Comparison of Feline Leukemia Virus-infected and Normal Cat T-Cell Lines in Interleukin 2-conditioned Medium

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ABSTRACT

Following mitogen stimulation (phytohemagglutinin or concanavalin A), feline lymphocytes were maintained in medium containing feline interleukin 2. Lymphocyte lines derived from the blood of feline leukemia virus-infected cats contained cells 50 to 90% of which expressed virus antigens, but lymphocyte lines derived from uninfected cats remained virus free. Leukemia virus-positive and -negative lymphocyte lines retained total dependence upon the presence of interleukin 2 for finite life spans of 20 to 40 cell divisions. Cell-doubling times and surface properties (membrane immunoglobulin negative, guinea pig red cell rosette positive) of all T-cell lines were similar. All cat lymphocyte lines rapidly developed strong but nonspecific cytotoxic effects against a variety of established cat target lines, including normal and leukemia virus-infected fibroblasts and virus-producing lymphomas. Attempts to infect virus-negative lymphocytes with leukemia virus in vitro produced lines containing 1 to 4% of infected cells; subsequently, this level of infection remained constant. Within observation limits, the characteristics of feline leukemia virus-infected and normal cat T-cells were similar. Leukemia virus infection did not predispose target lymphocytes to exhibit properties in vitro that might be associated with preneoplastic change, such as rapid or infinite cell division or development of independence from interleukin 2 regulation.

INTRODUCTION

FeLV3 is horizontally transmitted among cats (19, 21) via saliva which contains high titers of infectious FeLV when secreted by persistently viremic carrier animals (8). In suburban environments, at least 50% of all pet cats have received FeLV exposure by 3 years of age (13). The common outcome of infection is a transient infection which is rapidly terminated by the development of immunologic immunity, and as a result, only 1 to 2% of infected cats become persistently viremic (13, 14, 29). Leukemia and/or lymphoma is relatively common in cats, and the incidence is 5- to 10-fold greater than in dogs or humans (32). About 80% of feline leukemias appear in viremic cats, and the most common characteristics of preneoplastic or neoplastic cells when removed from in vivo homeostatic restraints. As some FeLV-producing feline lymphomas grow rapidly and are readily cultured in tissue culture. Evidence presented here suggests that FeLV-infected lymphocytes retain many characteristics of normal cat T-cells, thus indicating that FeLV infection per se is insufficient to cause FeLV-producing cat T-cells to assume a malignant phenotype.

MATERIALS AND METHODS

Animals. Cats were obtained through private donation and were housed in animal care facilities at the Harvard School of Public Health. All animals were screened for common pathogens, including FeLV infection, and were healthy and at least 6 months old when used as lymphoid tissue donors.

Preparation of Feline IL 2. Cats were sedated by ketamine injection (15 mg/kg i.m.) and exsanguinated via cardiac puncture, and the spleen and mesenteric lymph nodes were removed aseptically. Single-cell suspensions from lymphoid organs were prepared via mechanical disruption through fine wire mesh screens. Erythrocytes were removed from spleen cell preparations by layering washed cells over LSM (Litton Bionetics) and centrifugation. Lymphoid cell suspensions were washed 3 times before combining at a 1:5 spleen cell:mesenteric lymph node cell ratio.

Cells were then diluted to 2.5 x 10^6 nucleated cells/ml in RPMI Medium 1640 containing 2% FCS, 5 x 10^-5 M 2-mercaptoethanol, and 7.5 μg of Con A/ml (Sigma Chemical Co., St. Louis, MO) (25). After 24-hr incubation (37°, 5% CO₂) in 150-sq cm tissue culture flasks (Costar Plastics, Cambridge, MA), the supernatant was harvested following centrifugation at 600 x g for 30 min. After Millipore filtration (0.45 μm), conditioned medium containing IL 2 was stored at 4° or frozen at −70°.

In order to optimize growth factor yields, time-course studies were carried out on IL 2 appearance in culture supernatants sampled at intervals after addition of mitogen to feline lymphoid cell mixtures. Maximum IL 2 production was obtained after 10 to 20 hr, and subsequently, IL 2 secretion rapidly ceased; these findings were similar to those reported by others using mouse and human systems (1, 16).

Batches of conditioned medium were prepared individually from spleen and lymph node cell mixtures from 16 cats. Ten of these preparations contained IL 2 and supported the growth of test T-cell lines. We were unable to determine why spleen cell suspensions from 6 of the cats failed to yield active supernatants, although failure to produce IL 2 did not appear to be obviously associated with sex, breed, or age of the lymphoid cell donor cats.

There have been several reports that certain mouse, primate, and human leukemias secrete IL 2 after mitogen exposure (9-11, 27). We...
tested supernatants from 4 individual FeLV-producing feline lymphomas (designated FL74, F422, 3272, and 3281; see Ref. 15) for IL 2 activity both before and after stimulation with Con A. Cells from each lymphoma line were exposed to Con A (7.5 µg/ml) continuously for 48 hr during logarithmic growth from 2 × 10^5 cells/ml at initiation. Con A exposure produced marked agglutination of the single-cell suspension cultures. None of the lymphoma lines tested secreted detectable IL 2-like activity into their culture media before mitogen stimulation or at any time up to 48 hr after addition of mitogen.

**Initiation and Maintenance of IL 2-dependent Feline Lymphocyte Cultures.** Blood leukocytes from different cat donors were purified over LSM; thymocytes were prepared by mechanical disruption but were not LSM purified. After washing leukocytes 3 times in RPMI Medium 1640, individual cell suspensions were diluted to 1 × 10^6 cells/ml in growth medium (RPMI 1640:20% FCS:5 × 10^-5 M 2-mercaptoethanol:2 mM L-glutamine:1% bacterial antimycotic; Grand Island Biological Co., Grand Island, NY) and then incubated for 90 min or overnight with Con A (7.5 µg/ml) or PHA (0.375 µg/ml). After mitogen exposure, the treated cells were washed 2 times and resuspended at 5 × 10^5 cells/ml in a mixture of growth medium plus IL 2-containing medium in 25-cm^2 tissue culture flasks. Viable lymphocyte counts were made at daily intervals using a hemocytometer and phase-contrast microscopy. In cell cultures derived from FeLV-free cats, lymphoblasts were first observed 4 to 5 days after mitogen stimulation, but in cultures from FeLV-infected animals, large blast cells were evident after 2 to 3 days. Lymphocyte lines were diluted when necessary to maintain viable cells at concentrations between 1 × 10^6/ml and 6 × 10^6/ml. All IL 2-containing conditioned media were prepared following Con A activation of lymphoid cell mixtures, but lymphocyte lines which were initiated following preincubation with either PHA or Con A grew at equal rates in Con A-induced conditioned media.

**Titration of IL 2 Activity in Conditioned Media.** The activities of IL 2-containing culture supernatants were titrated using phase-contrast microscopy for viable cell counts. Serial dilutions of each IL 2 preparation were evaluated at daily intervals for 6 days to determine growth support of an original inoculum of 1 × 10^5 T-cells of feline thymocyte lines established previously. The 10 IL 2-positive supernatants varied in activity, but most supported maximal growth of feline lymphocytes when diluted 1:1 to 1:4 with fresh medium. Three of the conditioned media were more concentrated and supported maximum growth at dilutions of 1:6 to 1:8; at a concentration of 1:1, these highly active supernatants were toxic to established T-cell lines. Initially, we used ^3H-thymidine incorporation to provide an indicator of IL 2 activity, but a lack of correlation was occasionally observed in that some IL 2 preparations induced high levels of isotope incorporation, but a lack of toxicity, but most supported maximal growth when diluted to 1:10. For each IL 2-containing supernatant, the initial inoculum of T-cells was 1 × 10^5 cells/ml. The total count was determined by sodium dodecyl sulfate lysis of target cells; control counts represented release from target cells in medium alone, and this value varied according to the target line from 26 to 37% of total release at 18 hr.

**RESULTS**

**Establishment of Feline T-Lymphocyte Lines.** Heparinized jugulovenous blood (8 ml) was removed from 3 normal cats and from 3 persistent FeLV viremic individuals. Following separation of lymphocytes over LSM, the 6 individual cell samples were split. One-half of the cells were incubated overnight with PHA (1 µg/ml), then washed, and diluted in IL 2-containing media; remaining cells were not exposed to mitogen but were incubated directly in IL 2-conditioned media containing 10 µg of α-methyl-β-nanoside/ml to neutralize residual Con A. Cell viability in each culture was then monitored daily. In the absence of initial PHA stimulation, all lymphocyte cultures from both virus-negative and FeLV-positive individuals failed to grow. Conversely, following PHA stimulation, cell lines were established from the lymphocyte preparations from all cats. Results in Chart 1 summarize the differences noted during initiation of lymphocyte lines from virus-free and viremic individuals. Following PHA stimulation, the lymphocytes from normal donors exhibited no growth for the first 4 to 6 days of culture, and during this time, cell losses varied from 94 to 99% of the initial inoculum. Subsequently, blastogenesis of surviving cells was noted, and by Day 10, the 3 individual lines were growing in logarithmic phase. Lymphocytes from viremic individuals exhibited blastogenesis and logarithmic growth between Days 2 and 4, and initial cell losses in these cultures ranged from 70 and 87%. Following the induction period, the behavior patterns for all lymphocyte lines were similar as summarized in Table 1. Eventually, all cell lines died out despite a variety of attempts to maintain viability, including restimulation with PHA or Con A and maintenance in mitogen-free IL 2-conditioned media (containing an excess of α-methyl-β-nanoside). Prior to senescence, however, the virus-negative lines designated 1, 2, and 3 were cultured for 150, 110, and 120 days, respectively, and the FeLV-positive lines 4, 5, and 6 were maintained for 100, 134, and 83 days. For each line, the marked variations in mean cell doubling times resulted from slowing of growth as the culture interval progressed. Initially, all lines exhibited cell doubling at approximate 24-hr intervals, but after about...
Infection of FeLV-negative Lymphocytes with Virus in Vitro. Cell samples from 2 FeLV-free lymphocyte cultures were incubated for 24 hr with 10-fold dilutions of intact, infectious FeLV derived from culture supernatants of FL74 cells (15). Subsequently, the cells were washed and cultured for 4 weeks in parallel with the appropriate uninfected lines. Examination of infected lines by immunofluorescence (18) at 9, 16, and 30 days after incubation with virus revealed 1 to 4% of the lymphocytes were FeLV positive at all intervals. Virus nonexposed cultures remained 100% free of positive cells. Within the 1000-fold dilution range of the virus preparation used for T-cell exposure, there was no evidence to suggest a dose-dependent effect on the number of infected cells that resulted, for all exposed cultures contained a similar number of FeLV-positive lymphocytes.

Characteristics of FeLV-negative and -positive Lymphocytes. At 2-week intervals throughout the culture period, cell samples from each of the lymphocyte cultures (Table 1) were washed and resuspended in IL 2-free medium. In every case, the lines failed to grow when IL 2-conditioned medium was not supplied, although we commonly observed some cell division in the first 48 hr in normal medium. This initial increase in cell numbers rarely reached a doubling of the original cell inoculum (1 x 10^5/ml). Subsequently, all cells had died by 9 days after IL 2 deprivation.

Evidence that the cells cultured in feline IL 2 were lymphocytes was derived from morphology, for under phase-contrast examination, the cells were small, activated, mononuclear lymphoid cells. IL 2-maintained cells were also found to form spontaneous rosettes with GP-RBC, which is characteristic for feline T-cells (5). Both virus-infected and noninfected cells produced spontaneous E-rosettes, and although the percentage of E-rosette-positive cells remained consistent throughout culture, this number did not exceed 45% of the total cells (Table 1). In contrast, 65 to 80% of FL74 cells (a feline T-cell lymphoma) formed positive rosettes in parallel assays.

Lymphocytes obtained from virus-negative donors remained FeLV negative throughout culture, but the lines established from 3 FeLV-positive cats consistently produced FeLV in vitro. Each line was tested for FeLV infection at 21-day intervals; during the life of each line, the percentage of cells exhibiting virus production remained constant (Table 1).

Cytotoxic Effects of IL 2-maintained Feline Lymphocytes. The 6 cell suspension cultures from viremic and normal cats (Table 1) were tested for cytotoxic activity at both 4- and 18-hr incubation on a variety of cat target cell lines. Immediately after removal from donors, peripheral leukocytes exhibited low levels of cytotoxic activity (<25% specific release) at high E:T ratios (≥100:1). At lower E:T ratios (≤50:1), no cytotoxic effects were detected; this low-level cytotoxicity has been attributed to natural killer cell activity (23).

Following mitogen exposure and maintenance in IL 2-containing medium, the growing cells were tested for cytotoxicity after completion of 3 to 5 cell doublings. At this time, very high levels of cytotoxic activity were exhibited by all cell cultures as shown in Chart 2. Virus-producing and FeLV-free blood-derived lymphocyte cultures were equally cytotoxic to 3 individual FeLV-replicating feline leukemia target cell lines (designated FL74, FL422, and 3272). Cytotoxicity was also detected on normal feline killer cell activity (23).

Table 1

<table>
<thead>
<tr>
<th>Culture</th>
<th>FeLV infection status of donor</th>
<th>Total no. of cell divisions</th>
<th>Doubling time (hr)</th>
<th>% of cells FeLV infected</th>
<th>% of cells GP-RBC rosette positive</th>
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<tbody>
<tr>
<td>1</td>
<td>–</td>
<td>42</td>
<td>52 ± 20</td>
<td>0</td>
<td>29-40</td>
</tr>
<tr>
<td>2</td>
<td>–</td>
<td>42</td>
<td>46 ± 16</td>
<td>0</td>
<td>33-45</td>
</tr>
<tr>
<td>3</td>
<td>–</td>
<td>32</td>
<td>51 ± 16</td>
<td>0</td>
<td>26-36</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>30</td>
<td>44 ± 14</td>
<td>80-95</td>
<td>32-44</td>
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<tr>
<td>5</td>
<td>+</td>
<td>36</td>
<td>48 ± 18</td>
<td>50-60</td>
<td>19-39</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>23</td>
<td>46 ± 16</td>
<td>75-90</td>
<td>26-41</td>
</tr>
</tbody>
</table>

* Cell lines obtained after PHA stimulation of peripheral leukocytes.

b Cell growth determined by phase-contrast cell counts performed at 48-hr intervals.

c Determined by 3 fixed cell fluorescence (18) tests at 21-day intervals.

d Range from 3 tests performed at 18-day intervals. FL74 lymphoma cells used as controls and rosetted in parallel gave 65 to 80% rosettes (5).

e Mean ± S.D.
FeLV-infected Cat T-Cell Cultures

Embryo fibroblasts and also on the same fibroblast line that was productively infected with FeLV. The cytotoxic effects against lymphoma and fibroblastoid targets typified by the results in Chart 2 were obtained after an 18-hr incubation. At this time, specific cytotoxic effects against lymphoma targets were readily detected at E:T ratios of 3:1, for similar levels of cytotoxicity to be detected against fibroblast lines, it was necessary to increase the E:T ratio about 4-fold.

Cytotoxicity assays using an 18-hr incubation were repeated after the cell cultures had progressed through 7 to 10 and through 16 to 22 cell doublings; the cytotoxic effects obtained at each interval were similar. In every cytotoxicity test, the rate of cell killing was relatively slow; when assays were harvested after a short (4-hr) incubation, relatively little cytotoxic activity was evident.

DISCUSSION

FeLV infection and the establishment of persistent viremia commonly precede the emergence of overt leukemia or lymphoma in cats by a long interval often measured in years (7, 13). The oncogenic mechanism whereby T-cell infection eventually results in the emergence of virus-producing T-cell leukemias remains unknown. Others have described the conditions necessary for the successful long-term culture of normal cat T-cells in vitro (13). We wished to determine whether in vitro cultures of normal cat T-cells were susceptible to FeLV infection, or if lines of FeLV-infected T-cells could be isolated from persistently viremic animals in order to study a possible leukemogenic sequence of events in virus-infected cat T-cells. The experiments reported here revealed that the majority of uninfected T-cells in 3 individual normal donors were not readily infected with FeLV, although within each line, a small subpopulation (1 to 4%) of cells did express virus antigens after exposure to infectious virus. The infected:noninfected cell ratio in each culture remained constant for up to 20 cell doublings after in vitro infection, or up to 35 cell doublings after culture induction from viremic donors (Table 1). These observations indicate that FeLV infection does not predispose cat T-cells to grow at rates different from noninfected T-cells under the influence of feline IL 2, and as the T-cell lines which emerged exhibited essentially similar characteristics (finite life spans, division capacities, absolute dependency upon equal IL 2 concentrations, development of nonspecific cytotoxicity), we were unable to detect any overt characteristics of FeLV-infected lymphocytes that might be allied with a preleukemic state.

The one difference detected between noninfected and infected leukocytes was evident during initiation of the cell lines. After PHA stimulation, the great majority of lymphocytes from noninfected donors died, and in 2 of the 3 samples, the normal T-cell lines emerged from <1% of the original LSM-purified leukocyte inoculum. In the case of viremic donors, however, there was much less evidence for cell death, and by extrapolation (Chart 1), the cell lines developed from a much larger subpopulation of the original leukocyte preparation. Apparently, therefore, there exists a greater population of PHA-responsive cells in the blood of viremic cats than in blood from normal donors. In the context of the immune status of persistently viremic cats, this observation was unexpected, because there is no doubt that protracted FeLV infection leads to a state of generalized immunosuppression (13, 17, 22, 29, 34). Responses to PWM and Con A have been studied in detail in normal and viremic cats, and responses to Con A in particular are suppressed in infected animals (3, 4, 6, 20, 28). It appears, therefore, that an imbalance of lymphocyte subsets may accompany FeLV viremia, with strong suppression of Con A-responsive cells, weaker suppression of PWM responders, and elevation of the PHA-susceptible subset.

Development of nonspecific cytotoxic effects by normal PHA-stimulated lymphocytes is characteristic (26), but development of equal levels of killer activity by FeLV-producing T-cells indicates that, during virus production, the infected cells retain some normal lymphocyte membrane characteristics. Similarly, normal and FeLV-producing T-cell cultures expressed equal capacities to form spontaneous rosettes with GP-RBC, suggesting these membrane receptors remain unaltered during retrovirus replication.

Why <50% of the cells in each lymphocyte line formed spontaneous E-rosettes at each time point tested (Table 1) remains unclear. Viabilities exceeded 90%, and the low number does not reflect a property of FeLV-infected lymphocytes because a similar proportion of noninfected lymphocytes were E-rosette positive. It is possible, but unlikely, that the lines studied contained mixtures of cell types. Although only crude conditioned medium was used to support cell growth, the lines which emerged from the original PHA-stimulated LSM-purified leukocyte inocula were 100% surface immunoglobulin negative, and both morphology and histology indicated that each line was comprised of 100% activated lymphocytes. Possibly, the erythrocyte receptor is expressed on the lymphocyte surface at a specific stage of the division cycle, and therefore, at any one point in time, nonsynchronized cell populations contain only a minority of cells which are erythrocyte receptor positive.

Virus-producing feline lymphomas of T-cell origin (5, 15, 17) are not dependent upon exogenous IL 2 for proliferation in vitro, and unlike some lymphomas of other species (9–11, 27), these cell lines do not produce IL 2 (see "Materials and Methods"). The presumed precursors of these tumors are FeLV-producing T-
cells, such as the cells described here, and these cells retain absolute IL 2 dependence in vitro. It seems possible that FeLV-associated leukemogenesis is a multistep process in which FeLV infection is but the first stage. Although infected T-cell function is partly directed to retrovirus replication, the cells also appear to retain many normal characteristics. Leukemogenesis may therefore also involve secondary events, such as discrete mutations in single virus-infected cells. Provirus integration in the feline genome appears to be random (2), but it is likely that integration at certain sites would confer increased leukemogenic potential upon local secondary DNA damage, such as may be caused by environmental carcinogens. For example, provirus integration close to a gene governing expression of the IL 2 membrane receptor might predispose a secondary local mutation to result in an altered membrane receptor. If the altered receptor appeared in activated form on the membrane (such as it might normally assume only after complexing the IL 2), then a monoclonal FeLV-producing T-cell tumor, independent of exogenous growth control, would emerge from the milieu of FeLV-infected lymphocytes. The requirement for such secondary events would explain the variable, but usually protracted, latent period and the lack of appearance of leukemia in many persistently infected animals.

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