Macrophage Tropism of Feline Leukemia Virus (FeLV) of Subgroup-C and Increased Production of Tumor Necrosis Factor-α by FeLV-Infected Macrophages

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Erythroid aplasia is induced in cats by feline leukemia virus (FeLV) of subgroup C but not by FeLV of subgroup A. In an investigation of the role of macrophages in FeLV-C–induced diseases, the concentrations of FeLV and tumor necrosis factor-α (TNF-α) were compared between feline peritoneal macrophages incubated with FeLV of subgroup A or C. FeLV of both subgroups infected macrophages, but expression of FeLV-C was 21-fold higher than FeLV-A in peritoneal macrophages (P = .004). The supernatants of FeLV-C–inoculated macrophage cultures contained significantly higher levels of TNF-α (70 ± 14 U/mL) at 72 hours postincubation compared with FeLV-A–inoculated (38 ± 8 U/mL) and uninoculated (31 ± 8 U/mL) cultures. Moreover, a positive correlation was shown between cell-associated FeLV surface glycoprotein gp70 and TNF-α expression in FeLV-C–infected macrophages by immunofluorescence (r = .6; P = .001), measured with a computer-assisted, laser-based digital imaging system. The addition of TNF-α to a uniform population of FeLV-infected cells (feline embryonic fibroblasts) caused an enhancement of viral expression (P < .05). These results indicate that FeLV-C has tropism for macrophages. FeLV expression is positively correlated with TNF-α expression in macrophages, and TNF-α enhances FeLV replication in fibroblasts. We suggest that FeLV-C infection of macrophages and secretion of TNF-α may be important in hematopoietic suppression in FeLV-C–infected cats.

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were centrifuged at 400g for 10 minutes at 4°C to concentrate the cells. Concentrated cells were diluted with 10 mL minimal essential medium-α (MEM-α; Gibco Laboratories, Grand Island, NY) and 10 mL Hanks’ Balanced Salt Solution (HBSS, Gibco) and underlayered with 10 mL Percoll (Pharmacia). Samples were centrifuged at 400g for 20 minutes at 22°C. Interface cells were washed two times in HBSS and resuspended at 10 mL were incubated for 72 hours at 37°C in 75-cm² tissue-culture flasks (Costar, Cambridge, MA) in 15-mL MEM-α containing 0.5% bovine serum albumin (BSA; Intergen Co, Purchase, NY), glutamine (2 mmol/L), 0.5% penicillin-streptomycin (GIBCO), 33% horse serum (GIBCO), and 11% pokeweed-conditioned medium (PWMCM) from feline BM mononuclear cells. Nonadherent cells with medium were removed from flasks after 24 and 48 hours and the cultures were replenished with fresh medium. After 72 hours of incubation, adherent cells were collected by scraping with a rubber policeman, washed twice, and resuspended in MEM-α. Cells were stained with Giemsa for morphologic evaluation and with α naphthyl butyrate esterase (ANBE) for nonspecific esterase activity (Sigma, St Louis, MO). Positive staining for nonspecific esterase activity was detected in 81% ± 8% of the cells.

Virus inoculation. FeLVs of subgroups A and C derived from feline embryonic fibroblasts (FEA cells) were kindly provided by Dr Jennifer L. Rojko (The Ohio State University, Columbus). Viral inocula were standardized by virus infectivity assays. For in vitro FeLV infection of peritoneal macrophages, 1 X 10⁶ cells were incubated with biologically cloned FeLV-A or FeLV-C at a multiplicity of infection of 1.0 for 2 hours at 37°C. After 2 hours of incubation, cells were washed twice with PBS to remove free virus before culturing in 12-well plates.

TNF-α and FeLV determinations. Peritoneal macrophages were cultured at 1 X 10⁶ cells per well in 1.5-mL media in 12-well tissue culture plates (Costar) at 37°C in a humidified atmosphere containing 5% CO₂ and 10% O₂. Culture supernatants (110 μL) were collected after 2, 24, 72, and 168 hours postincubation for TNF-α and FeLV p27 determinations. Culture supernatants were centrifuged at 560g for 10 minutes at 4°C to remove cellular elements and stored at −70°C.

TNF-α was measured using a bioassay involving the TNF-sensitive L-929 cell line and the MTT tetrazolium assay as described below. A standard curve was made using recombinant human TNF-α (Genzyme Corp, Cambridge, MA). The 27,000-dalton core protein (p27) of FeLV was detected in cells on cytocentrifuged slides by use of an indirect immunofluorescent assay and in culture supernatants by a modified enzyme-linked immunosorbent assay (ELISA) (FeLV antigen test kit; Synbiotics Co, San Diego, CA).

Quantitative analysis of cell-associated FeLV and TNF-α in peritoneal macrophages. In selected experiments, peritoneal macrophages were collected from 12-well plates 72 hours after FeLV inoculation. Cells were dually stained for FeLV antigen and TNF-α. Briefly, cytocentrifuge preparations fixed in cold methanol were incubated with 1:20 dilution of mouse monoclonal anti-FeLV gp70 antibodies (kindly provided by Dr Jennifer L. Rojko) and 1:40 dilution of polyclonal rabbit anti-TNF-α antibodies (Genzyme) simultaneously for 1 hour at 37°C in a humidified atmosphere. After incubation, slides were washed twice in PBS for 5 minutes each, and rinsed in distilled water. Slides were then incubated with 1:80 dilution of phycoerythrin (PE)-labeled goat anti-mouse IgG antibodies and 1:80 dilution of fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG antibodies (Southern Biotech Assoc, Birmingham, AL) simultaneously for 1 hour at 37°C in a humidified atmosphere. After the second incubation, slides were washed twice in PBS for 5 minutes each, rinsed with distilled water, and mounted with 50% glycerine in PBS. Slides incubated with mouse anti-FeLV alone, polyclonal rabbit anti-TNF alone, PE-labeled anti-mouse IgG alone, and FITC-labeled anti-rabbit IgG alone were used as controls.

The ACAS 570 interactive laser cytometer (Meridian Instruments, Okemos, MI) is a computer-assisted, laser-based digital imaging system for quantitative and distributional analysis of fluorescence signals of anchorage-dependent cells. An inverted phase-contrast microscope with a microprocessor-controlled stage is used to rapidly transport cells past the microscope objective. At preselected intervals, a laser beam is pulsed and the resultant fluorescent emission is detected with a sensitive photomultiplier tube. The collected data are displayed on a color monitor as a fluorescent intensity curve of a cross-sectional profile or as a pseudocolor, two-dimensional image of a scanned area. FeLV-infected and control cells were analyzed with the ACAS 570 interactive laser cytometer with an argon laser tuned to 488-nm-exciting wavelength and three optical filters: 555 DR/LP, 530/30 BP, and 575 DF25 for PE and FITC. Cells were randomly selected and at least 40 cells per experiment were analyzed from each culture of FeLV-A-infected, FeLV-C-infected, and uninfected macrophages in four independent experiments. The average integrated fluorescence for PE and FITC for each cell was computed. The background fluorescence observed in control cells treated with PE- and FITC-labeled antibodies only was subtracted from the fluorescence observed in FeLV-A-infected, FeLV-C-infected, and uninfected cells treated with anti-gp70/anti–TNF-α antibodies and PE-labeled/FITC-labeled antibodies.

TNF-α treatment of FeLV-infected fibroblasts. FeLV-infected FEA cells were kindly provided by Dr Jennifer L. Rojko. To study the effect of TNF-α on FeLV expression, 1 X 10⁶ FeLV-A-infected fibroblasts or FeLV-C-infected fibroblasts were incubated with 0, 10, 100, or 1,000 U/mL of recombinant human TNF-α (Genzyme) in 200 μL media at 37°C for 2 hours in humidified atmosphere and cultured in 6-well tissue-culture plates in 2 mL media containing 84% Dulbecco’s MEM (GIBCO), 15% heat-inactivated fetal calf serum (FCS) (Hazelton Res Products, Lenexa, KS), and 1% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO₂ and 10% O₂. Culture supernatants were collected after 48 hours for assay of FeLV p27.

Colorimetric MTT tetrazolium assay. In selected experiments, the MTT tetrazolium assay was performed as described with modifications to measure viability of peritoneal macrophages and FEA cells. In brief, 1 X 10⁶ cells in 100 μL media were cultured in 96-well tissue culture plates (Costar) for 2, 24, 72, or 168 hours. After specific incubation periods, 20 μL of MTT solution (5 mg/mL) were added to each well and plates were incubated for 4 hours at 37°C for cleavage of MTT. After the incubation, 100 μL extraction buffer containing 20% sodium dodecyl sulfate (SDS) and 50% N,N-dimethyl formamide (Sigma) were added to each well and plates were incubated overnight at 37°C. Absorbance was measured using an ELISA plate reader ( Molecular Devices, UV Max) with a test wavelength of 580 nm and a reference wavelength of 650 nm.

Endotoxin screening. FEA cells and media were screened for endotoxin and were found to be negative at the 0.015 endotoxin unit (EU) level by the Limulus amoebocyte lysate assay (E-Toxate; Sigma).

Statistical analysis. Data were analyzed using one-way analysis of variance at 95% level of significance and simple linear regression and correlation. Multiple comparisons were made using the Bonferroni method of multiple comparisons.

RESULTS

Differential expression of FeLV by macrophages infected with FeLV-A versus FeLV-C. FeLV infection of macrophages was documented by increased p27 levels in the supernatants of cultures inoculated with FeLV. Both FeLV-A and FeLV-C...
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Fig 1. FeLV p27 levels in supernatants of FeLV-A- and FeLV-C-inoculated macrophage cultures and cell viability. Peritoneal macrophages were collected from normal SPF cats and were inoculated with FeLV-A or FeLV-C as described in Materials and Methods. The 27,000-dalton core protein (p27) of FeLV was measured using a modification of a commercially available ELISA kit (Synbiotics Co). The cell viability was evaluated using colorimetric MTT assay as described in Materials and Methods. Results represent mean ± SEM from six independent experiments. *P < .05 significantly different from FeLV-A. (♀) FeLV-A; (♀) FeLV-C; (□) control.

Fig 2. TNF-α levels in culture supernatants of macrophage cultures inoculated with FeLV-A or FeLV-C and uninoculated controls. Peritoneal macrophages were collected from normal SPF cats and were inoculated with FeLV-A or FeLV-C as described in Materials and Methods. TNF-α titers were measured in culture supernatants by a bioassay using the TNF-sensitive L929 cell line as described in Material and Methods. Results represent mean ± SEM TNF U/mL from six independent experiments. *P = .058 significantly different from FeLV-A and control. (♀) FeLV-A; (♀) FeLV-C; (□) control.
with the expression of TNF-α on an individual cell basis, immunofluorescent studies were performed on cultured macrophages after dual labeling with antibodies to FeLV gp70 and TNF-α. Cells of macrophage cultures inoculated with FeLV-A or FeLV-C and uninoculated control cells were treated with primary murine anti-FeLV gp70 antibodies and rabbit anti-TNF-α antibodies and stained with secondary antibodies labeled with PE (anti-gp70) or FITC (anti-TNF-α). Individual cells were analyzed for relative fluorescent intensity and distribution of both labels with a computer-assisted, laser-based digital imaging system. The average integrated fluorescence per cell attributed to FeLV gp70 was significantly higher ($P = .02$) in FeLV-C-inoculated cells compared with FeLV-A-inoculated cells (Fig 3). Similarly, average integrated fluorescence per cell related to TNF-α was significantly higher in FeLV-C-inoculated cells compared with FeLV-A-inoculated ($P = .04$) and uninoculated cells ($P = .014$). Although the average integrated fluorescence per cell for cell-associated TNF-α was higher in FeLV-A-inoculated cells compared with uninoculated cells, the difference was not statistically significant (Fig 3). There was a significant and positive correlation between FeLV expression and TNF-α expression in FeLV-C-inoculated macrophages ($r = .6; P = .001$). When the FeLV-negative cells were excluded from the analysis, the correlation between FeLV expression and TNF-α expression was stronger ($r = .8; P = .001$).

Effects of TNF-α on expression of FeLV in infected fibroblasts. The immunofluorescent studies of coexpression of FeLV and TNF-α showed a correlation between TNF expression and FeLV protein concentration in macrophages. It was not clear whether TNF-α enhanced FeLV expression. This issue was addressed in fibroblasts by characterization of FeLV expression in FEA cells that were uniformly infected with FeLV-A or FeLV-C. The addition of TNF-α (1,000 U/mL) to FeLV-infected fibroblasts (FEA cells) caused a significant enhancement of viral expression as indicated by increased levels of p27 antigen in culture supernatants ($P < .05$). Similar enhancement was detected in fibroblasts infected with FeLV-A versus those infected with FeLV-C (Fig 4). No...
significant effect on growth of fibroblasts was observed with these concentrations of TNF-α. A similar dose-dependent enhancement of virus replication was observed with 10 and 100 U/mL of TNF-α in FeLV-infected fibroblasts (data not shown).

**DISCUSSION**

In this study we showed that macrophages are target cells for FeLV of subgroup C. FeLV-C was expressed at 21-fold higher levels in peritoneal macrophages compared with FeLV-A. The finding that FeLV-A was expressed only at low levels in peritoneal macrophages is in agreement with previous studies of Hoover et al. with the Rickard strain of FeLV (FeLV-R) that contains subgroups A and B. In their experiments with FeLV-R, treatment with hydrocortisone was required to make macrophages permissive to FeLV-R infection. In contrast, in these studies, infection of untreated peritoneal macrophages in vitro with FeLV-C resulted in higher levels of virus expression. Recently, we showed that macrophages from the BM also are susceptible to infection with FeLV-C in vitro. Persistent infection of BM macrophages is associated with other retroviral infections including HIV-1 and SIV.12,21,22

TNF-α, primarily produced by activated macrophages, occurs in soluble and cell-associated forms.23 The cell-associated form of TNF-α kills tumor cells and virus-infected cells by cell-to-cell contact.24 FeLV-C-infected macrophages produce greater amounts of both forms of TNF-α than do FeLV-A-infected macrophages. The increased TNF-α expression by FeLV-C-inoculated macrophages compared with FeLV-A-inoculated macrophages and uninoculated macrophages correlates with our previous findings of higher TNF-α levels in cocultures of BM macrophages and FeLV-C-infected fibroblasts.13 Increased TNF-α secretion has been associated with HIV and SIV infection of macrophages.9,10,28

In the present study, TNF-α levels were higher early in the infection, which is similar to results of in vitro studies of other viral infections including HIV-1 and Sendai virus.35,36 A significant and positive correlation was present between FeLV expression and cell-associated TNF-α expression in peritoneal macrophages. A similar association between increased TNF-α and productive viral infection of macrophages has been reported with influenza A virus.27 Treatment of FeLV-infected fibroblasts with TNF-α enhanced FeLV replication in a dose-dependent fashion. These findings are consistent with similar stimulatory effects of TNF-α on HIV-1 replication.28,29

Increased production of TNF-α by FeLV-C-infected macrophages may play a role in the suppression of hematopoiesis in FeLV-C-infected erythroid aplasia in cats. Colony-forming units-erythroid (CFU-E) and burst-forming units-erythroid (BFU-E) are suppressed in FeLV-infected cats, whereas colony-forming units-granulocytes/monocytes (CFU-GM) are unaffected.30,31 We recently have shown that feline erythroid progenitors are extremely sensitive to the inhibitory effects of TNF-α.25 The erythroid progenitors have been shown to be infected with FeLV-C in experimentally induced feline erythroid aplasia.33 FeLV-C-infected thymic lymphoma cells exhibit increased palmitate acid proportions,34 which has been shown to potentiate TNF effects.35 FeLV-C-infected erythroid cells may acquire enhanced susceptibility to the inhibitory effects of TNF-α through an increased palmitate production. Macrophage dysfunctions, including defects in monocyte to macrophage maturation, have been reported in human retroviral infections.36 Similar functional abnormalities in FeLV-infected macrophages, in addition to increased TNF-α production, may impair erythroid progenitor and macrophage interactions. A defect in monocyte maturation has been suggested to play a role in the pathogenesis of aplastic anemia in humans.27

In this study, we showed that both FeLV of subgroup A and FeLV of subgroup C infect macrophages, but FeLV expression in macrophages is 21-fold higher with FeLV-C than with FeLV-A. The high level of expression of FeLV in FeLV-C-infected macrophages correlates with higher levels of TNF-α compared with FeLV-A-infected macrophages. It is suggested that FeLV-C is a monocytotropic virus and that increased expression of TNF-α by FeLV-C-infected macrophages may play a role in the pathogenesis of erythroid aplasia in FeLV-infected cats.

**REFERENCES**

5. Testa NG, Onions D, Jarrett O, Frassoni F, Eliason JF: Hae-matopoietic colony formation (BFU-E, GM-CFC) during the development of pure red cell hypoplasia induced in the cat by feline leukemia virus. Leuk Res 7:103, 1983


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