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Feline Immunodeficiency Virus Vaccination: Characterization of the Immune Correlates of Protection

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Whole inactivated virus (WIV) vaccines derived from the FL4 cell line protect cats against challenge with feline immunodeficiency virus (FIV). To investigate the correlates of protective immunity induced by WIV, we established an immunization regimen which protected a proportion of the vaccinates against challenge. A strong correlation was observed between high virus neutralizing antibody titers and protection following challenge. To investigate further the immune mechanisms responsible for immunity, all of the vaccinates were rechallenged 35 weeks following the initial challenge. Results of virus isolation from peripheral blood mononuclear cells indicated that 9 of 10 vaccinates were protected from viremia following the second challenge, suggesting that vaccine-induced immunity to FIV persisted for at least 8 months. However, more stringent analysis for evidence of infection revealed that 5 of 10 vaccinates harbored virus in lymphoid tissues. Unlike the protection observed immediately following vaccination, which correlated positively with virus neutralizing antibody titer, the ability to resist a second challenge with FIV was more closely correlated with the induction of Env-specific cytotoxic T-cell activity. The results indicate that both virus-specific humoral immunity and cellular immunity play a role in the protection induced in cats by WIV immunization but their relative importance may be dependent on the interval between vaccination and exposure to virus.

Vaccination has been the most effective intervention for the prevention of disease spread, in particular the spread of viral diseases. Since it is clearly not possible to challenge volunteers immunized with candidate vaccines with virulent human immunodeficiency virus type 1 (HIV-1), animal models have a crucial role to play in the development of a vaccine against HIV-1 and in identifying immune correlates of protection. Models for HIV-1 have been based on a number of systems, including chimpanzees infected with HIV-1, macaque monkeys infected with either simian immunodeficiency virus or HIV-2, and cats infected with feline immunodeficiency virus (FIV).

The design of effective HIV vaccines would benefit from an understanding of the cellular and humoral immunological mechanisms involved in the protection or control of disease progression. Infection with HIV-1 is associated with the development of both virus neutralizing antibodies (VNA) and virus-specific cell-mediated immune responses (31–33, 40), which may represent important components in the initial control of HIV-1 replication. However, the relative roles played by humoral and cell-mediated immune effector mechanisms in the maintenance of the symptom-free state are not clearly understood. Following initial infection, the fall in viremia correlates most closely with the appearance of HIV-1-specific major histocompatibility complex class I-restricted $CD8^+$ cytotoxic T lymphocytes (CTL) in the peripheral blood (4, 23, 35). The recent findings of HIV-1-specific CTL responses in seronegative, exposed individuals, including sex workers (39) and children born to HIV-1-infected mothers (5, 7, 30, 38) who have failed to become infected themselves, provide evidence suggesting that the CTL response may be responsible for the clearance of the virus and the observed protection.

In the macaque model systems, protection can be induced by immunization with whole inactivated virus (WIV) (6, 11, 29). However, the protection observed in macaques immunized with simian immunodeficiency virus grown in human cells appears to correlate more closely with an immune response directed to cell membrane antigens rather than a virus-specific immune response (44). While recent studies in macaques have demonstrated virus-specific correlates of immunity (8, 48), it has been possible also to protect macaques from challenge with simian immunodeficiency virus by immunization with murine cells expressing human major histocompatibility complex class II molecules (1). However, in the FIV system, the protection observed following immunization with WIV vaccines appears to correlate only with virus-specific parameters (19, 50), suggesting that this is an appropriate model in which to investigate further the correlates of protection.

Protection against FIV infection has been achieved by immunizing cats with a WIV vaccine produced from the FL4 cell line persistently infected with the Petaluma isolate of FIV (FIV/PET) (19, 50, 51). The observed protection appears to be associated with a high threshold of VNA, and indeed passive antibody transfer can confer protection (18). FIV Gag- and Env-specific CTL responses are also induced by WIV immunization (13), suggesting that cell-mediated immunity may also be involved in viral clearance. However, the protective immune responses induced by such WIV vaccines appear to be isolate specific, and although there has been one report of protection extending to a closely related isolate (FIV/Dixon) (50), it does not extend to the antigenically distinct isolates FIV/GL-8 (19) and FIV/Shizuoka (22).

The relative contributions of humoral immunity and cell-mediated immunity to the observed virus-specific protection afforded by WIV immunization are unknown. To investigate these issues, we established an immunization regimen that would elicit suboptimal protection, protecting only a proportion of the vaccinates from subsequent challenge, the remainder succumbing to FIV infection. Thirty-five weeks after the initial challenge, the vaccinated cats were rechallenged with a

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higher dose of virus to assess the duration of immunity conferred by vaccination. Throughout the study, VNA titers, antibodies recognizing immunodominant epitopes of Env proteins, antiviral core (p17 and p24) antibodies, and FIV Gag- and Env-specific CTL responses were monitored in vaccinated protected, vaccinated unprotected, and control cats in an attempt to elucidate the immune correlates of protection. Our findings indicate that both VNA and Env CTL responses appear to play important roles in protection.

MATERIALS AND METHODS

Immunization of cats. The WIV vaccine was prepared from the culture fluid of the FL4 feline lymphoblastoid cell line, which is persistently infected with FIV/PET (49). The vaccine was prepared in a way that had been shown previously to protect cats from challenge with homologous virus (19).

VNA. Plasma samples were tested for the presence of VNA by using a focus reduction assay in CrFK cells (9) which has been described previously (34).

ELISA. Titers of antibodies recognizing FIV p17 or FIV p24 were determined by enzyme-linked immunosorbent assay (ELISA). Microtiter plates (high binding; Greiner Laboritechnik, Dursley, Gloucestershire, United Kingdom) were coated overnight with either the synthetic peptide RAISSWKQRNRWEWRPD, representing an immunodominant linear neutralization site in the third variable (V3) region of FIV gp120 (10, 24), or with an immunodominant epitope in the transmembrane glycoprotein (TM) (2, 41) represented by the synthetic peptide CNQNQFFCK. Antibodies recognizing these FIV peptides were detected as described previously (14).

Isolation of FIV. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous peripheral blood by centrifugation over Ficoll-Hypaque (Pharmacia LKB Biotechnology Inc., Piscataway, N.J.). Then 10^6 PBMC were cocultivated with 10^6 MYA-1 cells, which are highly sensitive for FIV replication (28). The cultures were maintained in RPMI 1640 medium (Gibco Biocult, Paisley, United Kingdom) containing 10% fetal bovine serum (Biological Industries Ltd., Cumbernauld, United Kingdom), 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 100 IU of penicillin per ml, and 100 μg of streptomycin per ml (complete RPMI 1640 medium) supplemented with 5% culture supernatant from the Ltk^-IL-2.23 cell line producing human recombinant interleukin-2 (kind gift of T. Miyazawa, University of Tokyo, and M. Hattori, University of Kyoto). Samples of culture supernatant were tested at intervals for the presence of FIV p24 by ELISA (IDEXX Laboratories, Portland, Maine), and cultures were maintained for 21 days before being scored as negative.

Preparation of lymphocyte effector cells. Lymphocytes were prepared from the peripheral blood, spleens, and lymph nodes of vaccinated and control cats. Peripheral venous blood was collected into an equal volume of Alsever's solution (Scottish Antibody Production Unit, Carluke, United Kingdom), and PBMC were prepared by centrifugation over Ficoll-Hypaque. Vaccinated and control cats were euthanized at 16 weeks following the second challenge. At postmortem examination, blood was collected and prepared as described above. Spleen and lymph nodes (mesenteric and peripheral) were also collected, and a single-cell suspension of each of these tissues was prepared by gentle manual homogenization. Lymphocytes were isolated by centrifugation over Ficoll-Hypaque, washed twice, and either used immediately or stored in liquid nitrogen until used in the assays.

Detection of FIV Gag- and Env-specific CTL. Virus-specific CTL were assayed in the peripheral blood, spleens, and lymph nodes of vaccinated and control cats. Effector cells derived from fresh or in vitro-restimulated lymphocytes were assayed for CTL activity against autologous or allogeneic skin fibroblast target cells derived from biopsy material collected prior to vaccination (14). Unstimulated, fresh cells were thawed, washed, and cultured for 18 to 24 h in complete RPMI 1640 medium supplemented with 100 IU of human recombinant interleukin-2 (a kind gift from Cetus, Emeryville, Calif.) per ml before use. Effector cells with a viability of less than 99% (as assessed by trypan blue exclusion) were not used. Skin fibroblast target cells were labelled with 100 μCi of sodium ^{51}chromate (Amersham International plc., Amersham, United Kingdom) per/10^6 cells for 18 h at 37°C, washed three times, and then infected at 5 to 10 PFU per cell with recombinant vaccinia virus expressing either the *gag* (14) or *env* (43) gene product of FIV/GL-14 or FIV/PET, respectively, or with wild-type vaccinia virus for 1 h at 37°C. Unbound virus was washed away, and the cells were incubated for a further 2 h to allow optimal expression of the FIV Gag and Env products (3). Microcytotoxicity assays were performed as described previously (14). The results shown represent the mean lysis of triplicate cultures from which the allogeneic values have been subtracted. The allogeneic response did not exceed 5%.

Preparation of antigen-presenting stimulator cells. Antigen-presenting stimulator cells were generated by using a modification of a previously described technique (3, 26, 45). Briefly, autologous lymphocytes were stimulated in vitro by cultivation in complete RPMI 1640 medium supplemented with 7.5 μg of concanavalin A (Sigma, Poole, Dorset, United Kingdom) per ml for 48 h at 37°C. The lymphoblasts were then washed and infected at 5 to 10 PFU per cell with recombinant vaccinia virus expressing either FIV Gag or Env as described above. The cells were incubated for 3 h at 37°C to allow optimal expression of the FIV proteins and then fixed in 0.1% paraformaldehyde for 18 to 24 h at 4°C. The fixed, vaccinia virus-infected blasts were then washed three times with phosphate-buffered saline and either used immediately or stored at 4°C until used.

In vitro restimulation of effector cells. Lymph node lymphocytes were cocultivated with paraformaldehyde-fixed autologous antigen-presenting stimulator cells at a ratio of 20:1 (effectors to stimulators) in complete RPMI 1640 medium for 7 days at 37°C in a humidified atmosphere containing 5% CO_2. T-cell lymphoblasts present in the effector cell population were then expanded in complete RPMI 1640 medium supplemented with 10% culture supernatant from the Ltk^-IL-2.23 cell line for a further 7 to 10 days before assaying for FIV-specific lymphocytotoxicity.

TABLE 1. Antibody responses and outcome of challenge following two or three immunizations with WIV

Cat no.	No. of immunizations	Titer of Antipeptide antibody				VNA titer (log)	Infection of PBMC
		p17	p24	V3	TM		
1	3	125	125	10	10	4.12	−
2		625	125	10	30	3.90	−
3		125	125	20	10	4.02	−
4		125	625	10	30	3.87	−
5		625	125	20	40	4.20	−
6	2	125	125	10	10	3.42	−
7		5	5	10	10	3.23	+
8		125	125	20	20	3.37	+
9		125	25	60	10	3.26	−
10		625	5	20	80	3.58	−
11	0	0	0	0	0	0	+
12		0	0	0	0	0	+
13		0	0	0	0	0	+
14		0	0	0	0	0	+
15		0	0	0	0	0	+

RESULTS

Two immunizations with WIV vaccine protect only a proportion of cats against infection. In a preliminary experiment, two groups of five specific-pathogen-free domestic cats were immunized with a vaccine consisting of 250 μg of WIV in 250 μg of tMDP/SAF-M adjuvant (kindly provided by Chiron Corporation) per dose. The first group were immunized three times at intervals of 3 weeks, starting at 11 weeks of age. The second group were immunized twice, beginning at 14 weeks of age. The control group were left unimmunized. Three weeks after the final immunizations, when both groups of cats were 20 weeks old, the 10 vaccinates and 5 age-matched unvaccinated controls were challenged intraperitoneally (i.p.) with 10 50% cat infective doses (ID_{50}) of the homologous FIV/PET, grown in Q201 cells (46). Using virus-negative PBMC cultures as the criterion for protection, all of the cats which received three immunizations were protected, whereas virus could be isolated from two of five cats which received two immunizations (Table 1). The titers of antibodies recognizing FIV p17, p24, the V3 peptide, and the TM peptide were similar in the two groups of cats, but the VNA titers were significantly lower in the group which received only two immunizations (Student's t test; $P < 0.005$). These results revealed VNA as a correlate of protection.

Association between VNA and protection. We then immunized a larger group of cats with two doses of the WIV vaccine in order to produce protected and unprotected cats following challenge. The immune correlates of protection were then determined. Ten 11-week-old specific-pathogen-free cats were inoculated with the WIV vaccine at 0 and 3 weeks. Five age-matched control cats were inoculated with tMDP/SAF-M adjuvant alone. The cats were challenged i.p. 3 weeks after the second immunization with 10 ID_{50} of the homologous FIV/

TABLE 2. Virus isolations after first challenge

Cat no.	Virus isolation at indicated wk p.c.								
	3	6	9	12	15	19	21	24	26
A321	−	−	−	−	−	−	−	−	−
A322	−	−	−	−	−	−	−	−	−
A323	−	−	−	−	−	−	−	−	−
A324	−	−	−	−	−	−	−	−	−
A325	−	+	+	−	−	+	+	+	+
A326	−	+	−	−	−	+	−	−	−
A327	−	−	−	−	−	−	−	−	−
A328	−	−	−	−	−	−	−	−	−
A329	−	−	−	−	−	−	−	−	−
A330	−	−	−	−	−	−	−	−	−
A331	−	+	+	−	−	+	+	+	+
A332	−	−	+	+	+	+	+	+	+
A333	+	+	+	+	+	+	+	+	+
A334[a]	+	+	+						
A335	−	+	+	+	−	+	+	+	+

[a] Control cat humanely destroyed following an intussusception.

PET, grown in Q201 cells (46). On the day of challenge and at intervals of 3 weeks thereafter, the cats were tested for evidence of FIV infection by virus isolation. As shown in Table 2, all of the control cats were infected by 9 weeks postchallenge (p.c.). Virus could be isolated from the PBMC of two vaccinated cats (A325 and A326) by 6 weeks p.c., whereas the remaining eight vaccinated cats were negative for virus isolation at all time points examined.

Plasma samples taken on the day of challenge from the vaccinated and control cats were examined for VNA; the results are shown in Table 3. The titers of VNA in the two cats which became infected following the first challenge (A325 and A326) were significantly lower than the titers of the eight protected cats (Student's t test; $P < 0.001$).

Antibody responses to FIV TM peptide induced by vaccination. The antibody responses to FIV p24 and p17 and to immunodominant regions within the V3 domain of the FIV envelope glycoprotein and the TM glycoprotein were determined by ELISA on the day of challenge. The results are shown in Table 3. Immunization induced antibodies recognizing FIV p24 and p17 in all vaccinates, with titers ranging from 25 to 625. Similarly, an antibody response to the TM peptide was detected in all of the vaccinated cats, with titers ranging from 125 to 625. In marked contrast, there was a negligible response

TABLE 3. Serological responses of cats immediately prior to first and second challenges

Cat no.	Antibody titer								VNA titer (log)	
	p24		p17		V3 peptide		TM peptide			
	1[a]	2	1	2	1	2	1	2	1	2
A321	125	25	125	625	0	0	125	625	3.15	3.17
A322	125	25	625	125	25	5	125	125	3.70	2.95
A323	125	125	625	125	0	0	125	125	3.79	3.08
A324	125	25	25	25	0	0	125	25	3.64	3.01
A325	125	125	125	125	0	5	125	125	2.67	3.04
A326	125	25	125	25	0	0	625	625	2.43	2.60
A327	125	5	125	125	0	0	125	25	3.59	2.81
A328	125	5	25	25	0	0	125	25	3.28	2.91
A329	125	25	125	25	0	0	625	125	3.80	2.80
A330	125	25	625	125	0	0	625	125	3.75	2.64

[a] Day of challenge.

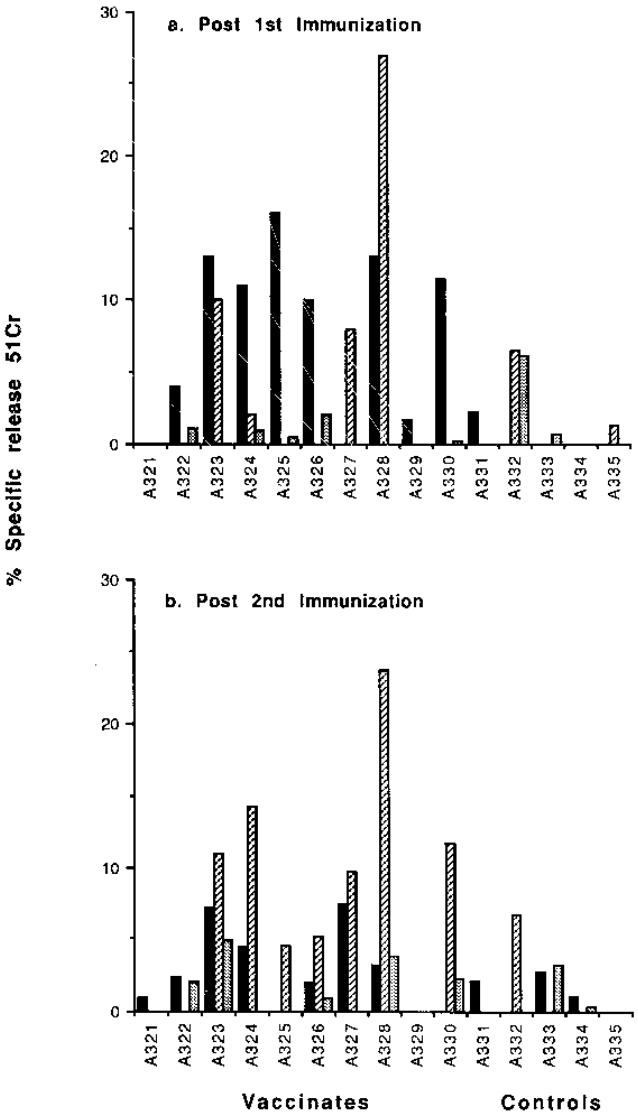

FIG. 1. Detection of FIV Gag- and Env-specific CTL following vaccination. The FIV-specific CTL activity was assayed directly in fresh PBMC prepared from vaccinated cats (A321 to A330) and adjuvant immunized control cats (A331 to A335) 2 weeks after the first immunization (a) and again 2 weeks after the second immunization (b). Autologous or allogeneic skin fibroblasts infected with recombinant vaccinia virus expressing either FIV Gag (■) or FIV Env (▨) or with wild-type virus (▫) and labelled with ^{51}Cr were used as target cells in the assay. The release of ^{51}Cr into the culture supernatant was detected after 4 h of incubation at 37°C. The results shown represent the mean values for triplicate cultures at an effector/target ratio of 35:1 from which the allogeneic values have been subtracted.

to the V3 domain of the envelope glycoprotein. No antibodies were detected in control cats (data not shown).

Vaccination elicits FIV Gag- and Env-specific CTL. Virus-specific lymphocytotoxicity was assayed in the fresh peripheral blood of vaccinated and control cats prior to vaccination and then 2 weeks after each immunization. No FIV-specific CTL activity could be detected before vaccination (data not shown). Following the first immunization, significant FIV Gag-specific CTL activity was detected in 6 of 10 immunized cats, and FIV Env-specific CTL response was also detected in 3 cats (Fig. 1a). Following the second immunization, the high levels of FIV Gag-specific CTL activity observed in 6 of the 10 cats following

TABLE 4. Virus isolation after second challenge[a]

Cat no.	Virus isolation at indicated wk after rechallenge								Comparison of titers before and after rechallenge	
	3	6	9	12	16					
					PBMC	Spleen	MLN	PLN	Anti-p17	VNA
A321	−	−	−	−	−	−	+	+	D	I
A322	−	−	−	−	−	−	+	−	D	D
A323	−	−	−	−	−	−	−	−	D	D
A324	−	−	−	−	−	−	−	−	NS	D
A325	−	−	−	−	−	−	−	−	NS	I
A326	−	−	−	−	−	+	ND	−	NS	I
A327	−	+	+	−	+	+	+	+	I	I
A328	−	−	−	−	−	−	−	−	NS	D
A329	−	−	−	−	−	−	−	+	NS	D
A330	−	−	−	−	−	−	−	−	D	D
A351	−	+	+	+	+	+	+	+	I	I
A352	−	+	+	−	+	+	+	+	I	I
A353	−	+	−	+	+	+	+	+	I	I
A354	−	−	+	−	+	+	+	+	I	I

[a] MLN, mesenteric lymph nodes; PLN, peripheral lymph nodes; D, decreasing titers; I, increasing titers; NS, no significant difference in titers; ND, not determined.

the first immunization were no longer detectable, although lower levels of FIV Gag-specific activity were still observed in the peripheral blood of 2 vaccinated cats (A323 and A327). However, five of the vaccinated cats did have detectable FIV Env-specific CTL responses (Fig. 1b). With the exception of A327, these cats (A323, A324, A328, and A330) all had Gag-specific CTL activity after the first immunization. No CTL activity was observed in the peripheral blood of control cats immunized with adjuvant alone. The FIV-specific CTL responses were observed only when autologous skin fibroblast cells were used as target cells, suggesting that the response was major histocompatibility complex restricted. There was negligible recognition of either autologous or allogeneic skin fibroblast target cells infected with wild-type vaccinia virus, indicating the specificity of the response.

Immunity to FIV persists for 8 months in vaccinated cats. To determine the duration of protective immunity afforded by two immunizations with WIV vaccine, and the contribution of FIV-specific CTL activity and VNA in this immunity, the 10 cats which were vaccinated with two doses of WIV, together with 4 age-matched unvaccinated control cats, were rechallenged 35 weeks following the initial challenge by i.p. inoculation of 25 ID_{50} of the same virus used for the first challenge.

Following this second challenge, although virus was readily detectable in PBMC prepared from the control cats from as early as 3 weeks p.c., it was possible to detect virus in PBMC from only one of the vaccinates (A327), suggesting that the immune protection conferred by two doses of WIV vaccine were still able to clear the 25 ID_{50} FIV/PET at the second challenge (Table 4). However, we cannot rule out the possibility that occult infection with FIV following the initial challenge prevented superinfection.

Effects of vaccination on virus load in lymphoid tissues after challenge. In previous vaccination studies, the efficacy of WIV vaccination was defined solely by the failure to detect viable FIV in the PBMC of vaccinated cats following challenge (19, 50, 51). In the present study, we examined the vaccinates more stringently for the presence of virus following the second challenge. Accordingly, the cats were euthanized 16 weeks after the second challenge, and at postmortem examination, PBMC were prepared and higher numbers (10^7) of cells were cocultivated with 10^6 MYA-1 cells, to increase the sensitivity of virus detection. Similar cocultivations were performed with the same number of splenocytes, peripheral lymph node cells, and mesenteric lymph node cells from each cat. The cultures were maintained for 21 days, and the presence of virus in each tissue was detected by measuring FIV p24 in the culture supernatant by ELISA (FIV antigen detection kit; IDEXX). These virus isolation results obtained postmortem, shown in Table 4, revealed that viable FIV could be isolated from at least one of the tissues tested in 5 of the 10 vaccinates and from every tissue tested from all of the controls.

Persistence of serological responses following vaccination. FIV-specific serological responses were measured on the day of rechallenge and compared with the titers measured on the day of first challenge. This comparison revealed that the most marked decreases occurred with the anti-p24 antibody titers, with no significant changes in the titers of antibodies recognizing p17 or the V3 or TM peptide (Table 3). The VNA titers decreased between the 2 days of challenge in six of the vaccinates, four of which remained virus negative following the second challenge. Increased VNA titers were detected in the remaining four vaccinates, three of which became infected following the second challenge. Thus, the earlier correlation between VNA titer and outcome following the first challenge was less evident with the rechallenge, suggesting that some other immune response might play an important role in protection.

Changes in antibody responses following rechallenge. The titers of anti-FIV p17 antibodies and VNA in samples taken on the day of rechallenge and at postmortem examination 16 weeks p.c. were compared since increasing titers might be associated with any viral replication following challenge. As shown in Table 4, the titers of both anti-p17 antibodies and VNA of all of the control cats increased, indicating that all became infected. Only one of the vaccinates (A327) displayed increasing anti-p17 titers, and virus was readily isolated from all of the tissues from this cat tested postmortem. Decreasing anti-p17 titers were observed in 4 of 10 vaccinates, and although virus could not be isolated from the PBMC of these cats, virus was detected in two of the cats in at least one of the tissues tested. There were no significant changes in the titers of anti-p17 antibodies in 5 of 10 vaccinates, virus being detected in the tissues of two of these five cats. Changes in VNA titers were more closely related to the results of virus isolation postmortem. Six of the vaccinates displayed decreasing titers, and

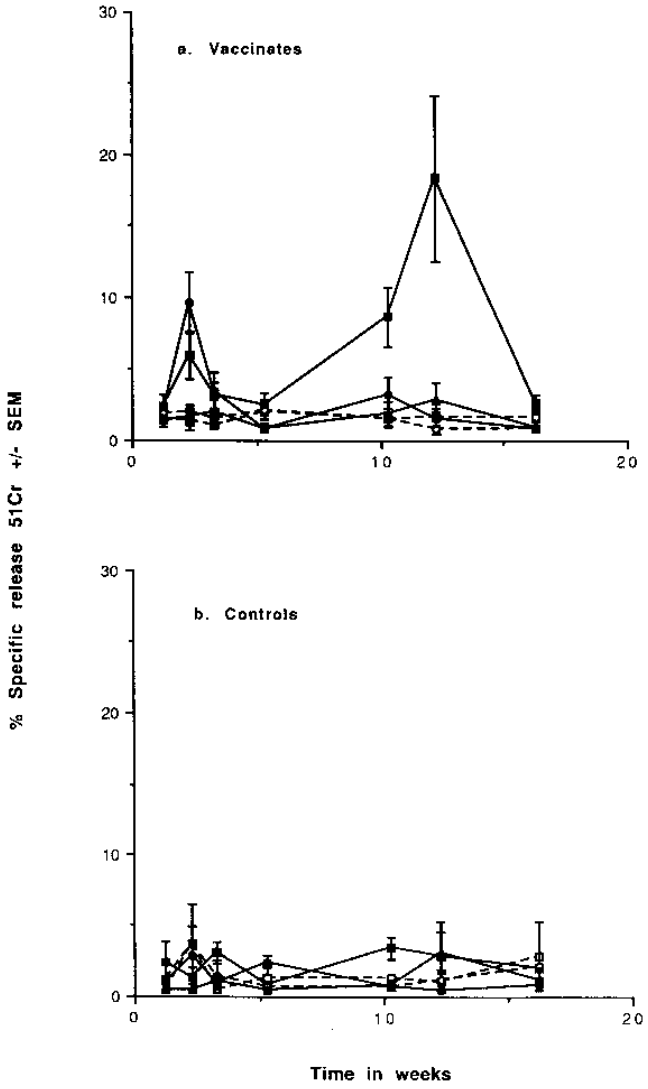

FIG. 2. Detection of FIV-specific CTL activity in vaccinated cats following rechallenge. Virus-specific CTL responses were assayed directly in fresh PBMC prepared from vaccinated cats (A321 to A330) and age-matched control cats (A351 to A354) at weekly intervals for 3 weeks after rechallenge with 25 ID_{50} of FIV/PET i.p. and thereafter at 2-week intervals. Autologous (——) or allogeneic (– – –) skin fibroblasts infected with recombinant vaccinia virus expressing either FIV Gag (■) or FIV Env (●) or with wild-type virus (▲) and labelled with ^{51}Cr were used as target cells in the assay. The release of ^{51}Cr into the culture supernatant was detected after 4 h of incubation at 37°C. The results shown represent the mean values (±) standard errors of the means [SEM]) for triplicate cultures of PBMC from the vaccinated (a) and the control (b) cats at an effector/target ratio of 35:1.

virus could be isolated from just two of these cats and then from only one tissue. The remaining four vaccinates showed increasing titers, and indeed virus was isolated from three of them.

CTL responses increase following rechallenge. It was not possible to detect FIV-specific CTL in the PBMC on the day of second challenge (data not shown), and the evolution of this activity was monitored in the peripheral blood at weekly intervals following rechallenge for 3 weeks and at 2-week intervals thereafter. The results are shown in Fig. 2. At 2 to 3 weeks following rechallenge, a small but significant increase in both Gag- and Env-specific CTL was detected in the peripheral blood of the vaccinated cats. The activity then returned to baseline levels, before a second peak of activity was observed at 10 to 12 weeks p.c., although at this time point higher levels (up to 27%) of specific lysis were observed. This second peak of CTL activity was exclusively FIV Gag specific. The activity returned to baseline levels by 16 weeks p.c. No significant increase in FIV Gag- or Env-specific CTL activity was observed in any of the unvaccinated control cats following challenge.

FIV-specific memory CTL are present in the lymph nodes of vaccinated cats. The previous experiments show clearly that FIV-specific CTL were induced in the peripheral blood of cats immunized with two doses of inactivated whole virus vaccine and that 8 of 10 vaccinated cats were protected from challenge by the homologous FIV/PET isolate. Following rechallenge with 25 ID_{50} of FIV/PET 35 weeks after the initial challenge, CTL activity was detected in the peripheral blood of all vaccinated cats, and 5 of 10 of these animals were protected from this challenge. The lymphoid tissue distribution of the virus-specific CTL response was assayed in the spleen, mesenteric lymph nodes, and peripheral lymph nodes 16 weeks after the second challenge. It was not possible to detect any FIV-specific CTL activity in any of the fresh lymphoid tissues examined at this time (data not shown). These assays were then repeated by using either mesenteric or peripheral lymph node cells from vaccinated and nonvaccinated control cats which were stimulated in vitro with autologous stimulator cells infected with recombinant vaccinia virus expressing either the FIV *gag* or *env* gene product, in an attempt to detect the presence of memory FIV-specific CTL. The results (Fig. 3a) reveal the presence of high levels of Gag-specific CTL activity in the mesenteric lymph nodes of 6 of 10 vaccinated cats and additional Env-specific CTL responses in 3 of these cats (A322, A325, and A330). No such CTL activity was detectable in the mesenteric lymph nodes prepared from infected control cats.

In marked contrast to the relatively high levels of FIV-specific cytotoxicity observed in the mesenteric lymph nodes, FIV-specific CTL activity was observed only in the peripheral lymph nodes of three vaccinated cats (A321, A325, and A330) (Fig. 3b). In this tissue, the presence of Env-specific CTL predominated and was detectable in all three responding cats, whereas Gag-specific CTL activity was observed in only one cat. No FIV-specific CTL responses were observed in the control cats.

DISCUSSION

In this study, we measured the FIV-specific humoral and cell-mediated immune responses induced by immunization with a WIV vaccine to search for correlates of protective immunity. Our previous studies of the immunity induced in cats following immunization with WIV vaccines have shown clearly that protection from homologous FIV challenge correlates with VNA and that a threshold level of VNA is required to afford protection (19, 21). Likewise, recent studies of macaques found that protective responses were associated with the appearance of cross-reactive VNA (8, 48). In the present study also, the titers of VNA measured on the day of challenge accurately predicted the outcome of the first challenge, since there appeared to be a threshold below which cats were not protected. However, this correlation was less evident on rechallenge, indicating that VNA may not be the sole factor involved in protection.

The assay for VNA used CrFK cells as a substrate. Unlike lymphoid cells, which are the natural target cells for FIV replication in vivo, CrFK cells are not susceptible to infection with

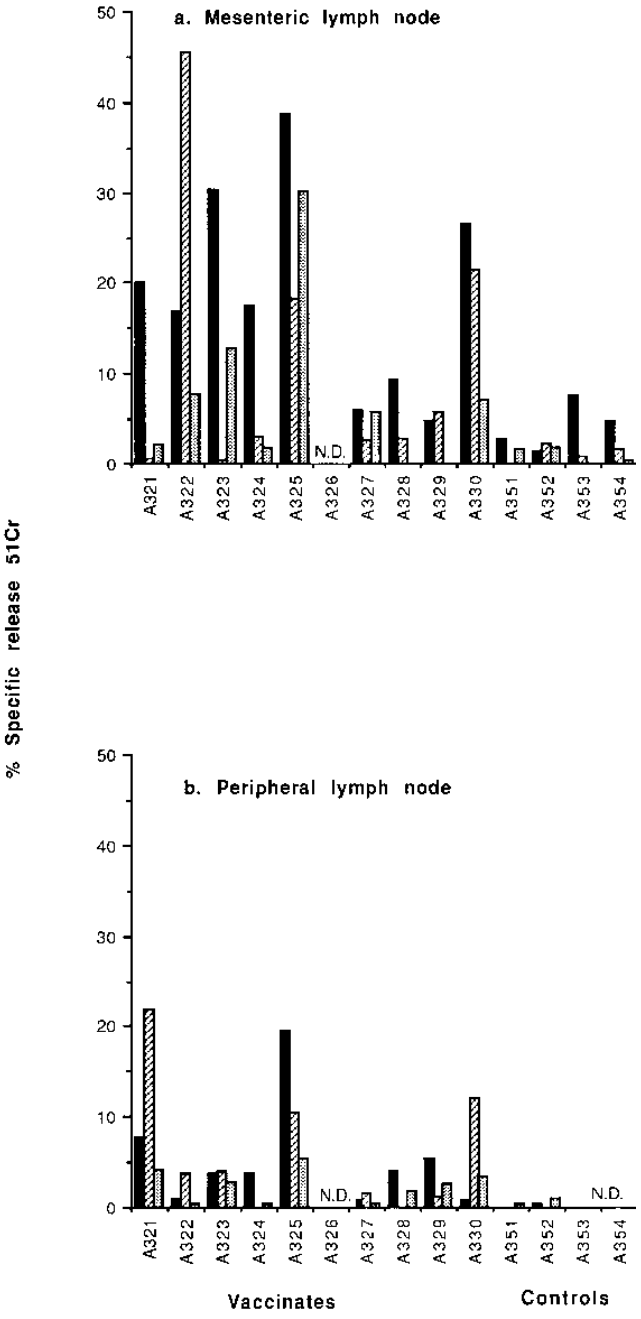

FIG. 3. Vaccination induces FIV-specific memory CTL in the lymph nodes. FIV-specific lymphocytotoxicity was assayed in mesenteric (a) and peripheral (b) lymph node samples collected at postmortem examination of vaccinated cats (A321 to A330) and control cats (A351 to A354) 16 weeks following rechallenge with FIV/PET. Autologous or allogeneic skin fibroblasts infected with recombinant vaccinia virus expressing either FIV Gag (■) or FIV Env (▨) or with wild-type vaccinia virus (▣) and labelled with ^{51}Cr were used as target cells in the assay. The release of ^{51}Cr into the culture supernatant was detected after 4 h of incubation at 37°C. The results shown represent the mean values from triplicate cultures at an effector/target ratio of 50:1 from which the allogeneic values have been subtracted.

all isolates of FIV (47). Furthermore, it is possible that with repeated in vitro propagation of FIV, alterations in the viral phenotype occur, making the virus more susceptible to neutralization. Indeed, it has been demonstrated that a single amino acid substitution in the surface glycoprotein allows a molecular clone of FIV to escape neutralization by the serum of a cat infected with this clone (42). For these reasons, it may be preferable to measure VNA by using an assay based on lymphoid cells. However, in this study, VNA titers measured in a neutralization assay based on the feline T-cell line MBM (27) could not be related to the outcome following the initial challenge (26a). This result may indicate that the mechanism of virus neutralization in the CrFK cell system is in some way related to the mechanism of protection against the FIV/PET isolate in vivo.

To characterize further the fine specificity of the VNA response, we examined the recognition of synthetic peptides from the V3 domain of the FIV envelope glycoprotein. A significant proportion of the neutralizing activity of serum in the CrFK system may be inhibited by a peptide from the immunodominant V3 domain (10, 24). However, immunization with this peptide appears insufficient to confer complete protection (15, 25, 37) and indeed may enhance infection in vaccinated cats (25). Although the vaccinated cats in this study exhibited high VNA titers, only two cats developed antibodies recognizing the V3 peptide, suggesting that neutralization epitopes other than the immunodominant V3 region may be important in protection. Previously we have shown that immunization with a denatured preparation of the FIV envelope glycoprotein (Env) failed to confer protection, despite inducing equally high titers of VNA as WIV immunization (20). This result could be associated with differences in the fine specificity of the VNA responses induced. The Env-specific antibodies elicited by WIV immunization appeared to be directed principally to conformational or glycosylation-dependent epitopes, whereas those induced by the affinity-purified Env recognized linear or conformation-independent epitopes. Thus, the findings of this study highlight the need to characterize epitopes outwith the immunodominant V3 region that are involved with virus neutralization.

The duration of protective immunity conferred by WIV vaccines had not been evaluated prior to this study, and cats have been assumed to be protected solely on the basis of the failure to isolate FIV from the peripheral blood. To investigate the duration of immunity and the mechanisms for the possible retention of immunity, we rechallenged the vaccinates with homologous FIV/PET 35 weeks following the primary challenge, that is, 38 weeks after the final immunization. Results of virus isolations performed on the peripheral blood indicated that 9 of 10 vaccinates were protected from the rechallenge. Following the rechallenge, the unvaccinated, age-matched controls were not consistently virus isolation positive at every time point tested, suggesting that the viral load may have been close to the detection limit of the virus isolation assay. Therefore, a more extensive search for virus was conducted postmortem, 16 weeks after rechallenge. Virus isolations were performed on peripheral and mesenteric lymph nodes and spleen in addition to peripheral blood. These assays revealed the presence of FIV in the lymph nodes of four vaccinates (A321, A322, A327, and A329) and the spleens of two others (A326 and A327), whereas virus was detected in the peripheral blood of only one vaccinate (A327). In contrast, virus was isolated postmortem from the peripheral blood, spleen, and lymph nodes of all four of the control cats. Furthermore, while the titers of anti-p17 antibodies and VNA increased in all of the control cats between the day of rechallenge and the time of postmortem examination, 16 weeks later, increasing titers of anti-p17 antibodies and VNA were demonstrable in only 1 of 10 and 4 of 10 vaccinates, respectively, providing evidence of suppressed viral replication in the vaccinates following rechallenge.

It is possible that the apparently lower viral load induced in the controls by the rechallenge was related to the age of the cats. Since we have observed that young adult cats may be less susceptible than kittens to infection with FIV (our unpublished data), we increased the dose of the rechallenge to 25 ID_{50} to ensure that all of the controls became infected. While this goal was clearly achieved, the age-related lack of susceptibility may have led to the slightly lower rate of viral detection in the year-old controls in the rechallenge study compared with the 20-week-old controls in the first challenge, which were consistently virus positive following challenge with 10 ID_{50} of the same viral stock.

These results raise a number of important issues. We have demonstrated that FIV may be detectable only in the lymphoid tissues, not in the peripheral circulation, and therefore caution should be exercised in interpreting data from vaccine studies in which vaccine efficacy is based solely on the failure to detect virus in the peripheral blood. Furthermore, as studies with HIV have revealed that virus is sequestered in lymph nodes during clinically latent stages of the disease (12, 36), it is conceivable that the immune responses elicited by WIV immunization were sufficient to remove virus from the circulation but not to clear virus from the lymphoid organs. The levels of VNA in vaccinated cats at the time of rechallenge do not correlate with the subsequent virus isolation pattern, in contrast to the results of the primary challenge and our previous studies (19, 21). It is tempting to speculate that complete protection involves a combination of immunological functions: VNA to clear virus from the bloodstream to the lymphoid organs, with a virus-specific CTL response to subsequently eliminate virus evading initial neutralization. Perhaps high levels of VNA can induce a sterilizing type of immunity capable of affording protection from the relatively low doses of virus encountered at challenge. However, when suboptimal levels of VNA are present, virus may gain access to the lymphoid organs, continuing to replicate unless cleared by a virus-specific CTL response. Indeed, a role for VNA in transport of infectious HIV to the lymph nodes has been identified (17). Our results provide some support for this speculation, since the four cats displaying the highest levels of FIV Env-specific activity immediately following vaccination (A323, A324, A328, and A330) remained virus negative. Furthermore, our recent studies have shown that WIV-vaccinated cats have high levels of Env-specific memory CTL activity present in the lymph node, a phenomenon not observed in unvaccinated, FIV-infected cats, suggesting that this immune mechanism may be important in the maintenance of protective immunity (16). In the present study, the CTL assays performed on the lymph nodes at postmortem examination revealed higher levels of lymphocytotoxicity in the mesenteric lymph nodes than in the peripheral lymph nodes. The reasons for the observed differences in the distribution and predominating specificity of the CTL responses in these two lymphoid sites is unclear but may be related to the i.p. route of challenge used in this study. However, it is impossible to distinguish between CTL activity induced by the vaccine and that induced by the challenge virus.

Two vaccinated cats (A325 and A326) became viremic following the initial challenge, virus being isolated from the peripheral blood on several occasions. Yet following reexposure to FIV at the time of rechallenge, it was no longer possible to isolate FIV from either the blood or the other lymphoid tissues. This result suggests that an equilibrium may exist between the host immune response, in particular CTL, and virus load (52). Perhaps the low levels of CTL activity and VNA observed in A325 and A326 were insufficient to eliminate the initial challenge virus, with reexposure to virus at the second challenge resulting in a temporarily higher virus load which elicited a vigorous anamnestic response that subsequently eliminated the virus. Clearly, further experiments are required before firm conclusions can be drawn regarding this phenomenon.

In conclusion, we have demonstrated that both VNA and FIV-specific CTL appear to be important immunological components of the protective response. VNA are possibly more important in the clearance of virus following challenge early after vaccination, while CTL have a role subsequently in clearing virus-infected cells. Future studies will aim to optimize vaccination protocols to clear the potential reservoir of infectious virus in the lymph nodes of vaccinated cats.

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