

Studies of AIDS Vaccination Using an Ex Vivo Feline Immunodeficiency Virus Model: Protection Conferred by a Fixed-Cell Vaccine against Cell-Free and Cell-Associated Challenge Differs in Duration and Is Not Easily Boosted

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Cats immunized with cells infected with a primary isolate of feline immunodeficiency virus (FIV) and fixed with paraformaldehyde were challenged with cell-free or cell-associated homologous virus obtained ex vivo. Complete protection was observed in animals challenged with cell-free virus 4 months after completion of vaccination (p.v.) or with cell-associated virus 12 months p.v. In contrast, no protection was observed in cats challenged with cell-free virus 12 or 28 months p.v. or with cell-associated virus 37.5 months p.v. Prior to the 28- and 37.5-month challenges, the animals had received a booster dose of vaccine that had elicited a robust anamnestic immune response. These results show that vaccine-induced protection against ex vivo FIV is achievable but is relatively short-lived and can be difficult to boost.

Feline immunodeficiency virus (FIV) is a lentivirus that produces persistent infections of its natural host, the domestic cat, that are characterized by progressive deterioration of immune functions, neuropathological changes, and opportunistic infections similar to those associated with AIDS following human immunodeficiency virus (HIV) infection of humans. FIV is widely used as a model for AIDS vaccine studies, as it may help develop a rational basis for the selection and presentation of effective immunogens (for reviews, see references 3, 11, 21, and 33). Previous investigations have shown that immunization with crude inactivated-FIV-infected cell and whole-virus vaccines can induce protective immunity in cats while subunit vaccines have been, at most, marginally beneficial and in some instances have even exerted infection-enhancing effects (5, 18, 20, 25, 27, 29, 38, 40, 45, 48). The information provided by these studies, together with similar studies using simian immunodeficiency virus (SIV), is extremely valuable because it has indicated that, at least under certain conditions, vaccine-induced protective immunity against immunodeficiency-inducing lentiviruses is achievable. However, there are still many aspects that require elucidation (6, 16).

In a previous report (29), we demonstrated that a fixed, infected-cell vaccine prepared with a fresh isolate of FIV resulted in 100% protection against intravenous challenge with homologous cell-free ex vivo virus performed 4 months after completion of vaccination (p.v.). Here we have used the same protocol to investigate the duration of the protection conferred by the same vaccine, its efficacy against cell-associated versus cell-free virus, and its capacity to restimulate protective immunity once this has declined.

MATERIALS AND METHODS

Animals. Female specific-pathogen-free (SPF) cats, received 7 months old from Ifa Credo (L'Asbregue, France), were housed individually in our climatized animal facility under European Community law conditions, allowed to adapt to the new environment for 1 month, and assigned randomly to experimental groups. In each experiment, vaccinated and naive cats were the same age. They were clinically examined once per week and bled under slight anesthesia periodically. All of the animals were antibody-, virus isolation-, and PCR-negative for FIV at the start of the experiments.

Vaccine, mock vaccine, and immunization schedule. The vaccine consisted of MBM cells infected with a stock of the Pisa-M2 strain of FIV (FIV-M2), a member of the B clade of FIV (34). This virus stock can be considered representative of a primary isolate since it was isolated in mitogen-stimulated feline peripheral blood mononuclear cells (PBMC) and then passaged a maximum of five times in MBM cells. Production of infected cells (>60% virus positive by surface immunofluorescence) and their fixation with paraformaldehyde for vaccine preparation were done exactly as previously described (29). Inactivation of virus infectivity was validated by inoculating 10^6 fixed cells onto fresh MBM cells that were then maintained in culture for 6 to 7 weeks without recovery of virus. Furthermore, neither virus nor proviral FIV DNA was detected in the vaccinated cats before challenge during observation periods of up to 12 months. Each immunizing dose contained 3×10^7 fixed infected cells mixed 1:1 with Freund's incomplete adjuvant in a total volume of 2 ml. The vaccine was injected subcutaneously at weeks 0, 3, 6, 9, and 21. The mock vaccine consisted of uninfected MBM cells prepared, formulated, and administered exactly as for FIV vaccination.

Virus challenges. Three different types of challenge were used: cell-free FIV-M2, cell-associated FIV-M2, and cell-associated FIV-Petaluma (FIV-Pet). Preparation and titration of the cell-free FIV-M2 challenge stock (pooled plasma from infected SPF cats) have been described previously (29). This stock contained $10^{3.0}$ 50% cat infectious doses (CID_{50}). Cell-associated virus consisted of pooled, Ficoll-separated PBMC collected directly, without culture, from SPF cats experimentally infected with ex vivo FIV-M2 or FIV-Pet 2.5 to 3.5 years previously. In the weeks preceding the harvest of challenge PBMC, donor cats had been repeatedly monitored for infectious cells present in the circulation by limiting-dilution reisolation in MBM cells. As preliminary studies had indicated good agreement between the results of titrations in vitro and in intravenously (i.v.) injected cats (data not shown), this monitoring was considered a good guide in the selection of the numbers of PBMC to use for challenge. All challenges were administered i.v. in 1 ml of pyrogen-free saline.

Criteria for protection from virus challenge. Challenged animals were monitored virologically and serologically at selected time points starting 1 month postchallenge (p.c.), except in experiment 3, in which monitoring was initiated at 3 months p.c. Duration of follow-up varied between 3 and 22 months in different experiments, depending on the goals and outcome. Criteria used to define complete protection were absence of virus reisolation from PBMC, inability to detect

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FIV proviral sequences in PBMC, and absence of an anamnestic antibody response. Numbers of infectious PBMC, proviral loads in PBMC, and levels of circulating CD4⁺ T lymphocytes were used instead to assess partial protection. Plasma viremia levels were also determined at selected times in one experiment.

FIV reisolation. Presence of infectious FIV in challenged cats was assessed by cocultivating 10⁶ PBMC with MBM cells and testing the cultures for reverse transcriptase (RT) twice per week. Infectious cell loads in the PBMC of the cats that were used as a source of cell-associated FIV challenge or that were monitored following challenge were determined by limiting-dilution reisolation. Cultures regarded as negative showed no evidence of RT in any sample collected during the 5-week culture period (15).

FIV provirus detection and quantitation. Diagnostic nested PCR using *gag* and *env* was performed on PBMC DNA as previously described (29). Sensitivity was 10 copies of the p34TF10 plasmid containing the whole FIV-Pet genome (kindly provided by J. E. Elder, La Jolla, Calif.). DNAs from uninfected cats and reagent controls were run in parallel, and the positive control (DNA from FIV-Pet-infected FL4 cells or from FIV-M2-infected MBM cells) was included in the second step only. Samples positive for the *gag* gene were further examined by competitive PCR to quantitate the FIV genomes by using an internal standard derived from the *gag* gene (35). The results are expressed as the number of proviral copies in 1 µg of PBMC DNA.

Discrimination between FIV-M2 and FIV-Pet provirus in infected cats. Restriction fragment length polymorphism (RFLP) analysis exploiting restriction site differences in the *gag* p25 region between FIV-M2 and FIV-Pet isolates was applied to nested *gag* PCR products. Amplicons were digested with the enzymes *Hind*III and *Sac*II (New England Biolabs, Beverly, Mass.), selected because of the presence of unique restriction sites in the *gag* gene of FIV-Pet and FIV-M2, respectively (7). Briefly, 15-µl PCR samples were diluted to 50 µl in an appropriate restriction buffer and digested with the two enzymes at 37°C for 2 h. The samples were then run on a 2% agarose gel and stained with 0.5 µl of ethidium bromide per ml.

FIV whole-antibody ELISA. Enzyme-linked immunosorbent assay (ELISA) microwells were coated overnight with 100 µl of 2-µg/ml gradient-purified, disrupted, whole FIV-M2 grown in MBM cells or, as a control, uninfected MBM cell lysate. After a postcoating step with skim milk, serially diluted sera were added to the plates in duplicate. Bound immunoglobulin G (IgG) was revealed with a biotinylated mouse anti-cat IgG serum, followed by an antibiotin peroxidase conjugate. Absorbance was read at 450 nm. To minimize plate-to-plate variability, the results were normalized by including a positive control serum with a known titer in each plate and correcting the titer of each sample based on the titer of that serum. The titers reported in the figures are the reciprocal of the highest dilution of serum that gave optical density readings higher than the average values obtained with 20 control FIV-negative serum samples plus three times the standard deviation. Sera that proved unreactive at a 1/100 dilution, the lowest dilution tested, are indicated as having titers of <100.

Lymphocyte proliferation assay. Ficoll-separated PBMC (1.5×10^5) obtained at the time of challenge were incubated for 4 days with 1 µg of purified and sonicated FIV-M2 grown in MBM cells or with 1 µg of mock antigen (MBM cell lysate obtained by sonication) in 200 µl of RPMI 1640 containing 10% heat-inactivated, AB-positive human serum and 2 mM L-glutamine and then pulsed with [³H]thymidine for 18 h. The stimulation index (SI) was calculated as the ratio of radioactivity incorporated by PBMC in the presence of FIV antigen to that in the presence of mock antigen. Only SIs of ≥ 2 were considered indicative of FIV-specific lymphoproliferation.

Lymphocyte subset composition analysis. The absolute counts of CD4⁺ and CD8⁺ T lymphocytes were obtained by flow cytometry as previously described (29). CD8⁺ T-lymphocyte counts are not reported because they do not provide important information for this study.

RESULTS

Experiment 1: cats challenged with cell-free virus at 4 months p.v. were still infection free over 2 years p.c. We previously reported that all six SPF cats vaccinated with the vaccine used in the present study and challenged with 10 CID₅₀ of cell-free ex vivo FIV were infection free 18 months p.c., while control animals—either unvaccinated or mock vaccinated with uninfected substrate MBM cells—had all become infected (29). Since we planned to use the six protected cats for further studies, their follow-up was prolonged. Vaccinated animals remained infectious virus and provirus negative, whereas challenged mock-vaccinated and unvaccinated control cats were constantly virus positive (data not shown). In addition, in the vaccinees, the ELISA antibodies to whole FIV induced by vaccination continued to decline progressively, albeit slowly (Fig. 1A), and lymphocyte subset counts remained within the normal range, while challenged control cats demonstrated a

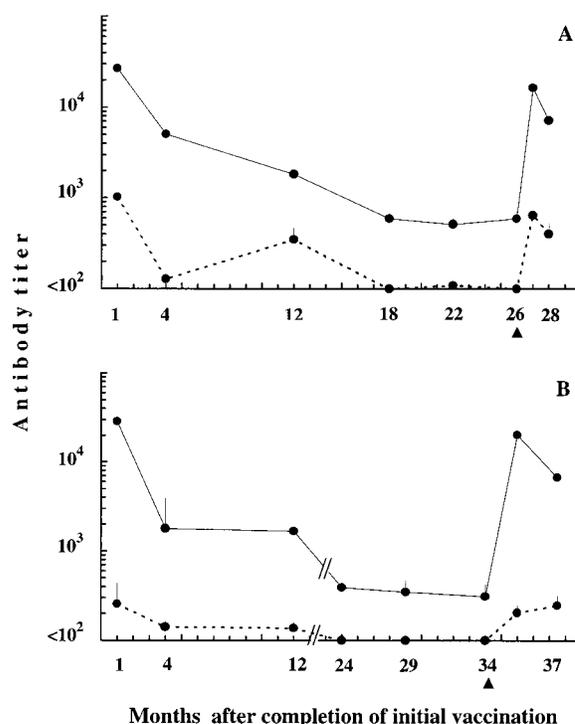


FIG. 1. ELISA antibody titers to whole FIV and control cell antigen in two groups of vaccinated cats at different time points. Titers of IgG antibodies to disrupted purified FIV-M2 grown in MBM cells (continuous line) and against MBM cell lysate control antigen (broken line) are shown. (A) Six vaccinated animals were challenged with cell-free FIV-M2 at 4 months p.v. and, since they remained infection-free, given a booster dose of vaccine at 26 months p.v. (arrowhead). (B) Nine vaccinated animals were challenged with cell-free or cell-associated FIV-M2 at 12 months p.v.; the three animals that became infected were removed from subsequent time points; the remaining animals received a booster dose of vaccine 34 months p.v. Symbols represent 95% geometric means, and bars represent confidence limits. For symbols without bars, the limits lie within the symbols. Only key time points are shown, but the animals were monitored many times over the observation period and the results were in complete agreement with those shown.

sustained antibody response to FIV and a progressive, marked reduction of circulating CD4⁺ T lymphocytes (data not shown). As reported below (experiment 3), at 26 months p.c., the animals in this group received a booster dose of vaccine in Freund's incomplete adjuvant that markedly stimulated the immune system and, nonetheless, they continued to show no evidence of infection by the multiple parameters used. Previous studies have demonstrated that immune stimulation of lentivirus-infected hosts can induce a marked increase of viral expression (32, 41, 43). It was therefore concluded that vaccination had resulted in effective, long-lasting control of the challenge virus and not simply in delayed development of infection.

Experiment 2: cats challenged 1 year p.v. are protected against cell-associated but not against cell-free virus. Nine SPF cats were immunized with the same vaccine lot and schedule as in experiment 1, left untreated for 1 year, and then challenged. Periodic monitoring of antiviral antibody showed an FIV-specific immune response that closely resembled that observed in experiment 1 in both kinetics and strength (Fig. 1B). At the time of challenge, antibody titers had declined significantly relative to those at 1 month p.v., when the response was at its peak level, but were nearly unchanged relative to those at 4 months p.v. (that is, the time when vaccinated cats in experiment 1 had proved 100% protected). Moreover, the

TABLE 1. Proliferative responses of PBMC from vaccinated cats to whole FIV and control MBM cell lysate antigens at the time of challenge

Expt	Lymphoproliferative response ^a		
	Vaccinated cats		Unvaccinated cats (FIV antigen)
	FIV antigen	MBM antigen	
1	4/6 (3–26)	0/5	0/12
2	3/6 (2.2–8)	0/6	ND ^b
3	4/6 (3–23)	0/6	0/4
4	5/6 (4–101)	1/6 (2)	1/8 (3)

^a Number of animals with an SI of ≥ 2 /number of animals examined (in parentheses, the range of SIs observed).

^b ND, not done.

proportion of cats that reacted against whole virus in lymphoproliferation was also similar to that at the time of challenge in experiment 1 (Table 1).

Two types of challenge were used. Three animals were inoculated with 10 CID_{50} of cell-free FIV-M2 (the same plasma stock and dose as in experiment 1), and the others were inoculated with pooled, viable PBMC freshly harvested from three FIV-M2-infected cats at a dose of 10^4 or 10^3 (three cats per group). As determined by quantitative isolation at the time of harvest, the latter inocula contained 10 and 1 FIV-infected cells, respectively. Groups of age-matched naive cats received the same challenges and served as unvaccinated controls.

The outcomes of challenges were determined over 22 months of follow-up. Because, as judged by all of the criteria used, none of the vaccinated and control animals inoculated with 10^3 PBMC became infected, only the results of challenges with cell-free virus and with 10^4 PBMC are reported. With both of these challenges, two of three unvaccinated control animals were already reisolation positive at 2 months p.c. and proved to be infected at every time tested thereafter with similar provirus and infectious virus loads, while one animal in either group was occasionally PCR positive during the early months p.c. but was not productively infected (Table 2). This indicated that the infecting strengths of the two challenges were similar and were not higher than that of experiment 1, in which four of six unvaccinated animals were reisolation positive since the first month p.c. (29).

The vaccinated groups behaved differently, depending on the type of challenge. The three vaccinees given cell-free virus were all reisolation positive at 1 month p.c. and remained so throughout the observation period (Table 2). Their ELISA anti-FIV antibody titers also rose severalfold above preexisting levels, indicating an ongoing active infection (Fig. 2A). In addition, their infectious virus loads, determined as the time needed for the virus reisolation cultures to become positive and as the proportions of infected PBMC at 4 and 22 months p.c., appeared to be in the same range as those of the controls (Table 2). Only the proviral loads in the PBMC during the early stages p.c. were somewhat lower in the vaccinees than in the naive animals. In any case, circulating CD4^+ T-lymphocyte numbers declined at similar rates in the vaccinees and the controls (Fig. 3A). Thus, the vaccinees challenged with cell-free FIV exhibited an infection course that was, in general, similar to that of unvaccinated control animals. In marked contrast, the three vaccinees challenged with cell-associated virus showed no evidence of FIV infection; with the exception of one animal that was found to be PCR positive with a low proviral load 1 month p.c., they remained virus isolation negative and PCR negative (Table 2) and showed no anamnestic

antibody response to FIV throughout the 22 months of observation (Fig. 2B).

From these results, it was concluded that vaccine protection against cell-free FIV, that had proved highly efficacious at 4 months p.v., had almost, if not entirely vanished by 1 year p.v., that the vaccine used was effective also against cell-associated virus, and that protection against the latter type of challenge was actually longer lasting than protection against cell-free virus.

Experiment 3: vaccine protection against cell-free virus is not easily boosted. Experiment 2 had shown that vaccination-induced protection against cell-free FIV was absent or negligible by 1 year p.v. It was therefore of importance to ascertain whether protective immunity in vaccinees could be recalled. Thus, the six vaccinated animals of experiment 1 (protected against challenge with cell-free virus at 4 months p.v.) were given one further dose of vaccine. All animals responded with a robust antiviral antibody response to whole FIV antigen, which was indicative of an anamnestic response (Fig. 1A). At the time of challenge, levels of antibodies to two synthetic peptides representing the V3 region of the surface glycoprotein and the immunodominant region of the transmembrane glycoprotein of FIV were higher than those detected at 4 months p.v. while neutralizing antibodies remained sporadic when tested in fibroblastoid CrFK cells and undetectable when tested in lymphoid cells with no evidence of enhancement (data not shown). The lymphoproliferation assay results were positive for four animals (Table 1). As already mentioned, after boosting, the animals remained free of FIV infection, as determined by PBMC culture and diagnostic PCR (Table 3).

Because we expected the boosted cats to be protected, we used a cumulative challenge. Thus, 2 months after the booster, the six vaccinated animals and four age-matched, unvaccinated controls were given 10 CID_{50} of FIV-M2 plasma, followed by 30 CID_{50} after an additional 3 months. For the same reason, the animals were not monitored for FIV infection until the time of the 30- CID_{50} challenge. Contrary to expectations, at this time, three of six vaccinees were already reisolation positive, versus one of four in the control group, and 1 month after the 30- CID_{50} challenge, five of six animals in the vaccinated group and four of four in the control group were reisolation positive. This situation remained unchanged at 12 months p.c., when the experiment was terminated. The lack of significant residual protection in the five infected vaccinees was confirmed by the other parameters considered: diagnostic and quantitative PCR and quantitative reisolation (Table 3). Antibodies to FIV were also augmented in these animals p.c., although the increase was moderate due to preexisting high levels of antibody (data not shown). Circulating CD4^+ T lymphocytes also declined in these animals following challenge (Fig. 3B). Also, at 12 months p.c., plasma viremia levels, determined by competitive RT-PCR, ranged between 15,000 and 94,000 RNA equivalents per ml with no differences between vaccinated and unvaccinated animals (data not shown). The one cat that proved to be protected despite the drastic challenge protocol used (cat 733) was still infection free more than 18 months p.c.

These results suggest that, once it has waned, vaccine-induced protection against cell-free FIV is not easily restored, at least not by using a single-dose booster administered over 2 years p.v. Because the boosted cats exhibited levels of anti-cell antibodies higher than those detected at 4 months p.v., when the cats had effectively resisted challenge (Fig. 1A), indicating that the booster had elicited an anamnestic immune response also to the substrate cells used for vaccine preparation, the results also confirmed the conclusion of our previous study

TABLE 2. Detection of FIV infectivity and amplifiable genomes in vaccinated and unvaccinated control cats after challenge with cell-free or cell-associated FIV performed 12 months p.v.

Cat category and no.	Presence of FIV postchallenge ^a																			
	1 mo		2 mo		4 mo		7 mo		9 mo		12 mo		16 mo		22 mo					
	Culture	PCR	Culture	PCR	Infectious virus load ^b	Culture	PCR	Culture	PCR	Culture	PCR	Culture	PCR	Culture	PCR	Infectious virus load ^b	PCR			
Cell-free challenge																				
Unvaccinated																				
748	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
3543	+	(1,200)	+	(3,900)	+	(7)	+	(2,600)	ND ^c	+	(4,300)	+	(10)	+	(2,200)	+	(8)	+	(13)	
3592	+	(900)	+	(2,100)	+	(11)	+	(1,600)	ND	+	(1,400)	+	(10)	+	(1,300)	+	(8)	+	(16)	
759	+	(14)	+	(680)	+	(7)	+	(770)	ND	+	(900)	+	(10)	+	(1,500)	+	(8)	+	(16)	
3558	+	(11)	+	(320)	+	(7)	+	(420)	ND	+	(800)	+	(10)	+	(1,500)	+	(8)	+	(16)	
3587	+	(11)	+	(580)	+	(11)	+	(780)	ND	+	(5,200)	+	(10)	+	(6,800)	+	(8)	+	(16)	
Cell-associated challenge																				
Unvaccinated																				
3549	-	-	-	<100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
3579	+	(2,100)	+	(1,800)	+	(11)	+	(3,200)	+	(14)	+	(2,200)	+	(10)	+	(2,800)	+	(12)	+	(13)
3593	-	-	+	(24)	+	(14)	+	(5,700)	+	(17)	+	(6,600)	+	(48)	+	(8,500)	+	(20)	+	(20)
Vaccinated																				
727	-	<100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
824	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
3607	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

^a PBMC obtained at the indicated times were examined for FIV infectivity by culture on MBM cells and proviral FIV gag sequences (gag and env sequences at the last two time points) by nested PCR. +, virus was isolated or PCR was positive; -, no virus was isolated from culture after 5 weeks or PCR was negative. Numbers in parenthesis indicate days of incubation at the time cultures first became positive or the proviral load present in 1 µg of PBMC DNA as assessed by competitive PCR.

^b At this time, PBMC were also examined for infectious virus load by quantitative isolation. Results are expressed as numbers of infected cells per 10⁶ PBMC.

^c ND, not done.

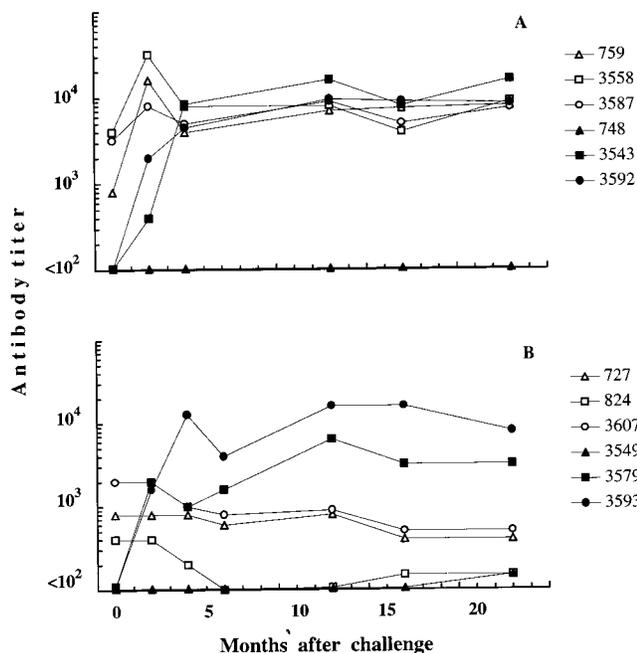


FIG. 2. ELISA of antibody to whole FIV antigen in individual vaccinated (open symbols) and unvaccinated control (solid symbols) cats after cell-free (A) or cell-associated (B) virus challenge performed 1 year p.v. Sera collected at the indicated times p.c. were tested for IgG antibody to disrupted, purified FIV-M2.

(29) that vaccine protection was not mediated by immunity to cellular antigens.

Experiment 4: vaccine protection against cell-associated virus also wanes with time and is not easily boosted. Experiment 2 had shown that vaccine-induced protection against cell-associated FIV is longer lasting than that against cell-free FIV. To further investigate the duration of protection against cell-associated virus, 34 months after primary vaccination, the six animals used in experiment 2 that had not become infected following challenge with cell-associated FIV were given one booster vaccine dose and rechallenged with cell-associated virus 3.5 months later. The booster elicited a prompt immune response, so that by the time of rechallenge, the cats showed high titers of antiviral antibody (Fig. 1B) and five of six cats were reactive in the lymphoproliferation assay (Table 1).

Because we also wished to investigate the breadth of immunity to cell-associated FIV conferred by the vaccine, three vaccinated cats were challenged with FIV-M2-infected PBMC and three were challenged with FIV-Pet-infected PBMC. These FIV strains are classified into two different clades and are 22% heterologous in regions V3 through V5 of the Env protein (34) but share a surface neutralization epitope(s), as shown by cross-neutralization assays with CrFK cells (44). However, this attempt was completely frustrated because all of the vaccinated cats became infected as readily as the controls, regardless of the viral strain used for challenge (Table 4), thus showing that at 37.5 months p.v., they were no longer protected against cell-associated virus challenge.

Since the vaccinated animals used for this experiment had previously resisted challenge with FIV-M2-infected cells, we also examined the possibility that they harbored FIV-M2 in a dormant state and that this viral strain was reactivated following challenge with FIV-Pet. Thus, the FIV strain present was characterized in the animals at the end of the experiment by using an RFLP method that has recently been shown to dis-

criminate FIV-M2 and FIV-Pet in dually infected cats and to detect as little as 1% of either strain present within the total proviral burden (7). As shown in Fig. 4, the cats challenged with FIV-Pet cells were found to harbor only the homologous provirus, thus excluding the possibility that the FIV-Pet challenge had reactivated a latent FIV-M2 infection.

DISCUSSION

Ideally, anti-FIV vaccines to be used in the field should protect against the broad range of virulent viral strains that circulate in nature and confer long-lasting or rapidly recallable protection after a few doses. Furthermore, since FIV transmission among domestic cats appears to be through biting (47) and possibly through sexual activities and the saliva and semen of infected cats may harbor virus-infected cells, as well as cell-free virus (3, 24), it is generally believed that an effective vaccine will have to protect against both cell-free FIV and viable infected cells. The experiments described here have addressed some of these issues.

Because of the discouraging (25, 27, 38, 40, 45) or modest (18) results obtained with simple immunogens, we used a fixed, infected-cell vaccine which in a previous study had effectively protected against fully virulent cell-free FIV (29). The vaccine was prepared with a low in vitro passage virus presumably representative of primary FIV isolates, whereas most similar experiments performed earlier with variable success used tissue culture-adapted virus. This might be an important difference because viral antigens need to be presented in a form that most closely resembles the native conformation in order to stimulate immune responses that will recognize the infectious virus, and it has been argued that the use of tissue culture-adapted virus in vaccine preparation might not lead to presentation of the epitopes most relevant for protection and/or may favor epitopes that divert the focus of the immune response

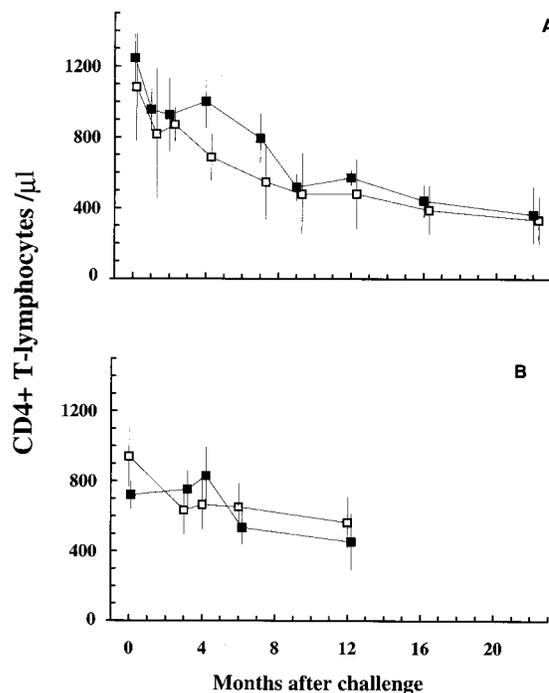


FIG. 3. Mean numbers of circulating CD4⁺ T lymphocytes in vaccinated (□) and unvaccinated (■) cats that became infected following cell-free challenge performed 12 (A) or 28 (B) months p.v. Bars represent standard errors.

TABLE 3. Detection of FIV infectivity and amplifiable genomes in vaccinated-boosted cats and controls after cell-free FIV challenge performed 28 months p.v.

Cat category and no.	Presence of FIV postchallenge ^a																	
	0 mo		3 mo		4 mo		6 mo			12 mo								
	Culture	PCR	Culture	PCR	Culture	PCR	Culture	Infectious virus load ^b	PCR	Culture	Infectious virus load ^b	PCR						
Unvaccinated																		
568	-	-/-	-	-/-	+	(10)	+/+	+	(16)	3 × 10 ²	+/+	(425)	+	(7)	10 ⁴	+/+	(2,220)	
3291	-	-/-	-	-/-	+	(10)	-/-	+	(28)	<2	+/+	(384)	+	(35)	10	+/+	(510)	
3363	-	-/-	+	(14)	+/+	+	(7)	+/+	+	(13)	2 × 10 ²	+/+	(1,769)	+	(7)	10 ⁴	+/+	(392)
3710	-	-/-	-	-/-	+	(7)	-/-	+	(28)	2	+/+	(390)	+	(28)	10 ²	+/+	(310)	
Vaccinated																		
733	-	-/-	-	-/-	-	-/-	-	-	-/-	-	<1	-	-	-	<1	-/-	-	
737	-	-/-	+	(14)	+/+	+	(10)	+/+	+	(20)	<1	+/+	(350)	+	(14)	10	+/+	(510)
806	-	-/-	+	(17)	+/+	+	(14)	+/+	+	(28)	<1	+/+	(330)	+	(14)	10	+/+	(330)
3532	-	-/-	-	-/-	+	(7)	+/+	+	(13)	8 × 10 ²	+/+	(1,724)	+	(7)	10 ²	+/+	(950)	
3535	-	-/-	+	(17)	+/+	+	(7)	+/+	+	(20)	3 × 10 ²	+/+	(2,040)	+	(7)	10 ³	+/+	(7,407)
3585	-	-/-	-	-/-	+	(14)	+/+	+	(20)	3 × 10	+/+	(869)	+	(14)	10 ³	+/+	(3,330)	

^a PBMC obtained at the indicated times were examined for FIV infectivity by culture on MBM cells and proviral FIV *gag* and *env* sequences by nested PCR. +, virus was isolated or PCR was positive; -, no virus was isolated from culture after 5 weeks or PCR was negative. Numbers in parentheses indicate days of incubation at the time when cultures first became positive or the proviral load present in 1 µg of PBMC DNA as assessed by competitive PCR.

^b At this time, PBMC were also examined for infectious virus load by quantitative isolation. Results are expressed as numbers of infected cells per 10⁶ PBMC.

from critical protective epitopes or even induce enhancing antibodies (36). In addition, in our study, all of the challenges were performed with virus obtained ex vivo. We believe this is a more appropriate challenge than virus grown in tissue culture

when testing the efficacy of prototype vaccines meant for field use. This is because tissue culture strains of FIV, as well as HIV type 1 and SIV, have been shown to present an abnormally elevated sensitivity to neutralizing antibody (2, 8), and it

TABLE 4. Detection of FIV infectivity and amplifiable genomes in vaccinated and unvaccinated control cats after challenge with cell-associated FIV-M2 and FIV-Pet performed at 37.5 months p.v.

Type of challenge, vaccination status, and cat no.	Presence of FIV postchallenge ^a									
	0 mo		1 mo		3 mo					
	Culture	PCR	Culture	PCR	Culture	Infectious virus load ^b	PCR			
FIV-M2										
Unvaccinated										
100	-	-/-	+	(11)	+/+	+	(8)	5 × 10 ³	+/+	(2,200)
540	-	-/-	+	(11)	+/+	+	(8)	5 × 10 ³	+/+	(1,070)
748	-	-/-	+	(7)	+/+	+	(11)	5 × 10 ³	+/+	(3,120)
3690	-	-/-	+	(7)	+/+	+	(8)	5 × 10 ³	+/+	(1,920)
Vaccinated										
727	-	-/-	+	(7)	ND ^c /+	+	(11)	5 × 10 ³	+/+	(1,980)
756	-	-/-	+	(14)	+/+	+	(8)	5 × 10 ³	+/+	(4,300)
3607	-	-/-	+	(11)	+/+	+	(8)	5 × 10 ²	+/+	(1,750)
FIV-Pet										
Unvaccinated										
60	-	-/-	+	(11)	+/+	+	(11)	5 × 10 ²	+/+	(950)
1329	-	-/-	+	(7)	+/+	+	(14)	5 × 10 ³	+/+	(1,100)
3549	-	-/-	+	(11)	ND/+	+	(29)	5 × 10	+/+	(630)
3711	-	-/-	+	(14)	+/+	+	(11)	5 × 10 ³	+/+	(1,830)
Vaccinated										
824	-	-/-	+	(7)	+/+	+	(29)	5 × 10	+/+	(730)
3530	-	-/-	+	(11)	ND/+	+	(11)	5 × 10 ²	+/+	(1,250)
3531	-	-/-	+	(18)	ND/+	+	(18)	5 × 10 ²	+/+	(1,050)

^a PBMC obtained at the indicated times were examined for FIV infectivity by culture on MBM cells and proviral FIV *gag* and *env* sequences by nested PCR. +, virus was isolated or PCR was positive; -, no virus was isolated from culture after 5 weeks or PCR was negative. Numbers in parentheses indicate days of incubation at the time when cultures first became positive or the proviral load present in 1 µg of PBMC DNA as assessed by competitive PCR.

^b At this time, PBMC were also examined for infectious virus load by quantitative isolation. Results are expressed as numbers of infected cells per 10⁶ PBMC.

^c ND, not done.

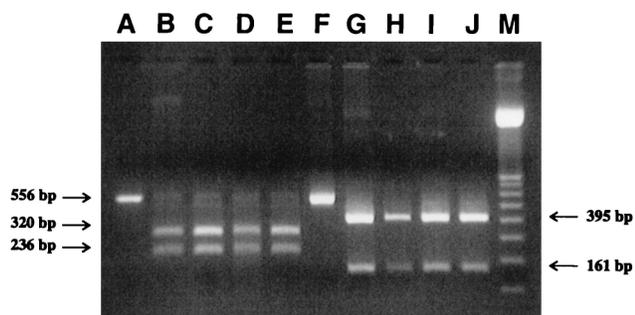


FIG. 4. RFLP of *gag* PCR products obtained from vaccinated cats following cell-associated challenge with two different FIV isolates (experiment 4). Lanes: A and B, undigested and *SacII*-digested amplicons from a control unvaccinated cat challenged with FIV-M2-infected PBMC, respectively; C to E, *SacII*-digested amplicons from three vaccinated cats challenged with FIV-M2-infected PBMC; F and G, undigested and *HindIII*-digested amplicons from a control unvaccinated cat challenged with FIV-Pet-infected PBMC, respectively; H to J, *HindIII*-digested amplicons from three vaccinated cats challenged with FIV-Pet-infected PBMC; M, 100-bp DNA molecular size marker.

is plausible that they are also abnormally susceptible to other immune effectors.

The outcomes of our experiments are summarized in Table 5. Despite the intensive immunization schedule adopted (five vaccine doses administered in Freund's incomplete adjuvant over a period of 21 weeks), vaccine protection against cell-free virus proved short-lived. While cats challenged *i.v.* with 10 CID_{50} of cell-free FIV at 4 months *p.v.* were still infection free over 2 years *p.c.*, cats that received the same challenge 1 year *p.v.* were as susceptible as unvaccinated cats. Thus, although further studies are needed to establish the precise duration of vaccine protection against cell-free challenge, we can conclude that it is measurable in terms of less than 1 year. It has recently been reported that cats immunized with a whole-virus vaccine prepared with high-passage FIV-Pet were still partly protected when challenged with cell-free virus 8 months *p.v.* (19). However, the different strain and passage history of the FIV used for challenge in this study do not permit extrapolation of that finding to our experimental situation.

Our study is the first to demonstrate that vaccines can protect against cell-associated, as well as against cell-free, FIV. Protection against cell-associated FIV was actually more persistent than protection against cell-free virus, since it was observed 1 year *p.v.* This was somewhat surprising because acquired immune resistance to cell-associated virus was deemed difficult or unattainable (39), but not unprecedented, as a similar observation has been made in SIV-vaccinated macaques (12) and in another report vaccinated chimpanzees were protected against HIV type 1-infected PBMC for at least 1 year *p.v.* (14). Whether the longer duration of protection against cell-associated virus is due to the fact that in this form FIV is more easily blocked than in the extracellular form or to the fact that the mechanism(s) responsible for controlling the two types of challenge is distinct or targeted via different viral antigens will have to await clarification of the immune effectors involved. In any case, protection against cell-associated virus also eventually waned, since cats at 3 years *p.v.* (and previously challenged at 1 year *p.v.* without infection) were fully susceptible to this challenge, despite the administration of a booster vaccine dose 3.5 months earlier.

Previous studies have shown that certain lentivirus-vaccinated hosts, although not fully protected, may nevertheless experience attenuated infections following challenge, as shown by reduced viral loads or delayed disease progression (1, 17, 18,

22, 26, 31, 46). In our study, there was little evidence that the initial *p.v.* phase of solid resistance was followed by an intermediate period of reduced susceptibility to challenge. Although the long incubation time prior to onset of feline AIDS, common to all but one FIV isolate (10), did not allow us to address the ultimate clinical outcome, unprotected vaccinated cats exhibited $CD4^+$ T-lymphocyte reductions during the observation periods similar to those seen in unvaccinated challenged cats, thus suggesting that, at least initially, virus-associated pathology progressed at unchanged rates in vaccinees. This might reflect the fact that our challenges consisted of *ex vivo* virus, which might be more difficult to contain once infection has been established.

The relatively short persistence of protection observed in these experiments indicates that the protective immunity conferred by the FIV vaccine tested here would need frequent restimulation to consistently prevent FIV infection in the field. In this perspective, the finding that, once it had waned, protection was not restored by administering a booster dose of vaccine prior to challenge is disturbing. It is possible that the protected state would have been maintained more effectively if boosters had been given at regular, closer intervals than in the present study. Although it is a considerable drawback, frequent boosting is a common practice for many inactivated-virus vaccines. The possibility that long-term protection may not be achieved, however, remains a concern.

The immune mechanisms responsible for vaccine protection against lentiviruses remain uncharacterized, and *in vitro* assays predictive of protection are lacking (6, 16, 30). One advantageous aspect of the present study is that animals immunized with the same vaccine were either totally protected or unprotected, depending on the time interval between vaccination and challenge, thus lending themselves to investigations on the characteristics of the host's immune response that are necessary for protection. In the present experiments, anti-FIV ELISA and neutralizing antibody titers and lymphoproliferative reactivity measured at challenge did not correlate with protection. This does not, however, mean that antibodies and cell-mediated immunity have nothing to do with protection, since it is likely that fine specificity, functions, and other variables of immune effectors evolve with time *p.v.* and that such evolution does not necessarily reflect itself in the above parameters. A fine dissection of the specificity and effector functions of sera obtained from protected and unprotected cats in the present study will be the subject of a separate report.

It is also likely that several immune effector functions worked synergistically to provide vaccine protection (4, 23, 28, 37). In a recent study, protective immunity observed in inactivated whole-virus-vaccinated cats was seen to be associated

TABLE 5. Summary of outcomes of challenges performed at different times *p.v.*

Type of challenge ^a	Result at <i>p.v.</i> time of:			
	4 mo	12 mo	28 mo ^b	37.5 mo ^b
Cell-free virus (15/16)	0/6 ^c	3/3	5/6	ND ^d
Cell-associated virus (10/11)	ND	0/3	ND	6/6 ^e

^a In parentheses, the efficiency of challenge in unvaccinated and mock-vaccinated cats is expressed as number of animals infected/number of animals challenged.

^b The animals were given a booster dose of vaccine 2 or 3.5 months before challenge.

^c Number of animals infected/number of animals challenged.

^d ND, not done.

^e Three animals were challenged with heterologous virus.

with high levels of Env-specific cytotoxic T-lymphocyte (CTL) activity (13). We have not measured CTL activity in the vaccinated animals; however, it seems unlikely that classical major histocompatibility complex-restricted CTL activity effected major resistance to the cell-associated challenge by eliminating inoculated infected cells before significant amounts of virus were released because the vaccinees and the donors of the PBMC used for challenge were not siblings and therefore the possibility that they shared identical major histocompatibility haplotypes is low. One possibility that appears to be excluded by the present findings is that the vaccine protection we have observed was dependent on immune effectors directed against cellular components similar to those that have been found to mediate protection against SIV in macaques immunized with xenogenic or allogenic cells (9, 42). First, our anti-FIV vaccine was produced in feline cells and the virus used for challenge was pooled plasma or PBMC from several infected cats. Second, immunization with a mock vaccine consisting of uninfected substrate cells treated exactly as for vaccine preparation failed to protect even marginally (29). Third, and possibly more important, the cats that were given a booster dose of vaccine in an attempt to re-establish protective immunity developed high levels of anti-cell activity as evidenced by ELISA-reactive antibody and nevertheless proved as susceptible to infection as naive controls.

In conclusion, this study confirms that a vaccine targeted to viral antigens and capable of protecting against fully virulent ex vivo FIV is feasible but also shows that the protected state may be difficult to maintain. Further studies should aim at the identification of means of extending and boosting protection, as well as of more refined immunogens than the one used in the present study. Understanding the immune mechanisms that mediate protection would obviously be of great help in these efforts. Solving such problems for FIV may provide important foundations for the design of AIDS vaccines for humans.

ACKNOWLEDGMENTS

We thank Giuliana Castellani for lovingly taking care of the animals and Marco Franchi for valuable technical help.

This work was supported by grants from the Ministero della Sanità-Istituto Superiore di Sanità, "Progetto Allestimento Modelli Animali per l'AIDS," and the Ministero della Università e Ricerca Tecnologica, Rome, Italy.

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