Infection of Peritoneal Macrophages In Vitro and In Vivo with Feline Immunodeficiency Virus

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Macrophages were harvested from the peritoneal cavities of healthy specific-pathogen-free cats by saline lavage. Three days before collection, the peritoneal cavities were stimulated with glutaraldehyde-fixed *Saccharomyces cerevisiae* cells to induce greater numbers of macrophages and to begin the activation sequence. Peritoneal macrophages from cats stimulated once with yeast consisted mainly of small macrophages and a smaller number of larger activated macrophages. After several days in culture, many of the small macrophages became activated and a portion of the activated macrophages developed into multinucleated giant cells. Peritoneal cells from cats that were stimulated twice or three times with yeast at 3-week intervals consisted of a higher proportion of activated macrophages initially and produced more and larger multinuclear giant cells with time. Cultures of peritoneal cells stimulated once with yeast were easily infected in vitro with feline immunodeficiency virus (FIV) and produced a transient burst of reverse transcriptase activity. After the initial burst of virus replication, the infection became latent. Even more multinucleated giant cells appeared after infection, and many of these cells fused with each other. Replicating virus could be rescued from the latently infected macrophages after 2 to 3 weeks of phorbol myristate acetate stimulation and cocultivation with T-lymphocyte-enriched peripheral blood mononuclear cells. Multiply stimulated peritoneal cells, which contained a much higher proportion of activated macrophages, could also be infected in vitro with FIV. The infection usually became latent, however, without going through an initial replicative stage. Peritoneal cells from chronically FIV-infected specific-pathogen-free cats contained a higher proportion of activated macrophages and were latently infected with FIV from the outset.

Initial research on human immunodeficiency virus (HIV) infection emphasized the tropism of the virus for the CD4+ subclass of T lymphocytes (8). The actual reservoir for HIV persistence is probably not T lymphocytes, however. HIV infection has been identified as a noncytolytic infection in macrophages in the brain, lungs, lymph nodes, and skin (1, 6, 7, 15, 17, 22). In some cases, infected macrophages form multinucleated giant cells (6, 10, 14, 16). On the basis of these and earlier studies of lentivirus infections of goats and sheep (1, 9, 12), the role of macrophages in HIV infection has become a major topic of research (1, 16).

Research into the role of macrophages in the pathogenesis of HIV infection in humans has relied heavily on tissues from patients with the acquired immunodeficiency syndrome, peripheral blood monocyte cultures, or chronic tumor cell lines such as the human promonocyte cell line U-937 or its derivatives (2, 3, 6, 10, 17, 22). There is a need, therefore, for either more readily available or natural models for macrophage-lentivirus interactions. Recently, a lentivirus has been isolated from domestic cats with an acquired immunodeficiency syndrome-like disorder (15, 23, 24). The causative agent, called feline immunodeficiency virus (FIV), is morphologically and structurally similar to HIV (15) but is antigenically and genetically distinct (21, 24). FIV infection is a promising model for HIV infection of humans, but more basic research is required to validate various aspects of the model. This study was concerned, therefore, with the susceptibility of feline macrophages to FIV infection and, in particular, how FIV infection of feline macrophages compares with HIV infection of human macrophages.

Ten-month-old specific-pathogen-free (SPF) cats were obtained from the breeding colony of the Feline Retrovirus

Research Laboratory of the School of Veterinary Medicine, University of California, Davis. Animals were housed in facilities of the Animal Resources Services, University of California, Davis. Cats were infected with the Petaluma strain of FIV as previously described (15, 24). The FIV-infected cats were in the chronic asymptomatic stage that follows the transient primary stage of the infection (24).

Peritoneal macrophages were harvested from the peritoneal cavities of adult SPF cats, essentially as previously described (4, 18, 20). Cats were injected intraperitoneally with 15 ml of a 1% suspension in phosphate-buffered saline of glutaraldehyde-fixed *Saccharomyces cerevisiae*. The yeast cells were grown in yeast extract-peptone broth with D-glucose, pelleted by centrifugation, washed three times in Hanks balanced salt solution (HBSS), and then fixed overnight at 4°C in 2% glutaraldehyde in Millonig buffer. The yeast cells were washed three times in HBSS, suspended in HBSS to a concentration of 10% (vol/vol), and stored at 4°C.

Three days after the yeast inoculation, the cats were sedated with ketamine hydrochloride (50 mg intramuscularly) and 300 to 600 ml of warm sterile saline was infused into the peritoneal cavities. The abdomens were gently massaged for 30 s, and the fluid was withdrawn through an 18-gauge 1-in. (2.54-cm) needle attached to a large syringe and a three-way valve. Multiply stimulated cats were inoculated with yeast at 3-week intervals and peritoneally lavaged 3 days after the final yeast injection. The peritoneal lavage fluid was centrifuged at $1,000 \times g$ for 10 min, and the cell pellet was washed three times in HBSS and then suspended in 5 ml of RPMI 1640 medium containing 10% fetal bovine serum and $1 \times$ penicillin and streptomycin. A 0.2-ml sample was centrifuged, and the cell pellet was suspended in 1 drop of fetal bovine serum. The cells were smeared onto a glass slide and stained with new methylene

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FIG. 1. Phase-contrast photomicrograph of 2-day-old culture of peritoneal leukocytes from a healthy SPF cat that was prestimulated once intraperitoneally with glutaraldehyde-fixed yeast cells. The predominant cell types at this stage of the culture are small macrophages and activated macrophages. Magnification, $\times 750$.

blue stain, and a differential leukocyte count was performed. The cell suspension was then adjusted to a concentration of 5×10^5 mononuclear cells per ml of tissue culture medium. Five milliliters of the adjusted cell suspension was placed in each 25-cm² plastic culture flask. The flasks were then incubated for 24 h at 37°C in an atmosphere containing 5% CO₂. Nonadherent cells were gently suspended, transferred to a new flask, and treated as lymphocytes. After 7 days, the adherent cells were washed twice in HBSS and exposed to a 1× trypsin solution for 10 min at 37°C to selectively remove adherent fibroblast-type cells. Macrophages were characterized by nonspecific α -naphthyl esterase staining (Sigma Chemical Co., St. Louis, Mo.) and by their ability to phagocytose glutaraldehyde-fixed yeast cells (as determined by phase-contrast light microscopy). Virtually all of the adherent cells phagocytosed yeast cells and stained positively for nonspecific esterase.

Peritoneal macrophage (7-day-old fibroblast-depleted) and peripheral blood mononuclear cell (PBMC) cultures were infected in vitro with the Petaluma strain of FIV (15) propagated in Crandell feline kidney cells (24). Tissue culture fluid was harvested when reverse transcriptase activity reached a minimum of 250,000 cpm, cells and subcellular debris were removed by low-speed centrifugation, and 1-ml portions were frozen at -70°C. Flasks (25 cm²) containing one-half-confluent monolayers of peritoneal macrophages were drained of medium, washed twice in HBSS, and then

overlayered with 1 ml of a 1:10 dilution in HBSS of the stock virus inoculum. The cultures were then incubated for 24 h at 37°C, washed three times with HBSS, and fed with complete medium.

Peritoneal lavage fluid from healthy SPF cats that had been stimulated once with intraperitoneally administered yeast cells contained many polymorphonuclear neutrophils and several types of mononuclear cells. Most of the polymorphonuclear neutrophils died during the first 48 h, leaving a nonadherent population of small mononuclear cells that behaved as lymphocytes when stimulated with interleukin-2 and two populations of adherent mononuclear cells (Fig. 1). These adherent mononuclear cells made up approximately 25% of the leukocyte population in the original lavage fluid (Table 1). The predominant adherent cell was a small macrophage about 10 to 15 μ m in diameter and containing a single nucleus. This cell stained positively for nonspecific esterase and was avidly phagocytic for yeast cells; five or more yeast cells were evident in each macrophage (data not shown). A second population (10 to 15% of the adherent cells) of macrophagelike cells was evident; they were about 20 μ m in diameter, had from one to three nuclei, and exhibited large veil-like membrane projections. These cells also stained positive for nonspecific esterase and were actively phagocytic for yeast cells. These cells were characteristic of activated macrophages.

After 2 to 7 days in culture, a small proportion (2 to 10%)

TABLE 1. Yield of various types of cells from peritoneal lavage fluid obtained from the abdomens of yeast-stimulated cats^a

Cat no.	No. of times stimulated	Total (10 ⁷)		
		WBC	MNC	Adherent MNC
22	1	10.8	4.3	2.6
3335	1	7.9	2.4	1.3
	2	45.4	5.4	3.3
	3	17.3	10.4	6.2
3337	1	13.0	4.2	2.5
	2	50.3	36.7	22.0
3051	1	22.7	5.0	3.0
3053	1	9.5	2.9	1.7
3156	1	11.5	2.6	1.6
4	1	4.1	0.9	0.5
	2	5.8	1.5	0.9

^a Healthy SPF cats were stimulated once, twice, or three times with glutaraldehyde-fixed yeast cells. WBC, Leukocytes; MNC, mononuclear cells.

of the activated macrophages developed into larger multinucleated giant cells. These cells were 40 to 60 μ m in diameter and contained 2 to 25 or more nuclei clustered near the center of the cytoplasm (Fig. 2). They stained weakly positive for nonspecific esterase and were weakly phagocytic for yeast; individual cells contained only one to five yeast particles (data not shown). Activated macrophages and multinucleated giant cells survived for >16 weeks in culture, while the smaller macrophages tended to disappear from the culture during the first 3 weeks.

Small macrophages made up 35 to 40% of the leukocytes present in the peritoneal cavity lavage fluid harvested from healthy SPF cats that were stimulated with yeast cells twice or three times at 3-week or greater intervals (Table 1). Unlike the macrophage population from cats stimulated once with yeast cells, macrophages in 7-day-old cultures from multiply stimulated cats were 25 to 50% or more activated macrophages. Many of the activated macrophages became multinucleated giant cells upon subsequent culture; these cells were larger and contained even more nuclei than cells of the same type obtained from once-stimulated cats. The various macrophage populations from multiply stimulated cats and cells of the same types obtained from cats stimulated only once with yeast behaved identically in terms of nonspecific esterase staining and yeast phagocytosis (data not shown).

Peritoneal macrophages from once-stimulated cats were easily infected with FIV in vitro. Reverse transcriptase

FIG. 2. Phase-contrast photomicrograph of an 8-day-old culture of peritoneal macrophages from the cat described in the legend to Fig. 1. The predominant cell types at this stage of the culture are activated macrophages and multinucleated giant cells. Magnification, $\times 750$.

TABLE 2. In vitro FIV infectibility of peritoneal macrophage cultures established from healthy SPF cats stimulated once, twice, or three times with yeast cells before cell harvesting

Cat no.	No. of times stimulated	No. of cultures with RT activity ^a		
		+	+/-	-
22	1	4	1	0
3051	1	6	0	0
	2	1	2	3
3053	1	3	0	1
	2	1	3	4
3156	1	2	0	2
3335	1	3	0	0
	2	1	1	7
	3	0	2	9
3337	1	2	2	2
	2	1	3	12

^a Reverse transcriptase (RT) activity was greater than three times (+), two to three times (+/-), or less than twice (-) the level in noninfected controls.

activity was detectable 7 to 14 days after infection in the supernatants of 71% of the cultures that were obtained from such cats (Table 2). Reverse transcriptase levels in positive cultures were generally low, ranging from 2 to 10 times the background levels for control cultures. A pronounced die-off of the small macrophages occurred during this period, but it was not possible to ascribe all of this cell death to virus-induced cytolysis because of the high spontaneous death rate in the small-macrophage population in noninfected cultures. Following the spontaneous or virus-induced death of the smaller macrophage cells, the reverse transcriptase activity in the cell supernatants declined rapidly to nonsignificant levels. Concurrent with the disappearance of reverse transcriptase activity from the culture supernatants, there was a progressive increase in the proportion of multinucleated giant cells. Some of the FIV-infected multinucleated giant cells became 5 to 10 times larger than similar noninfected giant cells (Fig. 3). This enlargement was associated with the fusion of adjacent multinucleated giant cells with each other, as determined by observing groups of adjacent cells by phase-contrast light microscopy over a 24-h period. Each fused multinucleated giant cell contained 100 or more nuclei arranged in distinct clusters scattered throughout the cytoplasm. Individual clusters contained 20 to 40 or more nuclei (Fig. 3). A remnant of intervening cell membrane was often visible between the clusters of nuclei, again suggesting that

FIG. 3. Phase-contrast photomicrograph of a 14-day-old culture of peritoneal macrophages from a healthy SPF cat that was prestimulated twice intraperitoneally with glutaraldehyde-fixed yeast cells. The cultures were infected on day 7 with FIV. A characteristic cell in FIV-infected cultures is the fused multinucleated giant cell. This cell arises from the fusion of adjacent multinucleated giant cells, as evidenced by the presence of groups of nuclei throughout the cytoplasm and remnants of intervening cell membranes. Magnification, $\times 750$.

TABLE 3. Reverse transcriptase (RT) activity in PMBC culture supernatants derived from FIV-infected and noninfected SPF cats^a

Cat no.	FIV status	RT activity (cpm/ml)
4	Noninfected	2,509
37	Noninfected	2,378
6	Infected	187,269
10	Infected	133,311
20	Infected	75,069
35	Infected	114,358
2433	Infected	115,052
2836	Infected	132,750

^a Cells were cultured for 14 days before the addition of concanavalin A- and interleukin-2-stimulated PMBC cultures established from the blood of FIV-infected and noninfected SPF cats.

these extremely large cells resulted from the fusion of several smaller multinucleated giant cells (Fig. 3).

Only 10% of the individual peritoneal macrophage cultures from healthy SPF cats stimulated twice with yeast cells yielded significant reverse transcriptase activity after in vitro infection with FIV, 23% yielded indeterminate or low levels of reverse transcriptase activity, and 67% yielded no reverse transcriptase activity whatsoever (Table 2).

Reverse transcriptase activity was even more difficult to detect in three-times-stimulated macrophage cultures that were infected in vitro with FIV; none of the cultures yielded high reverse transcriptase activity, 18% yielded indeterminate or low levels, and 82% were negative (Table 2). Morphological changes were even more obvious with multiply stimulated peritoneal macrophage cultures than in once-stimulated cultures. A greater proportion of the cells developed into multinucleated giant cells, and subsequent syncytium induction yielded cells that were even larger than comparable cells in FIV-infected, once-stimulated peritoneal macrophage cultures.

Peritoneal macrophage and PBMC cultures were established from SPF cats that had been infected with FIV 6 to 18 months earlier and stimulated once with yeast. PBMC cultures, enriched for T lymphocytes, were established by a previously described procedure (15, 24). Reverse transcriptase activity (15, 24) appeared within 8 to 15 days in the PBMC cultures and reached levels greater than 180,000 cpm/ml (Table 3). Peritoneal macrophage cultures from the same FIV-infected cats either did not spontaneously express reverse transcriptase or expressed it only at low levels (Table 4). Fused multinucleated giant cells did appear with time in the peritoneal macrophage cultures, in the same manner as in cultures of once-stimulated peritoneal macrophages infected in vitro with FIV.

Peritoneal macrophage cultures from cats stimulated once, twice, and three times were maintained for 3 to 12 weeks after in vitro FIV infection. Twelve of 22 once-stimulated cultures were transiently (1 to 2 weeks) virus positive after initial infection, compared with 3 of 26 twice-stimulated and 0 of 5 three-time-stimulated cultures. This initial burst of reverse transcriptase activity was low, however, and there were only background levels of activity at the time of the study. At this point, nonproductively infected macrophage cultures were stimulated with 10^{-7} M phorbol myristate acetate for 1 week. After 1 week of phorbol myristate acetate stimulation, the peritoneal macrophage cultures were cocultivated with concanavalin A and interleukin-2-stimulated PBMCs (2×10^6 cells per ml).

TABLE 4. Reverse transcriptase (RT) activity in peritoneal macrophage cultures derived from FIV-infected and noninfected SPF cats^a

Cat no.	FIV status	RT activity (cpm/ml) in:	
		Macrophages	Macrophages + PBMC
37	Noninfected	1,460	1,502
6	Infected	1,723	ND ^b
7	Infected	3,018	ND
10	Infected	4,394	32,441
20	Infected	2,152	23,176
26	Infected	3,164	28,722
35	Infected	2,097	18,114
2433	Infected	8,095	ND
2836	Infected	5,573	34,693

^a Cells were cultured for 14 days before the addition of concanavalin A- and interleukin-2-stimulated PBMC from healthy cats. After addition of the PBMC, the cultures were incubated for an additional 14 days before reverse transcriptase activity was measured.

^b ND, Not done.

Reverse transcriptase activity was detectable in the culture supernatants beginning 15 to 21 days after cocultivation (data not shown). Long-term in vitro FIV-infected peritoneal macrophage cultures from cats stimulated once with yeast cells had the highest rate of virus reactivation (12 of 22 [54%]). Cultures from twice-stimulated cats, which usually did not show virus production during acute in vitro infection, took longer to yield virus, and only 11 of 26 (42%) were positive. Only 1 of 5 (20%) of three-time-stimulated macrophages yielded reverse transcriptase after cocultivation with normal PBMCs. Mock-infected cultures, and FIV-infected cultures that were not cocultivated with PBMCs after phorbol myristate acetate stimulation, did not produce reverse transcriptase during similar periods.

The degree of peritoneal macrophage activation increased with consecutive bouts of yeast stimulation, indicating that immunologic mechanisms were responsible for macrophage activation. In support of this hypothesis, peritoneal macrophages obtained from the abdomens of saline-stimulated cats did not become activated following collection (20). Peritoneal macrophages from once-stimulated and from multiply stimulated SPF cats were sensitive to FIV infection in vitro, but the behavior of the infection depended on the differentiation status of the macrophages. Once-stimulated peritoneal macrophages, which contained more small macrophages, underwent a rapid but transient productive infection that may have been cytolytic to this cell type. After the replicative phase of infection, activation of the remaining infected macrophages appeared to be greater than that induced by yeast stimulation alone. This indicated that FIV infection, like yeast stimulation, was an additional activation stimulus for the macrophages.

Once peritoneal macrophages became activated, they would no longer support a replicative FIV infection. This was true whether the cells were well activated at the time of collection (multiply stimulated cats) or after culture for several days in vitro (once-stimulated cats). The tendency of FIV to cause latent infection in more-differentiated macrophages and active infection in nondifferentiated macrophages is consistent with what is known about HIV infection but different from what has been shown for the non-immunodeficiency-causing ungulate lentiviruses. Immature monocytic cells replicate HIV, whereas the infection is largely latent in differentiated macrophages (2). Conversely, imma-

ture macrophages are latently infected with CAEV and V-MV, whereas infection in differentiated macrophages is productive and lytic (13).

Many of the multinucleated giant cells in FIV-infected cultures fused with each other to become cells of extraordinary size. Cell fusion of this type is typical of lentivirus infections and is mediated by interactions between specific receptors on the cell membranes and viral envelope glycoproteins (17, 19). The fact that it occurred in latently infected cultures suggests that even nonproductively infected cells must elaborate some viral envelope glycoproteins on their surfaces.

Like HIV, FIV could be rescued from macrophages by stimulation with phorbol esters. Phorbols have been shown to induce macrophage differentiation and growth arrest and to stimulate NF- κ B-binding activity (3). NF- κ B is the transcription factor known to stimulate the HIV enhancer in activated T cells (11). The control of NF- κ B in macrophages is different from control in T cells, however. The NF- κ B-binding activity increases when T cells undifferentiate to blast cells and began to actively divide, whereas in macrophages, activity increases as the cells become more differentiated and cease to divide. Significantly, other lentiviruses that cause immunodeficiency (simian immunodeficiency virus and FIV) possess a functional NF- κ B binding site in their long terminal repeats, while similar binding sequences are not observed in the long terminal repeats of non-immunodeficiency-causing lentiviruses such as CAEV or V-MV (E. Sparger and P. Luciw, personal communication).

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