A Longitudinal Study of Feline Immunodeficiency Virus-Specific Cytotoxic T Lymphocytes in Experimentally Infected Cats, Using Antigen-Specific Induction

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The evolution of the virus-specific cytotoxic T-lymphocyte response in two cats experimentally infected with feline immunodeficiency virus (FIV) was monitored. Effector cells were derived from peripheral blood lymphocytes during the acute and chronic phases of infection (0 to 21 and 62 to 127 weeks, respectively) and from the spleen and lymph nodes at 127 weeks after infection. Lymphocytes were restimulated *in vitro* with paraformaldehyde-fixed, autologous lymphoblasts which had been infected with recombinant vaccinia viruses expressing FIV GAG or ENV proteins. Unstimulated lymphocytes were also used as effectors in some assays. ⁵¹Cr-labelled autologous skin fibroblasts infected with recombinant vaccinia viruses were used as targets. FIV GAG-specific cytotoxic precursors were detected in restimulated circulating lymphocytes during acute infection in both cats. The onset of this activity was as early as 2 weeks postinfection (p.i.) in one cat. From 62 weeks p.i. neither FIV GAG- nor ENV-specific precursors could be detected in the peripheral blood. However, at 127 weeks p.i., GAG- and ENV-specific cytotoxic precursors were detected in lymphocytes isolated from lymph nodes. The FIV-specific cytotoxic cells were predominantly major histocompatibility complex class I restricted. No cytotoxic activity was detected from unstimulated lymphocytes. These studies demonstrate the use of an assay system for dissecting the FIV-specific cytotoxic cell response and show that precursor cells appear in the circulation very early after infection and prior to a detectable antibody response. Our results also suggest that the persistent high-level circulating antiviral cytotoxic T-lymphocyte responses seen in human immunodeficiency virus-infected humans may not be a feature of FIV infections in cats.

Infection of cats with feline immunodeficiency virus (FIV) resembles infection of humans with human immunodeficiency virus (HIV) in its clinical course, pathological features, and humoral immune response (1, 13, 24, 48, 51, 56) and has become established, therefore, as an excellent experimental model for HIV infection in humans (17, 25, 28). In infections with either virus, immune mechanisms are presumed to be responsible for bringing initial virus replication under control and maintaining the asymptomatic period. Attention has focused on the role of cell-mediated immunity to HIV, in which several lines of investigation point to a protective role for HIV-specific cytotoxic T lymphocytes (CTL). First, it is the appearance of virus-specific CTL, often in the absence of neutralizing antibodies, which correlates best with the termination of acute viremia (7, 30, 45). Secondly, HIV infection is characterized by the persistence of a remarkably high-level CTL response throughout the asymptomatic period (9, 18, 22). More recently, circulating CTL precursors (pCTL) have been detected in uninfected individuals at high risk of exposure (10, 12, 32, 43, 44). Because this evidence is, necessarily, circumstantial, a more direct insight into the role of virus-specific CTL in immunity to immunosuppressive lentivirus infections by the manipulation of the response in experimental systems is advantageous, not least because the vigorous HIV-specific CTL response has also been implicated in the immunopathogenesis of the infection (2, 3, 36, 46).

We are interested in defining the role of FIV-specific CTL in the pathogenesis of FIV infection. A knowledge of the speci-

ficity of antiviral CTL generated in FIV infection will provide a rational basis for immunoprophylaxis and, potentially, immunotherapy. The presence of circulating FIV-specific pCTL from the ninth week after infection has been demonstrated (49), and the use of retroviral vector-transduced autologous lymphocytes to induce p24-specific CTL in chronically infected cats has been previously described (50), although transduced cells did not function as targets. In a previous study we described a target cell system designed to dissect the specificity of peptide-induced FIV-specific CTL (15). In this study we have expanded this technique to include the antigen-specific restimulation of cytotoxic precursor cells *in vitro* with endogenously processed FIV proteins presented by autologous cells. This system allows the *in vitro* generation of cytotoxic cells with specificity for individual virus proteins which can be clearly demonstrated at the level of the target cell. We have used this assay to monitor the evolution of the FIV GAG-specific response during acute infection, the GAG- and ENV-specific responses in the same cats during chronic infection, and the tissue distribution of pCTL with GAG and ENV specificity.

MATERIALS AND METHODS

Viruses. The Glasgow-14 isolate of FIV (FIV/GL14) was isolated from the peripheral blood lymphocytes (PBL) of an aged, entire male domestic longhair cat originating from Wales which presented with pyrexia, weight loss, and lymphadenopathy (23, 40). A stock of FIV/GL14 (GL14-A) was grown in Q201 cells (60) from the original isolate and stored at -70°C . The infectivity of GL14-A was determined to be 10^5 tissue culture infectious doses per ml for the interleukin 2 (IL2)-dependent, feline T-lymphoblastoid cell line MYA-1 (34). Two recombinant vaccinia viruses were used, one containing the *gag* gene of FIV/GL14, vFIV/GL14-*gag* (15), and the other containing an *env* construct from the Petaluma isolate (FIV/PET) (52), vFIV/PET-*env* (14). The wild-type strain of vaccinia virus (vWT) used in these experiments was Western Reserve (kindly pro-

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vided by M. Mackett, Paterson Institute for Cancer Research, Manchester, United Kingdom).

Experimental infections. Three female, adult, outbred, specific-pathogen-free (SPF) domestic shorthair cats were used. Cat F1 was bred at an isolation unit at the University of Glasgow Veterinary School, and cats F2 and S1 were purchased from a commercial source. Cats F1 and F2 were inoculated with 1 ml of a 1:100 dilution of GL14-A in sterile phosphate-buffered saline (PBS) by intraperitoneal injection. Cat S1 was given PBS alone as a control. FIV-infected cats were housed separately from the uninfected cat. Full clinical examinations were carried out at 6-week intervals throughout the experiment.

Serology. The appearance of circulating antibodies to FIV following experimental infection was detected by enzyme-linked immunosorbent assays (ELISA) and an indirect immunofluorescence assay. Antibodies to FIV p17 and FIV p24 were detected by ELISA as described elsewhere (38). Antibodies to the V3 and TM2 (4) regions of FIV ENV were detected using synthetic multiple antigenic peptides as capture antibodies in an ELISA described previously (15). These regions of ENV are conserved among FIV isolates Glasgow-8 (GL8), GL14, and PET. For the detection of anti-FIV antibodies by indirect immunofluorescence assay (64), Crandell feline kidney (CRFK) cells (11) chronically infected with FIV/PET were used as targets and fluorescein-labelled goat anti-cat immunoglobulin G (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) was used to develop the test. The reciprocal of the highest dilution at which fluorescent activity could be detected was recorded as the end-point titer of the sample.

FIV antigen detection. An ELISA (PetChek Feline Immunodeficiency Virus Antigen Test Kit; IDEXX) was used to detect FIV p24 antigen in tissue culture supernatants, untreated plasma samples from the acute stage of experimental infection, and untreated and acid-treated (21) plasma samples from the chronic stage.

Virus isolation. Quantitative virus isolation was carried out on plasma and on spleen and lymph node cells stored at 127 weeks postinfection (p.i.). One million MYA-1 cells were resuspended for 90 min at 37°C in 0.5 ml of neat plasma or plasma diluted 1:5, 1:25, or 1:125 in complete RPMI (RPMI 1640; Gibco, Paisley, United Kingdom) containing 10% fetal calf serum (FCS), 2 mM glutamine (Gibco), 100 U of penicillin (Gibco) per ml, 10 µg of streptomycin (Gibco) per ml, and 5×10^{-5} M 2-mercaptoethanol (Sigma, Poole, United Kingdom). Cells were washed once in complete RPMI, resuspended in 1.5 ml of complete RPMI containing 100 U of human recombinant IL2 (rIL2) (kindly provided by J. Nunberg, Cetus Corporation, Emeryville, Calif.) per ml, and cultured in 24-well plates. The plasma virus titer was calculated from the reciprocal of the highest plasma dilution to produce productive infection of MYA-1 cells. Spleen and lymph node cells were recovered from liquid nitrogen storage. Tenfold dilutions of viable cells, assessed by trypan blue dye exclusion, from 10^6 to 10^0 in 0.5 ml of complete RPMI, were added to 5×10^5 MYA-1 cells in 1-ml cultures in 24-well plates. Positive and negative controls, prepared by incubating MYA-1 cells with GL14-A or with medium alone, respectively, were included on each plate. At 3- to 4-day intervals, cultures were fed and supernatants were harvested and stored at -20°C for subsequent FIV antigen detection. Cultures were maintained for up to 25 days.

Stimulator cells. As a source of endogenously processed FIV antigens for the in vitro restimulation of cytotoxic precursors, fixed vFIV/GL14-gag- and vFIV/PET-env-infected autologous stimulator cells were prepared from PBL. Peripheral blood samples were taken from the cats, both prior to and following experimental FIV infection, by jugular venipuncture into preservative-free heparin (Monoparin; CP Pharmaceuticals Ltd., Wrexham, United Kingdom) in RPMI 1640 (Gibco) at a final concentration of 10 U/ml. PBL were separated by centrifugation over Lymphoprep (specific gravity, 1.077; Nycomed, Oslo, Norway). Lymphoblasts were generated by resuspending PBL in complete RPMI containing 7.5 µg of concanavalin A (Sigma) per ml. Cells were cultured at a cell concentration of 2×10^6 /ml in 25-cm² (surface area) tissue culture flasks (Costar Corporation, Cambridge, Mass.) at 37°C in an atmosphere of 5% CO₂ in air. After 24 h, cells were washed and resuspended in complete RPMI supplemented with 100 U of rIL2 per ml. Cultures were examined daily, fed with fresh culture medium, and subcultured as necessary to maintain a concentration of 2×10^6 cells per ml. After 6 days of culture, lymphoblasts were recovered by centrifugation and infected with vFIV/GL14-gag or vFIV/PET-env at 5 to 10 PFU per cell in a total volume of 50 to 100 µl for 1 h at 37°C. At the end of the virus adsorption period, cells were washed twice in complete RPMI and then incubated for a further 6 h at 37°C. After being washed twice in PBS-1% FCS, the cells were fixed in 0.5% paraformaldehyde in PBS at 4°C overnight and then washed four times. Stimulator cells were resuspended at 2×10^6 cells per ml in PBS-1% FCS containing 100 U of penicillin per ml and 10 µg of streptomycin per ml and stored at 4°C for up to 3 months. Expression of FIV gene products in stimulator cells was demonstrated by immunoblotting (24).

Effector cells. Effector cells for the cytotoxicity assays were bulk cultures of PBL which were restimulated in vitro. PBL were harvested from 10-ml blood samples as described above at 2 weeks prior to infection, at 1, 2, 7, 9, 14, and 21 weeks p.i. (acute stage), and at 62, 64, 87, 89, 91, 93, 103, 124, and 127 weeks p.i. (chronic stage). Plasma was stored at -20°C for subsequent serological analysis. For in vitro restimulation, PBL (responder cells) at 2×10^6 cells per ml were cultured in complete RPMI in 1-ml cultures in 24-well tissue culture plates (Costar). Autologous vFIV/GL14-gag stimulator cells were washed in complete RPMI and added to the responder cells at a stimulator-to-responder ratio of 1:10

to 1:20. From 62 weeks p.i., vFIV/PET-env stimulator cells, which had been previously unavailable, were included in addition to vFIV/GL14-gag stimulator cells in the restimulation cultures at the same stimulator-to-responder ratio. Cultures were incubated at 37°C in an atmosphere of 5% CO₂ in a humidified incubator. On day 2, cultures were supplemented with rIL2 at 200 U/ml. Cultures were fed with complete RPMI and rIL2 every 2 to 3 days. After 6 to 7 days the cultures were harvested, viable cells were counted by live-cell fluorescence under UV light (fluorescein diacetate [50 µg/ml] added 1:1 to cell suspension), and the cells were assayed for cytotoxic responses. At postmortem examination at 127 weeks p.i., lymphocytes, in addition to PBL, were also isolated from the spleen and pooled lymph node cells. The tissue was placed on a wire gauze in a sterile petri dish in 15 ml of complete RPMI and gently macerated with a rubber-ended plunger from a 10-ml disposable syringe. The splenic-cell suspension was then separated over Lymphoprep as described above. The lymphocytes were washed, resuspended in complete RPMI, and used as effectors in cytotoxicity assays. The remaining cells were cryopreserved. Freshly isolated lymphocytes were used in addition to restimulated lymphocytes as effector cells in the assays at 9, 93, and 127 weeks p.i.

Target cells. Target cells for the cytotoxicity assays were autologous skin fibroblast cells prepared prior to infection of the cats from a skin biopsy as described previously (15) and stored at the third passage in liquid nitrogen. Skin fibroblasts were recovered from storage and cultured for a further one or two passages in 10% MEM ALPHA (MEM ALPHA containing ribonucleosides and deoxyribonucleosides [Gibco], 10% FCS, 2 mM glutamine, 100 U of penicillin per ml, and 10 µg of streptomycin per ml) supplemented with human recombinant epidermal growth factor at 10 ng/ml (Sigma). Confluent monolayers were trypsinized, washed, and seeded into round-bottomed 96-well tissue culture plates at 8×10^3 cells per well in 10% MEM ALPHA without epidermal growth factor. Cells were labelled overnight with sodium [⁵¹Cr]chromate (Amersham, Amersham, United Kingdom) at 75 mCi per 10⁶ cells in 50 µl of 10% MEM ALPHA per well.

On the morning of the assay, skin fibroblast monolayers were washed four times with MEM ALPHA containing 2% FCS and then infected with vFIV/GL14-gag, vFIV/PET-env, or vWT at 5 PFU per cell. The plates were centrifuged at 250 × g for 5 min, and virus adsorption was carried out for 1 h at 37°C in a humid incubator in an atmosphere of 5% CO₂ in air. The inoculum was removed, and monolayers were washed once with 10% MEM ALPHA and then incubated for a further 2 h in 100 µl of 10% MEM ALPHA per well. These cells were then used as target cells in the cytotoxicity assays.

Expression of FIV proteins in the target cells was demonstrated by immunoblotting. Briefly, proteins were separated under reducing conditions on 10% polyacrylamide gels (24), transferred to an Immobilon-P membrane (Millipore) using a semidry system (Trans-blot SD; Bio-Rad), and blocked with 2% nonfat milk powder in Tris-buffered saline (TBS)-0.05% Tween 20 for 2 h. Blots were washed overnight at 4°C with TBS-0.05% Tween 20 and then probed with pooled high-titer serum from cats experimentally infected with FIV diluted 1:16 in 1% skimmed milk powder and 0.5% Tween 20 in TBS for 2 h. After being washed three times in 0.05% Tween 20 in TBS, bound antibodies were detected with affinity-purified goat anti-cat immunoglobulin G conjugated to horseradish peroxidase diluted 1:200 in 1% skimmed milk powder and 0.5% Tween 20 in TBS for 1 h. Blots were washed three times, and the peroxidase activity was detected by the enhanced chemiluminescence-Western blotting (immunoblotting) detection system (Amersham).

Cytotoxicity assays. The culture medium over the target cells was replaced with 100 µl of complete RPMI. Two twofold dilutions of the effector cells were performed in complete RPMI in 96-well plates. A volume of 100 µl of lymphocytes was added to each experimental well of target cells in triplicate at various lymphocyte-to-target (L:T) ratios. In order to determine the maximum and spontaneous isotope release from the target cells, on each plate 100 µl of 2% Triton X-100 (BDH, Poole, United Kingdom) in RPMI 1640 or 100 µl of complete RPMI was added to triplicate wells of each target cell type. The assay plates were centrifuged at 250 × g for 5 min. Cultures were incubated for 4 h at 37°C and then centrifuged again. A volume of 50 µl of culture medium was harvested from each well onto a 96-well plate containing a solid scintillator (LumaPlate; Canberra Packard Ltd., Pangbourne, United Kingdom). LumaPlates were dried overnight, and the ⁵¹Cr release was measured in a Topcount Microplate Scintillation counter (Canberra Packard Ltd.). The mean activity (counts per minute) for triplicate wells was calculated, and specific lysis for each sample was calculated as a percentage by using the following formula: (mean ⁵¹Cr release from test wells - mean spontaneous ⁵¹Cr release)/(mean total ⁵¹Cr release - mean spontaneous ⁵¹Cr release) × 100.

Flow cytometry. Analysis of CD4⁺ and CD8⁺ lymphocyte subsets by flow cytometry was carried out at 3 weeks prior to infection, immediately before infection, and at 3, 11, and 103 weeks p.i. as described previously (63).

The target cell surface phenotype was determined by flow cytometry. Culture medium was removed from skin fibroblasts at the seventh or eighth passage and replaced with EDTA solution (NaCl [40 g], KCl [1 g], Na₂HPO₄ [5.75 g], KH₂PO₄ [1 g], and EDTA [1 g] in 50 ml of 1% phenol red). Following incubation at 37°C for 10 min, the adherent cells were removed by gentle scraping, recovered by centrifugation, and then washed in buffer (PBS-1% FCS-0.1% sodium azide) and dispensed into polystyrene round-bottomed tubes (Falcon 2054; Becton Dickinson, Cowley, United Kingdom) at 4×10^5 cells per tube. The cells

FIG. 1. FIV p55 and gp95 expression in stimulator cells. Autologous lymphoblasts were infected with vFIV/GL14-gag (lane 5) or vFIV/PET-env (lanes 3 and 4) at 5 PFU per cell and analyzed by immunoblotting (approximately 10^6 cells per lane). Lane 1 contains uninfected lymphoblasts. Lane 2 contains FIV/GL14-infected lymphoblasts.

were then incubated with 50 μ l of primary antibody for 30 min at 4°C. The following primary monoclonal antibodies were used as supernatants: VPM 19 (Serotec MCA 897; anti-sheep class I major histocompatibility complex [MHC]) (61), VPG 3 (Serotec MCA1348; anti-feline class II MHC), and VPG 15 (Serotec MCA1345; anti-feline CD9). The anti-FIV p24 monoclonal antibody 43 1E2 (a gift from N. C. Pedersen, University of California, Davis) was used as an isotype-matched negative control. Cells were washed twice in buffer and then resuspended in 50 μ l of fluorescein isothiocyanate-conjugated F(ab')₂ fragment of rabbit anti-mouse immunoglobulin (Dakopatts, Glostrup, Denmark) diluted 1:20 with buffer for 30 min at 4°C. Cells were washed twice in buffer and then resuspended in 0.5 ml of buffer and run on a Coulter EPICS Elite flow cytometer. A gate was set around all cells on a cytogram of forward light scatter versus right-angle light scatter. A positive gate was determined by overlaying plots for negative-control samples with those of the test samples on a logarithmic frequency histogram.

RESULTS

Development of the CTL assay. A technique for the in vitro antigen-specific induction of FIV-specific cytotoxic precursor cells was developed. Stimulator cells were prepared by infecting autologous lymphoblasts with recombinant vaccinia viruses followed by paraformaldehyde fixation to provide a source of endogenously processed antigen for in vitro restimulation of lymphocytes from FIV-infected cats. The expression of FIV antigens in stimulator cells was confirmed by immunoblotting, by which a band corresponding to the GAG precursor polypeptide was seen at 55 kDa and a band corresponding to the signal and surface regions of the envelope glycoprotein was seen at 95 kDa (Fig. 1).

Following the in vitro restimulation, effector cells were used in ^{51}Cr release cytotoxicity assays. The target cells for the cytotoxicity assays, which were ^{51}Cr -labelled autologous skin fibroblast cells infected with vFIV/GL14-gag, vFIV/PET-env, or vWT (14, 15), were further characterized in this study. Expression of FIV proteins in the target cells was confirmed by immunoblotting (Fig. 2). The phenotype of feline skin fibroblasts was investigated by flow cytometry. The cells were found to be 99 to 100% positive for feline MHC class I antigens and 99 to 100% negative for feline MHC class II antigens (Fig. 3). Although skin fibroblasts were all positive for CD9, a putative cellular receptor for FIV (62), we have been unable to achieve productive infection of feline skin fibroblasts with FIV/GL14 in

FIG. 2. FIV p55 and gp95 expression in target cells. Skin fibroblasts were infected with vFIV/PET-env at 0.625, 1.25, 2.5, or 5 PFU per cell (lanes 1 to 4, respectively) or with vFIV/GL14-gag at 2.5, 5, 10, or 20 PFU per cell (lanes 6 to 9, respectively) and analyzed by immunoblotting (approximately 2×10^5 cells per lane). Lane 5 contains uninfected skin fibroblasts.

vitro, as determined by antigen detection from tissue culture supernatants, despite prolonged exposure to virus.

Spontaneous ^{51}Cr release from target cells was usually between 5 and 15% but never more than 30% of the maximum isotope release. Variability among triplicate samples did not exceed 10%.

Kinetics of FIV/GL14 infection of cats. Cats F1 and F2 became infected with FIV following inoculation with virus, as evidenced by seroconversion. In both cats, plasma antibodies to p17, p24, TM2, and V3 were detected by ELISA from 4 weeks p.i. (Fig. 4). Antibody levels rose steeply initially, with most reaching a peak at around 9 weeks p.i. After this, antibody levels plateaued or fell slightly. Uninfected control cat S1 remained seronegative for FIV throughout the experiment. The onset of seroconversion at 4 weeks p.i. was confirmed by indirect immunofluorescence assay. So that cytotoxic responses might be correlated with virus load, plasma samples were tested for the presence of FIV p24 antigen by an ELISA. Since the presence of antibody may interfere with antigen detection because of the formation of immune complexes (6, 26, 35), plasma samples from the chronic stage were treated with acid to dissociate any such complexes. However, plasma antigenemia could not be detected at any stage in either untreated or acid-treated samples. Although the techniques used in this study have been reported by others to be useful for this purpose (20, 21), they were not successful in our hands. CD4⁺ and CD8⁺ lymphocyte subsets were enumerated by flow cytometry (Table 1). Prior to infection, all three cats had a CD4/CD8

FIG. 3. Flow cytometric analysis of feline skin fibroblast cell surface marker expression. The isotype-matched negative control is shaded on the frequency histogram. Skin fibroblasts are negative for MHC class II antigens and positive for MHC class I antigens and CD9.

FIG. 4. Seroconversion to FIV following experimental infection with FIV/GL14. The development of plasma antibodies to FIV in experimentally infected cats F1 (squares) and F2 (circles) and mock-infected SPF cat S1 (triangles) was determined from serial plasma samples by ELISA. O.D., optical density.

ratio of >1.2 . In FIV-infected cats F1 and F2, the CD4/CD8 ratio became inverted by 11 weeks p.i. and had fallen further by 103 weeks p.i. The CD4/CD8 ratio was maintained in SPF cat S1. The three experimental cats remained clinically healthy throughout the experiment.

Virus load in FIV-infected cats. In order to relate any differences in virus load between the infected cats and the relative virus loads in the spleen and lymph node lymphocytes to the activity and tissue distribution of pCTL, quantitative virus isolation was performed. Dilutions of plasma and spleen and lymph node cells taken at postmortem examination were cocultured with MYA-1 cells. FIV-infected cat F1 had a greater virus load than F2. FIV antigen was detected earlier in MYA-1 cells cocultivated with plasma from F1 (day 11) than with plasma from F2 (day 18), and plasma from F1 had a higher titer than plasma from F2 (titers, 25 and 1, respectively). Recovered spleen and lymph node cells were found to be $>90\%$ viable by trypan blue exclusion. In both FIV-infected cats, the virus load in the spleen was higher than that in the lymph nodes since virus could be isolated from fewer spleen cells than lymph node cells. Virus could be isolated from 100 lymph node cells from cat F1 and 10,000 lymph node cells from cat F2. The smallest number of splenic lymphocytes required to isolate virus was 10 from cat F1 and 100 from cat F2. Virus was never detected in any tissues from SPF cat S1.

Development of FIV-specific CTL response. PBL were isolated from the FIV/GL14-infected cats, F1 and F2, and the SPF cat, S1, at 2 weeks prior to infection, then at intervals until week 21 of infection, and again from 62 weeks p.i. until the cats were euthanized at 127 weeks p.i. Lymphocytes derived from circulating PBL or from spleen or lymph node tissue were restimulated in vitro for 6 to 7 days prior to assay and then were tested for their ability to recognize a panel of autologous

and heterologous targets expressing FIV GAG (acute infection) and FIV GAG or FIV ENV (chronic infection) and autologous targets infected with vWT in 4-h cytotoxicity assays. In addition to restimulated lymphocytes, directly isolated lymphocytes were used as effectors in assays at 9, 93, and 127 weeks p.i.

In cytotoxicity assays carried out before and 1 week after infection, minimal lytic activity was detected. Antigen-specific cytotoxicity was first detected 2 weeks after infection. Restimulated lymphocytes from FIV-infected cat F1 killed autologous GAG-expressing targets but not heterologous GAG-expressing targets or autologous targets infected with vWT (Fig. 5). In the absence of MHC haplotype data, the discrimination between autologous and heterologous target cells in unrelated cats has been assumed previously to indicate MHC restriction (14–16, 49, 50). In line with this proposal and in light of the results for target cell phenotype presented above, the cytotoxic activity detected in this study represents a classical MHC class I-restricted CTL response. This response was maintained throughout the acute infection monitoring period up to 21 weeks p.i., with levels of specific lysis ranging from 38 to 64% at the highest L:T ratios. From 7 and 14 weeks p.i., heterologous vFIV/GL14-gag-infected targets were recognized, in addition to autologous targets, although at a level lower than that of autologous targets and only at the maximum L:T ratio. Effector cells from the uninfected cat, S1, gave minimal lysis on any target cell type (0 to 10%). GAG-specific pCTL from FIV-infected cat F2 were detected from 14 weeks p.i. (62 and 44.2% specific lysis at L:T ratios of 90:1 and 45:1, respectively) and also at 21 weeks p.i. (Fig. 6). Heterologous targets were not recognized by effectors from cat F2. During chronic FIV infection, neither GAG- nor ENV-specific pCTL activity could be detected with PBL from either cat F1 or F2. At 127 weeks p.i., FIV-specific pCTL activity was detected with lymphocytes isolated from the lymph nodes of both FIV-infected cats. In cat F1, this lymph node pCTL activity was ENV specific with no detectable GAG-specific response, whereas in cat F2, GAG-specific but not ENV-specific pCTL were detected from lymph node lymphocytes (Fig. 7). A low level of antigen-specific heterologous recognition was also seen. Splenic lymphocytes did not display FIV-specific pCTL activity.

Primary FIV-specific CTL activity could not be detected from PBL during acute infection or chronic infection or from lymph node or splenic lymphocytes at 127 weeks p.i.

DISCUSSION

Using an assay system capable of detecting secondary cytotoxic responses to individual FIV proteins, we report the first longitudinal study of antigen-specific cytotoxic precursor cell activity in FIV-infected cats.

This study represents the first series of experiments using the vFIV/GL14-gag construct, and although it might be expected

TABLE 1. Decline in CD4/CD8 ratio following experimental infection of cats F1 and F2 with FIV/GL14

Week p.i.	CD4/CD8 ratio		
	F1	F2	S1 (SPF)
–3	1.67	1.27	1.72
0	1.45	1.42	1.34
3	1.17	0.63	1.39
11	0.95	0.87	1.23
103	0.49	0.62	1.56

FIG. 5. FIV GAG-specific cytotoxic responses during acute infection in cat F1. Restimulated lymphocytes from cat F1 were tested against autologous (Aut) and heterologous (Het) targets infected with vFIV/GL14-gag (vGAG) or vWT at various L:T ratios. No vWT targets were included in the assay at 21 weeks p.i.

that recognition of the GAG product would be relatively conserved between FIV isolates, this could not be assumed. For this reason FIV/GL14 was used for the experimental infections in preference to FIV/GL8, which is most often used in our laboratory and for which animal titrated stocks are available. We have shown that FIV/GL14 infection was established with the inoculum used in this study by demonstrating rapid seroconversion and inversion of the CD4/CD8 ratio and later by performing virus reisolation. The usefulness of vFIV/GL14-gag to detect CTL raised against immunogens from FIV/GL8 and FIV/PET isolates has since been demonstrated (14, 15).

We detected GAG-specific cytotoxic precursors during acute infection with FIV. The onset of this response was very early in cat F1, occurring from the second week of infection and prior to seroconversion. This appearance of virus-specific pCTL is earlier than that demonstrated previously (49), in which pCTL were detected from 9 weeks p.i., and is similar to that seen in HIV and simian immunodeficiency virus infections (7, 30, 39, 45, 65), in which this response correlates with clearance of the initial viremia. The temporal association between the onset of the FIV GAG-specific response and virus load could not be established in this study, as the measurement of plasma antigenemia was unsuccessful. In cat F2, GAG-specific responses were detected later, from 14 weeks p.i. One possible reason for this difference between cats is that since we were monitoring only for GAG-specific responses during acute infection, pCTL directed against other virus proteins, which may have been present at an earlier time, would not have been detected. Such temporal differences in the appearance of HIV-specific CTL between individuals are seen after primary HIV infection (31).

For the same cats in which high levels of target cell killing were seen in acute infection, we were unable to detect either GAG- or ENV-specific precursors from PBL during the chronic infection monitoring period from 62 to 127 weeks p.i. This is in contrast to the results of others who have detected FIV-specific circulating cytotoxic precursors in cats chronically infected with FIV/PET in cross-sectional studies (20 to 22 and

30 to 56 months p.i.) (49, 50). This apparent disparity may represent a genuine difference in responses to the different isolates of FIV used in these studies, or it may be that pCTL in chronic FIV infection are present at a level below the limit of sensitivity of our assay. While this question remains to be resolved, our results show that the level of circulating FIV-specific pCTL in chronic infection at least declines compared with that in acute infection. Declining anti-HIV CTL activity is generally associated with a poor prognosis, although no clear correlation between HIV-specific CTL activity and markers of disease progression such as CD4⁺ cell counts, antigenemia, or viral load has been found (29, 41). Despite the apparent disappearance of GAG- and ENV-specific cytotoxic precursors from the periphery after acute infection, the FIV-infected cats remained clinically healthy and maintained their body weights throughout the study. The fall in the CD4/CD8 ratio between acute and chronic infection would indicate that infection was progressing normally in these cats. We were able to isolate FIV-specific cytotoxic precursors from the lymph nodes of chronically FIV-infected cats. Lymphoid tissues are a major

FIG. 6. FIV GAG-specific cytotoxic responses during acute infection in cat F2. Restimulated lymphocytes from cat F2 were tested as described in the legend to Fig. 5. Symbols are as defined in Fig. 5.

FIG. 7. FIV GAG- and ENV-specific cytotoxic responses from restimulated lymph node lymphocytes during chronic infection (127 weeks p.i.). Results for GAG-specific lysis by F1 effectors and ENV-specific lysis by F2 effectors were minimal and have been omitted for clarity. Abbreviations: Aut, autologous; Het, heterologous; vENV, vFIV/GL14-*env*; vGAG, vFIV/GL14-*gag*.

site for dissemination of FIV (5), and FIV has been found in association with mononuclear cells and follicular dendritic cells in germinal centers (5, 55). It is perhaps not surprising that virus-specific cytotoxic precursors were found in the lymph nodes, but it is more surprising that they were not also found in the spleen, particularly since we have shown that the virus loads in the spleen and lymph nodes were similar. The reason for this difference is not clear.

We did not detect cytotoxic activity in unstimulated PBL or lymphoid tissues. It has been shown elsewhere that this target system is capable of detecting fresh effector activity induced in vivo by immunization with FIV peptides (15, 16) and whole inactivated FIV (14). Although we cannot rule out the possibility that we failed to detect a fluctuating response or fresh cytotoxic activity specific for FIV proteins other than those tested for here, in light of similar results reported by others (49) it seems likely that, as in most virus infections, the majority of FIV-specific cytotoxic cells are isolated in the precursor form and require restimulation and expansion in vitro to achieve measurable levels of differentiated effector cells. This is in marked contrast to the virus-specific CTL response in HIV-infected humans, which is extremely active, with relatively high levels of circulating effector CTL persisting into the asymptomatic period (9, 18, 22, 37, 41, 58).

The recognition of autologous targets in this study is compatible with an MHC class I-restricted response, since the target cells were shown to be uniformly positive for feline MHC class I antigens and uniformly negative for feline MHC class II antigens. This implies that this response is mediated by CD8⁺ cells, although the relatively small numbers of lymphocytes available for these experiments have prevented the use of depletion experiments to phenotype the effector population thus far.

Killing of heterologous targets was seen in some cytotoxicity assays. That this response was generated in response to FIV infection, and not as an artifact of in vitro culture, was shown by the minimal levels of heterologous recognition with lymphocytes from the control cat S1 or any cat prior to infection. The results presented here are similar to those obtained in investigations of cytotoxic effector cells induced in response to equine influenza virus infection (19), in which cytotoxicity against autologous and heterologous targets was found, but only if the targets expressed viral antigens. Heterologous recognition may be mediated by classical MHC-restricted pCTL if class I alleles are shared between cats. The likelihood of common alleles increases with relatedness, so cats from the same

source are more likely to exhibit class I homologies. However, the cats used in this study originated from two distinct sources and heterologous recognition was seen only between cats from different sources; i.e., effectors from cat F2 did not recognize targets from cat S1. Although this does not preclude classical pCTL as effectors, MHC-unrestricted, antigen-specific cytotoxic cells must also be considered as potential mediators of heterologous recognition. Non-MHC-restricted HIV ENV-specific cytotoxic cells have been found in freshly isolated peripheral blood mononuclear cells from HIV type 1-infected patients (33, 41, 42, 57, 59), and armed killer cells and NK cells have been implicated as the effectors (41, 57, 59). Determination of the phenotype of the effector cells mediating the heterologous killing seen here awaits the use of purified lymphocyte subpopulations rather than bulk cultures as effector cells. Potential candidates are lymphokine-activated killer cells, or NK-like cells, characterized by a broad range of killing (66). Feline NK-like lymphokine-activated killer cells have been observed following long-term culture of lymphocytes in the presence of human rIL2 (54), and recently, CD8⁺ CD57⁺ lymphokine-activated killer cells have been induced from FIV-infected cats (67). However, cytotoxicity mediated by these cells is not antigen specific, whereas we never detected appreciable levels of lysis of vWT-infected target cells. Heterologous killing could be mediated by the same population which mediates MHC-restricted killing, but in a different differentiation state. Prolonged in vitro exposure of murine CTL to IL2 can result in a broader specificity of lytic activity (8, 27, 47, 53), although this was not seen before 8 to 9 days (47), whereas we cultured lymphocytes for a maximum of 7 days.

In conclusion, we have detected FIV GAG-specific MHC class I-restricted pCTL in PBL from 2 weeks after experimental FIV infection. This response declines in the peripheral blood as infection progresses, but cytotoxic precursors were detected in the lymph nodes at 127 weeks p.i. The role of FIV-specific pCTL in the containment of virus is currently being investigated.

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