. FIV_{χ} (dashed lines) and FIV_{PET} (continuous lines) plasma viremia and proviral loads determined with the M2*env*- and PET*env*-specific assays, respectively.

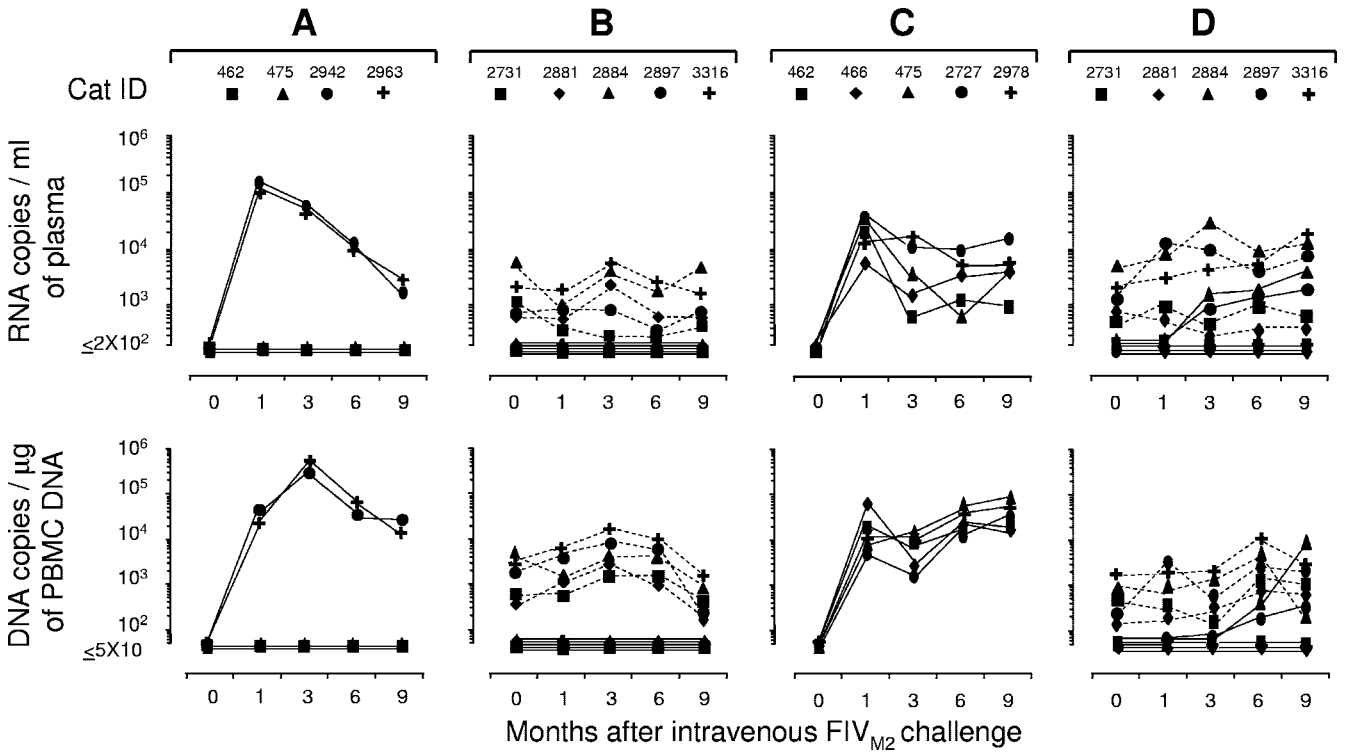

FIG. 7. Viral set points in FIV$_{\chi}$-preinfected and naïve control cats after i.v. challenge with FIV$_{M2}$. (A and B) First challenge with 10 CID$_{50}$. (C and D) Second challenge with 30 CID$_{50}$. (A and C) Naïve cats. FIV$_{M2}$ plasma viremia and proviral loads determined with the M2*gag*-specific assay. (B and D) FIV$_{\chi}$-preinfected cats. FIV$_{\chi}$ (dashed lines) and FIV$_{M2}$ (continuous lines) plasma viremia and proviral loads determined with the M2*gag*- and PET*gag*-specific assays, respectively.

FIV$_{\chi}$ was used to investigate the role of Env compared to the other viral gene products in the resistance of lentivirus-infected hosts to superinfection by a second strain of the virus (8, 50) in two sets of experiments. In the first set, it served as a challenge for cats that had been infected with either parental virus approximately 3 years in advance, while in the second, it served to preinfect cats that 10 months later were challenged with either parental virus. The outcomes of the challenges were determined by monitoring the animals for 9 months by using appositely developed real-time PCR and RT-PCR assays in appropriate combinations that permitted the independent detection and quantitation of FIV$_{\chi}$ and the parental viruses. Although we did not specifically look for the emergence of recombinant viruses in the cats that became superinfected, the results obtained in the assays targeting *gag* or *env* of the same viral strain were uniformly highly concordant, making it unlikely that recombinants were a major fraction of the total viral burden in such animals.

The overall results clearly demonstrated a general tendency of the preinfected cats to contain the challenge viruses: indeed, they were undetectable in the peripheral blood of many preinfected cats (Table 3) and, when detected, exhibited much reduced viral RNA and proviral DNA loads compared with those in naïve animals challenged in parallel as controls. Although this aspect was not specifically addressed, the fact that this extent of protection had not been observed in a previous superinfection study involving the same viral strains (45) suggests that the presence of identical components in the two viruses significantly facilitated containment of the second virus. Since plasma and PBMC were often found to be negative for the challenge viruses in cats that had clearly positive lymphoid organs when euthanized at the end of the experiments, the overall results also showed that characterizing the virus contents of a few blood samples may not suffice to rule out infection by a second virus. Similar discrepancies between the results of examining peripheral blood and lymphoid organs have been reported in several superinfection studies with wild-type and attenuated lentiviruses (15, 24, 30, 47), suggesting that containment of a second virus can occur not only at the level of establishment of the infection, but also at the level of how freely it may circulate in blood.

Another bit of clear evidence that emerged from the overall results is that the presence at challenge of NA capable of blocking the second virus in vitro was not a major determinant of resistance to superinfection. Indeed, the proportions of preinfected subjects who possessed such antibodies were approximately the same among the cats who scored as completely protected (defined as negative for the challenge virus in blood at all times and in the lymphoid organs at the end of the experiment) and among those who became superinfected (5/10 and 4/10, respectively). On the other hand, in no instance did the NA reach the titer of 1/512, which was previously shown to correlate with protection in FIV-vaccinated cats (19) and to be rarely, if ever, detected in infected cats (12). However, it is paradoxical that the group of preinfected animals that resisted

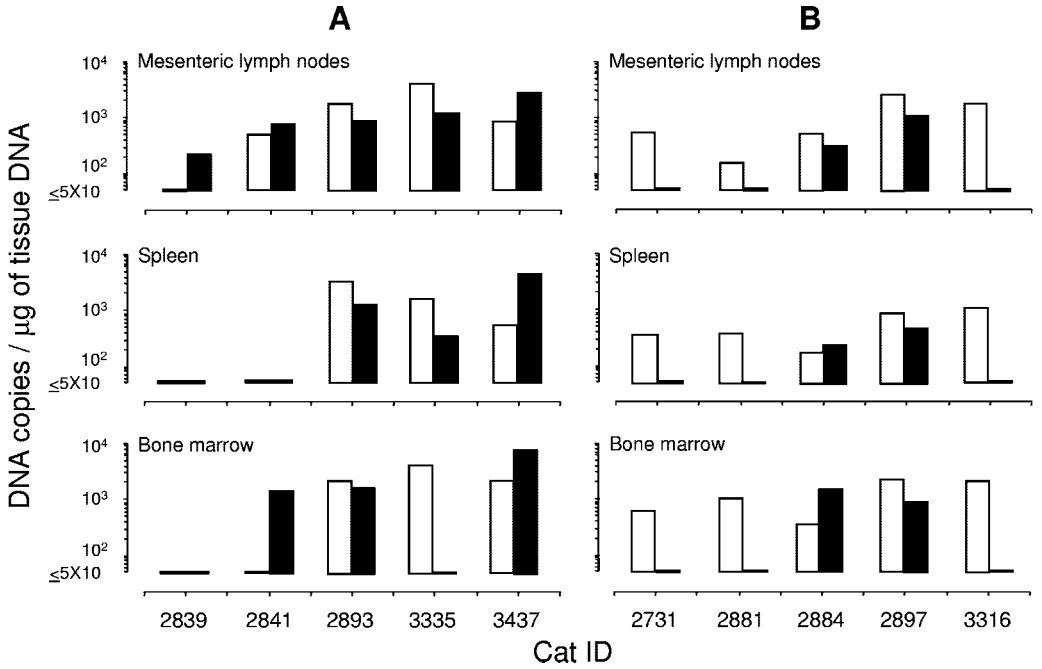

FIG. 8. Proviral loads of the preinfecting FIV_{χ} and of the challenge viruses in selected tissues of cats at the ends of the experiments shown in Fig. 6 and 7. (A) FIV_{PET}-challenged cats examined with the M2*env*- and PET*env*-specific assays. (B) FIV_{M2}-challenged cats examined with the M2*gag*- and PET*gag*-specific assays. Open bars, FIV_{χ}; closed bars, challenge viruses.

the challenge best was also the one in which NA to the challenge virus were the least frequent (Table 2).

If we examine the relative importance of a matched Env versus a matched non-Env component in resistance to superinfection by comparing the numbers of cases of complete protection that occurred, the results of the first part of the study fully support the above contentions, as well as previous SIV data showing that complete protection can be independent of a completely matched Env (20). Indeed, there were fewer cats completely protected against FIV_{χ} in the group preinfected with FIV_{M2}, which shared only Env with the challenge virus, than in the one preinfected with FIV_{PET}, which shared all of the virion except Env with FIV_{χ} (two of five versus five of five). Of note, no cat in the latter group allowed FIV_{χ} infection despite the fact that challenge was carried out twice, first systemically and then intravaginally. This might indicate that resistance to superinfection was particularly robust in this preinfection-challenge combination, although the fact that FIV_{χ} was molecularly cloned and grown in vitro, two circumstances known to weaken FIV challenge (14, 23, 25), may have contributed significantly.

The indications of the second set of experiments were less straightforward. First, all or a large fraction of the FIV_{χ}-preinfected cats became superinfected with FIV_{PET} or with FIV_{M2}, respectively, possibly due to the circumstances that these viruses, being ex vivo derived, had a more complex quasispesies than the FIV_{χ} used as a challenge in the experiment described above (14, 23) and that the time elapsed after preinfection was shorter than in the first part of the study. That the duration of this interval can influence the outcomes of superinfections has been observed in numerous studies with live attenuated SIV vaccines (reviewed in reference 32), as well as

TABLE 3. Summary of the outcomes of challenging preinfected cats

Preinfecting virus	Challenge virus	Route of challenge	Detection of the challenge virus in:			No. of cats completely protected from the challenge
			Plasma[a]	PBMC[b]	Lymphoid organs[c]	
FIV_{PET}	FIV_{χ}	I.v.	0/5	0/5		
		Vagina	0/5	0/5	0/4[d]	5/5
FIV_{M2}	FIV_{χ}	I.v.	0/5	1/5 (transient)		
		Vagina	1/5	2/5 (transient)	3/5	2/5
FIV_{χ}	FIV_{PET}	I.v.	0/5	5/5 (transient)	5/5	0/5
	FIV_{M2}	I.v.	2/5[e]	2/5[e]	2/5[e]	3/5

[a] Virus positive cats/examined cats, as determined by RT-PCRs at selected times PC.
[b] Provirus-positive cats/examined cats, as determined by PCRs at selected times PC.
[c] Provirus- positive cats/examined cats, as determined by PCRs at the end of the experiment.
[d] Cat 2682 was not available for autopsy.
[e] Cumulative results after the second challenge.

in an experiment with HIV-2 in which no macaques became superinfected when challenged 8 weeks after initial infection versus four of four and one of four challenged at weeks 4 and 2, respectively (41). Second, and most interestingly, three of five cats following challenge with FIV_{M2} and none following challenge with FIV_{PET} proved to be completely protected, suggesting that under the experimental conditions used in this part of the study, effector mechanisms targeting the viral Env but distinct from NA played an important role in blocking the second virus. The implicated mechanisms might include antibody-dependent cell cytotoxicity mediated by Env-specific nonneutralizing antibodies, Env-specific cell-mediated immune responses, and viral interference (24, 34). Thus, the possibility exists that not only the strength of resistance to superinfection, but also the effectors implicated, evolved with time after the first infection (52).

In summary, this investigation of the resistance of FIV-preinfected cats to superinfection by a second strain of the virus indicated that (i) in long-term-infected hosts, resistance is mainly mediated by effector mechanisms that do not target strain-specific sites of Env; (ii) if these sites play a dominant role, it is only for a limited time after the first infection; and (iii) in any case, the role of NA is minimal or nonexistent. Many efforts are being done to identify correlates of protection from lentiviruses. As recently discussed (22, 31), the findings with primate lentiviruses have emphasized the significance of the mediators elicited by internal proteins of the virion, while NA are thought to play an ancillary function, if any. By and large, the present results with a nonprimate lentivirus fully agree with this picture.

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