Acute virulent infection with feline immunodeficiency virus (FIV) results in lymphomagenesis via an indirect mechanism

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A R T I C L E   I N F O

Article history:
Received 6 September 2012
Returned to author for revisions
30 October 2012
Accepted 4 December 2012
Available online 3 January 2013

Keywords:
Feline immunodeficiency virus
FIV
Neoplasia
Lymphoma
Tumorigenesis
Lymphomagenesis

A B S T R A C T

Four cats (24%) experimentally infected with FIV unexpectedly developed neoplastic changes within four months of inoculation. While FIV has previously been associated with neoplasia, the rapidity and high attack rate seen here is highly unusual. PCR for antigen receptor rearrangements (PARR) detected clonally rearranged T cells in two animals diagnosed with B cell follicular lymphoma by classical means. All cats were negative for feline leukemia virus; gamma-herpesvirus DNA was not amplified using degenerate primers. FIV proviral load in neoplastic tissue was two orders of magnitude lower than in the periphery, lower in neoplastic vs non-neoplastic lymph node, and clonal integration was not detected. We hypothesize that neoplasia was secondary to FIV immune dysregulation, and show that PARR can augment our capacity to phenotype these tumors and distinguish follicular hyperplasia from lymphoma. Age of exposure and relative virulence of the inoculum likely contributed to this unusual presentation of FIV infection.

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Introduction

Feline immunodeficiency virus (FIV) is a lentivirus in the family Retroviridae that causes immune dysfunctions in cats similar to those observed in people infected with human immunodeficiency virus (HIV) (Gabor et al., 2001; Ishida et al., 1989; Pedersen et al., 1989; Pedersen, 1994; Zenger, 1990). FIV was first described in 1987 in a large multiple cat household experiencing immunodeficiency related diseases (Pedersen et al., 1987). Clinical symptoms during the initial few weeks of infection include fever, leucopenia, gingivitis, and generalized lymphadenopathy. Clinical signs usually regress by 1–4 months post experimental infection, concurrent with seroconversion, and a latent stage of variable duration occurs wherein viral load attains a steady state. End stage disease typically does not occur for several years post-infection, and is marked by loss of CD4 immunocytes and high circulating viral load. Clinical signs reported during this phase of disease include neurologic manifestations, enhanced susceptibility to opportunistic infections, and neoplasia (Ravi et al., 2010).

The association between FIV and an increased incidence of neoplasia has been frequently reported in the years since FIV was first described (Alexander et al., 1989; Barr et al., 1993; Callanan et al., 1992; Gabor et al., 2001; Graftydd-Jones et al., 1988; Hopper et al., 1989; Hutson et al., 1991; Magden et al., 2011; Pedersen et al., 1989; Pedersen, 1994; Poli et al., 1994; Poli et al., 1994; Shelton et al., 1994, 1999b; Terry et al., 1995; Yamamoto et al., 1989; Zenger, 1990). The incidence of FIV-associated neoplasms ranges from 1 to 21% of FIV-positive cats, consistent with the reported incidence in HIV-positive individuals, and neoplasia typically rises sporadically several years post-infection (Feder and Hurvitz, 1990; Sabine et al., 1988; Zenger, 1990). The most frequently reported type of neoplasia associated with FIV infection is lymphoma, typically a high grade B cell tumor, also consistent with reports of HIV-associated neoplasia (Callanan et al., 1996; Gabor et al., 2001; Poli et al., 1994; Rabkin et al., 1991; Terry et al., 1995; Wang et al., 2001).

Infectious etiologies other than FIV and HIV have been associated with increased incidence of neoplasia. Recent publications have implicated infectious diseases in up to 17% of reported new cancers (Fontham, 2009). In cats, feline leukemia virus (FeLV) is known to result in the development of lymphoma (Ahmad and...
Human and primate herpesviruses have been associated with a variety of cancers, often in association with lentiviral co-infection (Bruce et al., 2012; Carbone et al., 2008; Delecluse et al., 2007; Wen and Damania, 2010). Some other examples of agents that are associated with neoplastic transformation include human papilloma virus, hepatitis B and C viruses, and *Helicobacter pylori* (Fontham, 2009).

The ability of viruses to induce neoplasia typically relates to specific features of the viral replication cycle (Maeda et al., 2008). A potential direct role of FIV-induced lymphomagenesis, i.e. FIV clonal integration disruption of oncogene function, has been reported in two select cases (Beatty et al., 1998b, 2002; Wang et al., 2001). In one study, FIV sequences were detected in DNA isolated from a cat experimentally infected with FIV that developed lymphoma of the lymph nodes, liver, and omentum. The tumor was of high grade B-cell origin and demonstrated monoclonal integration of FIV (Beatty et al., 1998b). A second study demonstrated clonal FIV proviral integration in two of fourteen cases of feline lymphosarcoma by PCR and confirmed with Southern blot analysis (Wang et al., 2001). These two studies suggest that in a subset of cases, a direct oncogenic role of FIV in proto-oncogene stimulation may lead to malignant transformation (Rosenberg et al., 1991).

The majority of studies investigating mechanisms of FIV infection that underlie an increased rate of tumorigenesis have failed to demonstrate clonal integration of the FIV-provirus in lymphomas (Beatty et al., 1998a; Callanan et al., 1992, 1996; Terry et al., 1995). This implicates an indirect mechanism of tumorigenesis potentially involving impaired immune surveillance and removal of neoplastic cells secondary to FIV-induced immunosuppression. This theory is supported by observed increases in B cell proliferation following FIV infection, resulting in the production of large pools of circulating lymphocytes (Callanan et al., 1993). Acute FIV infection is consistently associated with lymphoid follicular hyperplasia and expansion of B cell regions resulting in a pre-neoplastic phenotype. Such vigorous lymphoid expansion may result in the enhancement of opportunities of malignant cell development and ‘escape’ (Callanan et al., 1992). While B cell lymphomas are the more frequently observed tumor type, T cell lymphomas can also occur with FIV infection (Endo et al., 1997; Gabor et al., 2001; Wang et al., 2001). Case reports of FIV-positive animals with multiple neoplasms, such as a spinal lymphosarcoma with concurrent disseminated mastocytoma (Barr et al., 1993), would also suggest a generalized mechanism of enhanced tumor susceptibility.

Cancer development during lentiviral infection has been associated with co-infection of a second viral agent. For example, co-infection with HIV and Kaposi’s sarcoma-associated herpes virus [KSHV or human herpes virus 8 (HHV8)] results in epidemic Kaposi’s sarcoma (KS), a cancer of lymphatic endothelium (Cesarman et al., 1995; Chang et al., 1994; Rabkin et al., 1991). AIDS patients are over 300 times more likely to develop KS than persons on immunosuppressive therapies (Beral et al., 1990). Non-human primates infected with both simian immunodeficiency virus (SIV) and simian gamma-herpesviruses (including the macaque equivalent of KSHV) have also been associated with increased incidence of gastrointestinal stromal tumor development and lymphomas (Bielefeldt-Ohmann et al., 2005, 2008; Bruce et al., 2012). These examples, and a recent report associating spontaneous B cell lymphoma in dogs to infection with an EBV-like gammaherpesvirus (Huang et al., 2012), suggests that an as-yet undescribed feline gamma-herpesvirus may be associated with lymphoma development in FIV-positive cats.

In this study we observed neoplastic changes in four of seventeen FIV-inoculated kittens as an unanticipated outcome in a protocol testing a novel anti-retroviral therapy. This
observation is unique in that neoplasia occurred with an unusual high frequency (24%) and was detected within four months of initial infection. Immunohistochemical analysis demonstrated that two cats developed classically defined B cell follicular lymphoma; evidence of T cell neoplasia or pre-neoplasia was detected by PCR for antigen receptor rearrangement (PARR) in both tumors (Avery, 2009; Lana et al., 2006). A third animal developed lymphoid leukemia, while a fourth cat demonstrated pre-neoplastic changes in the mesenteric lymph node.

We hypothesized that based on the highly virulent disease experienced in these animals, the rapidity of lesion development, and the young age of the kittens at the time of FIV inoculation, oncogenesis was most likely due to dysregulation of lymphoid expansion down-regulation following viral induction of highly proliferative B and T cells. Below we describe a series of experiments which support this hypothesis by ruling out FIV clonal integration and failing to detect a number of potential co-pathogens.

Results

High PBMC proviral loads and significant hematologic changes were detected in FIV-infected animals

Twenty-three cats were enrolled in a study to test new anti-retroviral therapies. Seventeen cats inoculated with virulent FIVC-36 were monitored to study completion (de Rozières et al., 2004; Sourvinos et al., 2000) and achieved high viral burdens: peak proviral load approximately $10^6$ viral copies/million peripheral blood mononuclear cells (PBMC), peak plasma viremia approximately $10^6$ viral copies/ml, and CD4:CD8 ratios < 2.0 at 3 weeks post-infection (data not shown). The six FIV-positive animals reviewed in this study included five positive control cats who did not receive anti-retroviral therapy, and one cat (#7) that did receive a novel nucleoside analogue therapy. No other cats in this study had lesions that were grossly or histologically consistent with neoplasia. These FIV-positive cats exhibited $3.29 \times 10^4 - 1.68 \times 10^5$ viral copies/million PBMC on day 56 post-inoculation (Table 1). Compared to six sham-inoculated control cats, these animals had early decreased CD4:CD8 ratio, significant CD4 depletion, and neutropenia at 28 days post-infection (Fig. 1A–C). Three of these six FIV-positive animals developed neoplastic lesions and one of the six developed pre-neoplastic lesions, which were extensively characterized (Table 1).

FIV proviral loads in mesenteric lymph nodes from the six FIV-positive animals reviewed further were approximately two orders of magnitude lower than peripheral viral loads ($3.18 \times 10^2 - 8.69 \times 10^5$ viral copies/million mesenteric cells). PBMC proviral loads trended lower in cats noted to have lymphoma ($p=0.10$) compared to three cats in this group without lymphoma. Mesenteric proviral load was significantly lower in three cats with neoplastic ($n=2$) or pre-neoplastic ($n=1$) changes than three cats without phenotypic evidence of lymphoma ($p=0.028$) (Fig. 1D). The fourth cat with evidence of neoplasia (cat 4) was not included in the analysis of mesenteric proviral load as this animal was euthanized early in the study.

Neoplasia or pre-neoplastic lesions developed in four cats within four months of inoculation

Four animals developed neoplastic or pre-neoplastic changes within four months of virus inoculation in mesenteric lymph nodes ($n=3$), intestinal mucosa ($n=1$), also represented in mesenteric lymph node group) or bone marrow ($n=1$) as determined by gross and histologic post-mortem examination (Table 1). Neoplastic changes manifested primarily as effacement of normal lymph node architecture (cats 5 and 7) and intestinal mucosa (cat 5) with multiple large coalescing follicles with no apparent marginal zone and moderate cellular pleomorphism including anisokaryosis, anisocytosis, high nuclear:cytoplasm ratio, and

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**Fig. 1.** FIV C36 inoculated cats ($n=6$) exhibited (a) decreased CD4:CD8 ratio; (b) CD4 depletion; (c) and severe neutropenia in comparison to FIV naive controls ($n=6$). FIV proviral loads performed on mesenteric lymph nodes for six FIV-positive animals were approximately two orders of magnitude lower than peripheral viral loads (d); (a) Mean CD4:CD8 ratio in FIV-positive cats is markedly decreased in comparison to FIV naive cats over the 91 days study duration. (b) FIV-positive cats showed a decreased mean CD4 cell count in comparison to naive cats from study day 14–70. (c) Neutropenia is evident based on the mean neutrophil count in FIV-positive cats in comparison to FIV naive controls. (d) Mean PBMC proviral loads from day 42 post-infection trended notably lower in cats with lymphoma ($n=2$) or pre-neoplastic lesions ($n=1$) ($p=0.10$) compared to three cats without lymphoma. Mesenteric proviral load (terminal time point, day 91) was significantly lower in three cats with lymphoma: mean $= 5.2 \times 10^2$ viral copies/million mesenteric cells vs. cats without lymphoma $4.5 \times 10^3$ viral copies/million mesenteric cells ($p=0.028$) via student’s t-test statistical analysis. Error bars represent ± 1 standard deviation.
increased numbers of mitotic figures—all features compatible with follicular lymphoma (Fig. 2). Cat 4 developed neoplastic changes that included effacement and replacement of normal bone marrow elements by sheets of markedly pleomorphic lymphoid cells with a high mitotic rate. Multifocal aggregates of these neoplastic cells were also observed in the pulmonary, splenic, and hepatic parenchyma and are consistent with lymphoid leukemia with involvement of the liver, lung, and spleen (data not shown). Cat 6 demonstrated pronounced follicular hyperplasia in the mesenteric lymph node with merging of germinal centers and many follicles with narrow or discontinuous marginal zones, indicating pre-neoplastic changes (data not shown).

**Table 2**

<table>
<thead>
<tr>
<th>Cat/Tissue</th>
<th>CD79a (B cell)</th>
<th>CD3 (T Cell)</th>
<th>Clonality (PARR assay)</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 1 — Naïve cat with normal tissue</td>
<td>Nd</td>
<td>Nd</td>
<td>No clonality</td>
<td>Normal tissue</td>
</tr>
<tr>
<td>Cat 5 — Intestinal Mass</td>
<td>3</td>
<td>2</td>
<td>Nd</td>
<td>B cell follicular lymphoma with presumptive concurrent</td>
</tr>
<tr>
<td>Cat 5 — Mesenteric Lymph node</td>
<td>3</td>
<td>0</td>
<td>Primarily B cell</td>
<td>T cell neoplasm; restricted to mesenteric LN and intestinal mass</td>
</tr>
<tr>
<td>Cat 5 — Colon, retropharyngeal, peripheral lymph node</td>
<td>Nd</td>
<td>Nd</td>
<td>No clonality</td>
<td>Pre-neoplastic changes with weak B cell staining</td>
</tr>
<tr>
<td>Cat 6 — Mesenteric Lymph Node</td>
<td>2</td>
<td>3</td>
<td>Nd</td>
<td>B cell follicular lymphoma with presumptive concurrent</td>
</tr>
<tr>
<td>Cat 7 — Intestinal Mass</td>
<td>4</td>
<td>2</td>
<td>T cell</td>
<td>T cell neoplasm; restricted to mesenteric LN</td>
</tr>
<tr>
<td>Cat 7 — Colon, retropharyngeal, peripheral lymph node</td>
<td>Nd</td>
<td>Nd</td>
<td>No clonality</td>
<td>T cell neoplasm; restricted to mesenteric LN</td>
</tr>
</tbody>
</table>

**Immunohistochemistry demonstrates predominance of B cell lymphocytes within transformed tissues**

Immunohistochemistry (IHC) was performed to determine the phenotype of neoplastic cell populations using a scoring system of 0–4 (Table 2). Intense B cell staining (CD79a) in neoplastic cells of the mesenteric lymph nodes and within the intestinal mass of cat 5 indicated primarily B cell expression and composition (Fig. 3A–D). CD3 positive T cells were primarily located in paracortical and sinusoidal areas, consistent with normal, resident or migrating lymphocytes (Fig. 3A–D). Nuclei of these cells were round to ovoid, with 0–1 nucleoli, and were minimally pleomorphic. Cat 7 expressed high levels of the B cell marker, CD-79a, with lower levels of CD3, the T cell marker (Fig. 3E and F). Cells of the presumptive lymphoid leukemia diagnosed in cat 4 gave inconclusive IHC results for B and T cell markers (data not shown).

A hypertrophic mesenteric lymph node with abnormal follicles from cat 6 demonstrated mild to moderate CD79a staining, while CD3 labeling was marked in intensity and positive cells were widely distributed. The T cell expansion in this node was characterized by normal small lymphocytes, in contrast to the B cells in the markedly hypertrophic and abnormal follicles, which were enlarged with cleaved nuclei and frequent mitoses. Immunolabeling for FIV p24 gag expression using the PAK2C1 monoclonal antibody was either negative or demonstrated only very rare positive cells in any of the tissues examined (data not shown).**

**Determination of lymphoma clonality as B and T cell**

A PCR-based analysis of antigen receptor rearrangements (PARR) was conducted to extend elucidation of the origin of cells involved in the neoplastic process. This technique amplifies across the Ig-2 VDJ locus of mature B cells or the T cell receptor (TCR) locus of mature T cells. A clonal population of B or T cells (indicative of an oncogenic process) results in a peak in DNA amplicons of a unique size, whereas a heterogeneous population of cells (indicative of a polyclonal expansion of cells from an infectious disease process) results in a mixture of amplicon sizes (Avery, 2009; Lana et al., 2006). A PARR assay detection system that can detect approximately 1 neoplastic cell in 100 non-neoplastic cells was used. DNA samples from FIV naïve control cat 1 exhibited polyclonal amplification as expected in a naïve control animal (Fig. 4A and B). DNA derived from the tumor from cat 5 demonstrated primarily B cell (Fig. 4D), with some evidence of T cell clonality (Fig. 4C). The tumor tissue from cat 7 demonstrated populations of clonal cells of T cell...
Fig. 3. Immunohistochemistry from cats 5 and 7 indicates primarily B cell with moderate T cell expression. (A) Intestinal mass; cat 5, 40× and 4× (inset). Mass is composed predominately of B lymphocytes (black arrows) arranged in multilobular pattern. Anti-CD79a immunohistochemistry (IHC) with diaminobenzidine (DAB) as chromogen and hematoxylin counterstain. (B) Intestinal mass; cat 5, 40× and 4× (inset). Scattered T lymphocytes (red arrows) are present throughout the mass in an interlobular pattern and are less numerous than B lymphocytes. Anti-CD3 (IHC) with DAB as chromogen and hematoxylin counterstain. (C) Mesenteric lymph node; cat 5, 10× and 4× (inset). Neoplastic B lymphocytes multifocally expand the normal lymph node architecture. Anti-CD79a IHC with DAB as chromogen and hematoxylin counterstain. (D) Mesenteric lymph node; cat 5, 40× and 4× (inset). Paracortical and medullary sinus T lymphocyte (red arrows) populations are within normal limits. Anti-CD3 (IHC) with DAB as chromogen and hematoxylin counterstain. (E) Mesenteric lymph Node; cat 7, 40× and 4× (inset). Neoplastic B lymphocytes (black arrows) multifocally expand the normal lymph node architecture. Anti-CD79a IHC with DAB as chromogen and hematoxylin counterstain. (F) Mesenteric lymph Node; cat 7, 40× and 4× (inset). Normal amounts of interlobular T lymphocytes (red arrows) are present throughout the lymph node. Anti-CD3 (IHC) with DAB as chromogen and hematoxylin counterstain. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Clonality assay results from naïve control cat, cat 5, and cat 7. DNA from cells extracted from naïve cat mesenteric LN (a and b), grossly neoplastic mesenteric LN from cat 5 (c and d), microscopically diagnosed lymphoma from cat 7 (e and f), and peripheral lymph nodes from cat 7 (g and h) were subjected to a test for T cell (TCR) or B cell (Ig-2) clonality. FIV naïve cat exhibited polyclonal amplification. Discreet peaks demonstrate primarily B-cell clonality with likely concurrent T-cell expression in cat 5. Mesenteric LN tissues from cat 7 demonstrate T-cell clonality, while the peripheral LN tissues from cat 7 show no evidence of either TCR or Ig-2 clonality (product must be 3 × the size of the background to qualify as a positive result).
relative to this low viral copy number in a highly cellular tumor, a significantly lower in the three cats with lymphoma. As anticipated viral loads. Additionally, mesenteric proviral load was significant-ly higher in the FIV-positive cats. The cases we describe here emerged at less than four months (Callanan et al., 1992, 1996; Poli et al., 1994; Terry et al., 1995).

To support an indirect mechanism of tumor development, we ruled out a direct role for FIV tumor induction by (1) determination of peripheral and tumor proviral load and (2) Southern blot analysis to identify unique FIV sequence insertion sites in the two solid tumors. As shown in Fig. 1D, FIV proviral loads performed on mesenteric lymph nodes for six FIV-positive animals were significantly lower in the three cats with lymphoma. As anticipated relative to this low viral copy number in a highly cellular tumor, a unique clonal integration site was not detected using standard Southern blot analysis of restriction enzyme digested tumor DNA and a labeled FIV-gag probe (data not shown).

Discussion

This report describes a thorough characterization of FIV-associated neoplasms that were unexpectedly encountered in cats enrolled in an anti-retroviral therapy study. While FIV has been previously associated with neoplasia by others, the high incidence rate of animals affected (4 of a total of 17 FIV inoculated cats in this study), and the extremely rapid development of these neoplasms is highly unusual. FIV associated lymphomas in cats naturally infected with the virus have been reported to occur between 5 and 13 years of age (Callanan et al., 1996; Court et al., 1997; Ishida et al., 1989; Shelton et al., 1989b; Terry et al., 1995), while experimental FIV infection has been associated with tumorigenesis nine months to two years post FIV inoculation (Callanan et al., 1992, 1996; Poli et al., 1994; Terry et al., 1995). The cases we describe here emerged at less than four months post-inoculation. A thorough characterization of tumor phenotype, FIV load in tumor cells compared to bone marrow cells, and search for potentially associated co-factors provides indication that immune dysregulation induced by early viral infection resulted in high levels of malignant transformations apparently independent of direct FIV effects.

Mechanisms of retroviral-induced tumorigenesis include: (1) acute transformation, associated with the presence of a viral oncogene (v-onc) within a viral genome; (2) non-acute transformation, associated with retroviral integration into a site that disrupts normal cellular oncogene (c-onc) function; (3) transformation via viral envelope protein disruption of cellular signaling pathways; or (4) trans-activation, in which viral accessory genes dysregulate cellular homeostasis that would typically mitigate transformation events that occur during cell division (Maeda et al., 2008; Magden et al., 2011). The FIV genome does not contain v-onc regions. Clonal proviral integration sites were not detected via Southern blot analysis, and proviral load per cell calculated by real time PCR using DNA from tumor tissue was <10^4 copies/million cells. Thus, our results suggest that the virus did not disrupt a tumor suppressor gene or activate an oncogene resulting in a clonal expansion of one cell. Further, proviral burden was significantly lower in mesenteric lymph nodes with neoplastic or pre-neoplastic phenotype compared to PBMC proviral loads, suggesting an overgrowth of tumor cells without

Table 3

Summary of PARR characterization from all cats analyzed using the clonality assay. All cats tested other than cats 5 and 7 demonstrated polyclonal T cell receptor and Ig gene amplification, indicating no evidence of clonality. Peripheral lymph node samples from cats 5 and 7 also demonstrated polyclonal T-cell receptor and Ig gene amplification, indicating no evidence of clonality.

<table>
<thead>
<tr>
<th>Cat ID</th>
<th>Status</th>
<th>Site Sampled</th>
<th>Ig rearrangement</th>
<th>TCR rearrangement</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Naïve</td>
<td>MLN</td>
<td>Polyclonal</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>2</td>
<td>FIV+ with no histologic evidence of neoplasia</td>
<td>MLN</td>
<td>Polyclonal</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>3</td>
<td>FIV+ with no histologic evidence of neoplasia</td>
<td>MLN</td>
<td>Polyclonal</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>4</td>
<td>FIV+ with rapid onset of pneumonia, lymphoid leukemia</td>
<td>MLN</td>
<td>Polyclonal</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>5</td>
<td>FIV+ with gross tumor (MLN)</td>
<td>MLN</td>
<td>Monoclonal</td>
<td>Oligoclonal</td>
</tr>
<tr>
<td>6</td>
<td>FIV+ with histologic evidence of pre-neoplastic lesions (MLN)</td>
<td>MLN</td>
<td>Polyclonal</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>7</td>
<td>FIV+ with histologic evidence of neoplasia (MLN)</td>
<td>MLN</td>
<td>Polyclonal</td>
<td>Monoclonal</td>
</tr>
<tr>
<td>8</td>
<td>Colonic</td>
<td>MLN</td>
<td>Polyclonal</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>9</td>
<td>Prescapular</td>
<td>MLN</td>
<td>Polyclonal</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>10</td>
<td>Popliteal</td>
<td>MLN</td>
<td>Polyclonal</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>11</td>
<td>Retropharyngeal</td>
<td>MLN</td>
<td>Polyclonal</td>
<td>Polyclonal</td>
</tr>
</tbody>
</table>

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integrated provirus. We were therefore unable to attribute tumorigenesis to a direct mechanism relating to proviral insertion disrupting oncogene function, suggesting an indirect mechanism for tumorigenesis in this case.

HIV-associated lymphomas have been associated with infection with a second oncogenic virus, such as EBV (Hernandez and Shibata, 1995). Thus, we screened for a variety of feline co-pathogens that we hypothesized had the potential to amplify FIV-associated transformation, including FeLV and herpesviruses (Huang et al., 2012; Shelton et al., 1989b; Wen and Damania, 2010). However, we were unable to prove any associations via serologic and PCR assessments. While we cannot definitively rule out an as-yet undescribed gamma-herpesvirus, or other viral infection in these animals based on this negative data, the most likely co-morbidities resulting in the high rate of tumorigenesis noted were not detected in this study.

Histopathologic analysis and immunohistochemistry supported a diagnosis of B cell follicular lymphoma in both cats 5 and 7. The PARR assay demonstrated that cat 5 had a clonal population of primarily B cells, consistent with immunohistochemistry findings. The fact that IHC staining in cat 5 was not robust suggests that B cell markers were down-regulated, a finding reported by others in classical Hodgkin’s lymphomas (Tzankov et al., 2003). Based on our definition of clonality exceeding 3 × polyclonal baseline, we cannot characterize the tumor from cat 7 as a B cell origin. The PARR assay detects only approximately 75% of histologically confirmed lymphomas in cats (A. Avery, unpublished observations) because the primers do not anneal to all possible immunoglobulin V and J genes. Therefore, one explanation for failure to detect a clonal B cell population in cat 7 is that the lymphoma used V or J immunoglobulin genes not detected by our assay. In certain situations, such as gastrointestinal lymphoma, those numbers may differ. For example, Moore et al. (2012) found clonally rearranged immunoglobulin genes in only 50% of feline gastrointestinal B cell lymphomas. Thus a negative clonality result does not negate the histologic diagnosis of lymphoma.

We also demonstrated clonal T cell populations in both cats 5 and 7. This observation was not consistent with the morphologic appearance of T cells identified by immunohistochemistry, which had a classical reactive lymphocyte appearance versus a neoplastic phenotype. One explanation for this finding is that malignant transformation of T cells was coincident with B cell transformation within the tumor parenchyma. Co-existence of two lymphoid neoplasms is rare, but can be seen in a setting of immunosuppression and is usually associated with EBV virus (Yin et al., 2005). Alternatively, given the lack of overt histologic evidence of T cell lymphoma, it is possible that these clonal populations represent a restricted, and perhaps pre-neoplastic, expansion of a T cell clone. With time, this clone may also have developed into histologically recognizable lymphoma.

Three studies have documented FIV-associated T cell lymphomas. These previous studies have described lymphomas of T cell origin in one out of five (20%), 13 of 50 (26%), or one of two lymphomas (Endo et al., 1997; Gabor et al., 2001; Wang et al., 2001). These T cell lymphomas were diagnosed using cross-reactive antibodies to classify phenotypes by IHC, and by using the Working Formulation Classification System for histologic characterization. To our knowledge, determination of lymphoma phenotype by other methods such as the PARR assay has not been evaluated in a case-study like this, and demonstrates the interesting possibility that FIV-induced lymphocyte proliferation, or an FIV-associated immunological deficiency, may in some cases result in multiple simultaneous tumor formation associated with more than one lymphocyte lineage.

FIV and other lentiviral infections have been reported to result in hypergammaglobulinemia and B cell expansion during acute disease (Ackley et al., 1990; Rosenberg and Fauci, 1989; Yamamoto et al., 1997). It is likely that this expansion occurs as a result of immune activation and chronic antigen stimulation via cytokine and chemokine pathways, as well as impaired capacity for normal immune down-regulation. In the process of rapid expansion, it is possible that a B or T cell clone experiences somatic cell rearrangements or mutations that disrupt a tumor suppressor gene or activate an oncogene, resulting in the pattern noted in these animals (Sourvinos et al., 2000).

Conclusions

Given the high level of replication and previously described virulence of FIV-C36 (de Rozières et al., 2004; Terwee et al., 2008), the young age of the cats at time of inoculation, and the rapidity with which the lesions developed, the observed neoplasms likely arose due to mutations originating in the highly proliferative maturing population of B and T cells. The fact that the predominance of tumors reported here and in the literature occurs in mesenteric lymph nodes suggests a unique role for this site in FIV infections, particularly since uninfected cats more commonly develop an alimentary lymphoma versus a mediastinal anatomical location (Barrs and Beatty, 2012). Since the experimental animals were procured from a commercial closed-breeding colony SPF cat vendor, it is also possible that genetic or epigenetic factors (i.e. microbiome, undetected co-morbidities) contributed to the unusual incidence of neoplasia in this cohort.

The mesenteric lymph node targeting in FIV positive cats may be related to normal immune function of this tissue, co-infection with commensals or potential pathogens that normally inhabit the gastrointestinal tract, or specific targeting or trafficking of activated B cells to this organ. Future studies will more exhaustively evaluate potential co-factors that may contribute as well as specific immune dysregulation that may enhance susceptibility to oncogenic transformation.

Materials and Methods

Animal model

Cats aged approximately two months were purchased from a commercial SPF vendor (Cedar River Laboratories, Mason City, IA) as part of an anti-retroviral testing protocol. The cats were shipped to Colorado State University and acclimated for two weeks. The animals were divided by gender and litter and group housed for the study duration. Eighteen cats were inoculated intravenously with 1.0ml of a 1:25 dilution of previously characterized FIV-C36 stock known to induce a high titer viremia (described in Terwee et al., 2008), six of which served as positive controls and did not receive anti-retroviral treatment. One positive control was removed from the study due to an unrelated health issue prior to the end of the study, resulting in seventeen cats followed for the duration of the study, five of which were positive controls. Six negative control animals received a sham inoculation of media only. All procedures were approved by the Colorado State University Institutional Animal Care and Use Committee.

Hematology

Baseline CBC (complete blood count) with differential was performed prior to FIV inoculation and then weekly starting two weeks post-FIV inoculation and at days 21, 28, 35, 42, 50, 56, 63, 70, 77, 84, and 91. Blood samples were collected for a variety of analyses weekly, including plasma viral load, PBMC viral load, and
CD4/CD8 T cell subset analysis. Approximately 6 ml of blood were collected at each blood draw using Vacutainer® Brand Blood Collection Sets with 25 3/4 gauge needles. Blood was collected into 3ml sterile tubes with 7.5% liquid EDTA (K3) additive. Biochemical analysis and CBCs were performed by the Colorado State University Clinical Pathology Diagnostics laboratory, unless noted below. Animals were not anesthetized during blood withdrawal.

T cell subset analysis

To determine percentage of cells positive for CD4 and CD8 surface antigens, EDTA anti-coagulated blood was incubated with FITC-labeled mouse monoclonal anti-CD4 and PE-labeled mouse monoclonal anti-CD8 (Southern Biotech, Birmingham, AL) diluted in FACS buffer as previously described (Terwee et al., 2008). Red blood cells were lysed by Q-prep (Beckman Coulter), and flow cytometry performed on a CyAn cell sorter (DakoCyтомation, Glosthrop, Denmark). We analyzed results using the Summit software package (Dako). A Z1 Series Coulter Counter (Coulter, XL D/T) was used to determine total leukocyte and RBC counts. Differential leukocyte counts were determined manually from stained smears. Absolute neutrophil and lymphocyte counts were calculated by multiplying the total leukocyte count by the percentages of neutrophils or lymphocytes for each cat at each time point. To determine absolute CD4+ and CD8+ cell counts, total lymphocyte counts were multiplied by percentage of FITC (CD4) or PE (CD8) fluorescing cells. We collected blood from all cats 24 days prior to infection to establish baseline values.

FeLV, feline herpesvirus, feline panleukopenia virus, feline calicivirus sero-assays

Serum samples were assayed for FeLV p27 antigenemia at the Colorado State University diagnostic laboratory (CSU VDL) via a SNAP Combo FeLV Ag/FIV Antibody test manufactured by IDEXX Laboratories, Inc. CSU VDL also tested samples for antibodies to feline herpesvirus (FHV) and feline calicivirus (FCV) via a serum neutralization assay, and feline panleukopenia virus (FPV) via hemagglutination inhibition assay.

FIV proviral DNA and plasma RNA quantitation

Real-time PCR was performed on an iCycler thermocycler (Bio-Rad, Hercules, CA) using the AmpliTaq Gold DNA polymerase-containing TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). A real time PCR standard curve was generated from serial dilutions of feline peripheral blood mononuclear cells (PBMC) from 1000 to 5 x 10⁶ subjected to real time PCR for the cellular house-keeping gene, Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) as described by Leutenegger et al. (1999). PBMCs from study animals were purified from heparinized whole blood samples from experimental animals using a Histopaque (Sigma, St. Louis, MO) gradient according to the product insert, and frozen at –70 °C until processing. RNA was purified from plasma using a QiAamp Viral RNA Mini Kit (Qiagen, Valencia, CA). DNA was extracted from 1 million PBMCs using the Qiamp blood mini DNA kit (Qiagen, Valencia, CA). DNA from each sample was eluted with 50–200 µl of buffer. Primers and probe for FIV-C gag (Pedersen et al., 2001) were used to quantitate FIV in PBMCs and plasma. The sensitivity of detection is a minimum of 5 copies. DNA samples were also subjected to GAPDH real time analysis and values normalized to GAPDH standard curve to determine the number of cell equivalents per DNA sample; proviral copy number per cell was calculated on this basis (Terwee et al., 2008).

Necropsy, histology, and immunohistochemistry

At 91 days post-inoculation, necropsies were performed on cats 1–3 and 5–7. Cat 1 was a FIV naïve control. Cats 2 and 3 were FIV inoculated study animals that did not develop neoplastic lesions and are included for histologic comparison. Cats 5, 6, and 7 were FIV-inoculated cats that exhibited neoplastic changes. Cat 4 was euthanized and necropsied at day 39 due to a declining clinical condition. The mesenteric lymph nodes from each animal were located and removed by sharp dissection, and then placed into standard tissue cassettes. A 1 x 1 x 3 cm³ mass was found on the mesenteric border of the mid-jejunum in cat 5 and similarly removed by sharp dissection, and placed in a standard tissue cassette. The cassettes were then placed in 10% neutral-buffered formaldehyde for tissue fixation for 24 hours prior to trimming and processing for histology. Five µm paraffin sections were collected on charged slides (Superfrost; Colorado Histo-Prep, Fort Collins, CO), with one slide of each tissue stained with hematoxyline and eosin (H & E) for light microscope examination. Immunohistochemistry (IHC) was performed on the remaining consecutive sections using previously described protocols (Bielefeldt-Ohmann et al., 2008; Tolnay et al., 2010), employing the following primary antibodies specific for: T cell receptor complex protein CD3 (Abcam; clone number SP7), B cell receptor complex protein CD79a (Abcam; clone number HM47/A9), and FIV p24 capsid protein (Dr. Chris Grant, clone number PAK2C1). IHC was scored semi-quantitatively as previously described (Bielefeldt-Ohmann et al., 2008), in which tissues were graded on a scale of 0–4 by using the number of positive cells and the overall intensity of labeling as criteria to characterize gene expression in the following manner: 0—no apparent labeling, 1—minimal positive cells and/or labeling, 2—moderately distributed positive cells and/or labeling, 3—marked labeling and/or presence of positive cells, 4—diffuse, high-intensity labeling throughout the tissue.

PCR for antigen receptor rearrangements (PARR) clonality assays

DNA was isolated from snap frozen lymph nodes using the DNeasy Blood and Tissue kit from Qiagen. Between 10 and 100 ng of DNA was amplified using Qiagen Multiplex Master Mix kit. The following primers were used at a concentration of 200 nM in 3 individual 25 µL reactions:

Positive control (rhodopsin gene): 5′-FAM-ACCACCCA-GAACTGCTAGA-3′ (forward), 5′-CCGGAGTGCATGAAATG (reverse).

Immunoglobulin genes: VIC-CCGGAGACACCGCCACATATT (forward), CTCTGAGACCCGTCACCAG and CTCTGAGACCATGGTACAT (reverse).

T cell receptor genes: PET-AAAGCGAGYAGGGGACTG (forward), CTGGACAGTGGCCAGSACC (reverse) (Moore et al., 2012).

The cycling conditions were as follows: 94 °C for 8 s, 60 °C for 10 s, 72 °C for 15 s for 40 cycles in an ABI 9600 Thermocycler. Fluorescent PCR products were separated on an ABI 3130 × L Genetic Analyzer, and analyzed using GeneMarker software (Soft-Genetics, State College, PA). A sample was determined to be clonal if there were one to four discrete PCR products greater than 3 x the height of polyclonal products in the same sample. The sample was interpreted as polyclonal (negative for clonality) if there were heterogeneously sized PCR products present, with no individual product greater than 3 x the height of the next highest peak.

The primers used in the PARR assay were estimated to detect approximately 75% of confirmed feline lymphomas. PARR negative lymphomas may be explained one of three ways: (1) the
using a MasterCycler (Eppendorf®), samples were heated to bromide at 100 V for 30 min. Gels were imaged with the Kodak® management, and CSU Laboratory Animal Resources animal care provided valuable assistance with diagnostic assays and cat management. We would like to acknowledge Davis Seelig and Wendy Sprague who with the pathology associated with these cases. We would also like to thank Debra Kamstock for her assistance with project and case management.

Herpes virus screening via PCR

Tissue samples were screened for a concurrent herpes virus infection via PCR using nested degenerate primer sequences designed to amplify the DNA pol region of herpes viruses as previously described (VanDevanter et al., 1996). An ovine rhadovirus was used as a positive control (K. Fox, Colorado State University, Fort Collins, CO). Primary amplification of 300 ng DNA was performed in a 50 μL reaction mix. Two upstream primers (DFA (forward) 5'-GATTTGCCAGCAGCAGT-3' and ILK (forward) 5'-CTCGGACACAGCARNYNCGNMTMNA-3') and one downstream primer (KGI (reverse) 5'-GCTTGTTACACAGTNTACNCCYT-3') were used in the primary amplification. Secondary amplification was performed by mixing one upstream primer (TGV (forward) 5'-TGTAATCCGTGCYTAYGMMTYACNGNGT-3') and one downstream primer (IVG (reverse) 5'-CACAGAGTCCGTRTNCRRDATAD-3'). The reaction mixtures contained 45 μL Platinum® PCR Supermix (InvitrogenTM), 100 μM each of 1 primer (1 μL) and 1.5 μM (25 mM) MgCl2. Using a MasterCycler (EppendorfTM), samples were heated to 94°C for 3 min, 60°C for 2 min, 72°C for 1 min, 94°C for 30 s, 46°C for 1 min, and 72°C for 20 s to complete the first cycle. This cycle was repeated a total of 45 times after which the samples were heated to 72°C for 7 min and then held at 4°C. Twenty μL from the secondary amplification was electrophoresed in 1.5% agarose gel in Tris-acetate (TAE) buffer with 2 μL ethidium bromide at 100 V for 30 min. Gels were imaged with the KodakTM Image Station and examined for product of an appropriate size (200–250 bp). Similar studies performed with these primers were able to detect between 10 and 100 gamma- herpesviral genomes per reaction (R. Troyer, personal communication).

Southern blot analysis

DNA from mesenteric lymph node samples from two cats with confirmed B and/or T cell lymphoma (cat 5 and 7), cat 6 that exhibited pre-neoplastic changes in the mesenteric lymph node, and one control cat confirmed negative for FIV (cat 1) were digested. Genomic DNA (10 μg) was digested with EcoRI, fractionated on a 1% agarose gel, transferred to nitrocellulose and hybridized with a FIV-C36 tag-specific sequence probe.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

We would like to thank Debra Kamstock for her assistance with the pathology associated with these cases. We would also like to acknowledge Davis Seelig and Wendy Sprague who assisted with the clinical pathology analysis. Kelly Anderson provided valuable assistance with diagnostic assays and cat management, and CSU Laboratory Animal Resources animal care and veterinary staff assisted with project and case management.

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