Cytopathogenic Test for Feline Leukemia Virus Infections

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Received for publication 29 October 1971

A mixed culture cytopathogenic effect with the development of multinucleated giant cells in feline leukemia virus-infected cultures after cocultivation with XC cells is described. This cytopathogenic effect has been utilized as a convenient visual marker for the detection and assay of feline leukemia virus in feline and canine embryo fibroblasts as well as in a canine tumor cell line, M-132-1. Comparison of the cytopathogenic reaction in the three cell lines indicates that the reaction is more rapid and pronounced in M-132-1 cells than in the embryo fibroblast cultures.

In vitro infections of susceptible cell cultures by murine, avian, and feline leukemia viruses are not generally accompanied by observable changes in these cultures. However, serological techniques such as complement fixation (7) and immunofluorescence (10) have been developed for the detection and assay of non-cytopathogenic viruses. Klement et al. (2) and Rowe et al. (6) have developed and refined an indirect cytopathogenic test for the quantitation of murine leukemia viruses (MuLV).

In a preliminary communication, we described a mixed cell culture cytopathogenic (MC) method using XC cells for the detection and assay of feline leukemia virus (FeLV) in a canine tumor cell line, M-132-1 (5). Here we report the use of feline and dog embryo fibroblasts (FEF and DEF), which are generally used in FeLV studies, for the assay of FeLV by the MC method. Characteristics of the syncytial giant cells formed in the MC test using embryo fibroblasts and M-132-1 cells are also compared.

MATERIALS AND METHODS

General tissue culture methods. All stock cell cultures were maintained in 32-oz (ca. 960 ml) screw-capped glass bottles at 37°C. All culture media were supplemented with 10% inactivated fetal calf serum, 100 units of penicillin, 100 μg of streptomycin, and 5 μg of fungizone per ml of medium. Petri dish cultures were incubated at 37°C in a humidified 5% CO₂ atmosphere.

FEF and DEF cultures. These were obtained from Flow Laboratories, Rockville, Md., as primary cultures and were maintained in Eagle’s minimum essential medium (MEM—Eagle’s base) (Grand Island Biological Co., New York). The medium for feline embryo cultures contained 1% tryptose phosphate broth.

XC cells. XC cells, a cell line of a rat tumor induced by an avian sarcoma virus (8), were obtained from W. P. Rowe, National Institutes of Health, Bethesda, Md., and maintained with serial subpassage in MEM.

M-132-1 cell line. This canine tumor cell line was originally derived from a canine fibrosarcoma in a female coonhound by John R. Mitchell (Michigan State Public Health Department) and had undergone undetermined tissue culture subpassages. The cell line was grown and maintained in modified McCoy’s 5A medium (Grand Island Biological Co.) and underwent 30 subpassages in our laboratories. The culture is characterized by rapidly dividing cells of relatively small size which readily attach to glass or plastic substrate and appear stellate, fusiform, or round (Fig. 1). The cells initially grow as a dispersed monolayer of cells, but with increasing cell density spherical clumps of cells of varying sizes are formed throughout the monolayer. In very heavy cultures, the individual cells and clumps grow as a floating culture in addition to the monolayer on the substrate. Karyological examination of the cells from passage 29 showed chromosome numbers ranging from 53 to 88 with a modal chromosome number of 73. The spreads examined consisted primarily of telocentric chromosomes, with frequent meta- or submetacentric chromosomes. This pattern is characteristic of most long-term canine tumor cell cultures (3, 4). The canine origin of the M-132-1 cell line was further confirmed in an immune cytotoxic test using an antiserum to canine thymus cells prepared in horses and an antiserum to tissue cultured cells of a canine lymphosarcoma prepared in rabbits. Both sera were highly toxic to the M-132-1 and DEF cells but not to mouse embryo fibroblasts. Electron microscopic examination of the M-132-1 cells did not reveal virus-like particles. In utero inoculation of M-132-1 cells produced fibrosarcomas in dogs.
M-132-1 (FeLV). When M-132-1 cells were inoculated with a stock of FeLV prepared from a chronically infected feline lymphoblast culture, FL-74, the culture became chronically infected by about day 25, as confirmed by electron microscopy, complement fixation, and fluorescent antibody techniques using FeLV-specific antisera. The infected culture had undergone several serial subpassages and served as a source of canine cell-derived FeLV. Morphologically, the cells resemble the uninfected M-132-1 cultures in all respects.

FL-74. This is a floating culture of feline lymphoblasts chronically infected with FeLV (9). A stock culture of this cell line was obtained from Pfizer, Inc., Maywood, N.J. Electron microscopic examination of the cells in thin sections revealed large numbers of extracellular C-type particles and particles budding from the cell membrane.

Virus stocks. A stock of FeLV no. 41 was prepared from FL-74 culture. Two other FeLV stocks, 184 and 461, were prepared from M-132-1 (FeLV) cultures. Details on the preparation of virus stocks 41 and 184 have been described elsewhere (5). Virus stock 461 was prepared identically to the other stocks but was concentrated 100 times. Possible murine leukemia virus (MuLV) contaminants in stocks 41 and 184 were excluded by the micro-Ouchterlony gel diffusion method (5). Virus stock 461 was similarly tested and confirmed to be free of (MuLV) contaminants. A preparation, no. 186, from uninfected M-132-1 cultures was made in a manner identical to the FeLV stocks and used as a control inoculum in virus infection studies.

Antisera. For complement-fixation (CF) and neutralization tests, antisera specific against FeLV prepared in guinea pigs and antisera specific against MuLV prepared in rabbits were obtained from Electro-Nucleonics Laboratories, Inc. For immunofluorescence tests, antisera specific against FeLV prepared in goats were obtained from Huntington Laboratories, Maryland.

Virus infection. Petri dish cultures of M-132-1, FEF, and DEF were made in 60-mm Falcon plates at an initial cell inoculum of 3 x 10⁴ cells from trypsinized stock cultures and maintained in MEM. Twenty-four hours later, 0.4 ml of serial 10-fold dilutions of the virus stocks or control preparation were allowed to adsorb onto the cells at 37 °C for 45 min. The cultures were refed and maintained routinely for a period of 21 days with successive subpassages at 3- to 4-day intervals before being tested for virus infection. Occasionally, virus-inoculated cultures were tested earlier or later than 21 days.

Virus detection. As a routine, virus-inoculated and control cultures were subpassaged in such a manner that duplicate cultures derived from the same parental petri plate were available for parallel CF and MC tests.

CF test. Cells from cultures to be tested were dislodged with a rubber policeman and suspended in 2 ml of the spent culture medium, frozen and thawed three times in a dry ice-alcohol mixture, and kept frozen at -70 °C until used. Culture harvests were tested at a dilution of 1:2 in micro-CF plates, as described for the COCAL test (7). Positive virus controls at 1:10 or higher dilutions were included in each test. A sample was considered positive for viral CF antigens if a 3+ or greater CF reaction was observed in the absence of anticomplementary reactions and in the absence of positive reactions with negative tissue culture antigen specimens.

RESULTS

Mixed culture cytopathogenic reaction. In an initial experiment, FeLV 41-inoculated FEF cultures, which had undergone one subpassage during the 12 days postinoculation, were used. Strips of monolayer cells from the control and virus-treated petri dish cultures were removed in two perpendicular directions with a rubber policeman as described by Klement et al. (2). These cultures were overlaid with 10⁶ freshly trypsinized XC cells in MEM supplemented with 10% noninactivated fetal calf serum and were incubated at 37 °C in a humidified 5% CO₂ atmosphere.

During the next 48 hr, multinucleated giant cells were observed in the virus-treated cul-
tures along the border and in the channels where the monolayer had been removed. The giant cells, however, were not confined to the channels but were present between the fibroblasts of the intact monolayer. Four to five days later, the cytopathic reaction of multinucleated giant cell development was conspicuous and clearly visible upon microscopic examination at 35 or 80× magnifications in methanol-fixed, Giemsa-stained cultures. The giant cells, in addition to being observed frequently, were made prominent by their unusual large size and shape. Some of these cells contained one or more prominent vacuoles. The number of nuclei in the giant cells varied from as few as three to over a dozen in many. Control FEF cultures processed in an identical manner were devoid of such large numbers of multinucleated cells.

Even though syncytial giant cells appeared in confluent and heavy monolayers of virus-treated cultures upon mixed cultivation with XC cells, it was difficult and time-consuming to score the cytopathic effect. Hence, the procedure for MC tests was modified, based on controlled experiments using M-132-1 (FeLV) cells.

Results of these experiments indicated that the seeding concentration of FeLV-infected cells was an important factor in rendering the MC reaction clearly visible. Best results were obtained when cultures were initiated with approximately $3 \times 10^3$ M-132-1 (FeLV) cells and the 50 to 75% confluent cultures were overlaid with $10^4$ XC cells 24 hr later. Other parameters, such as temperature of incubation or use of inactivated fetal calf serum in the growth medium, did not significantly alter the MC reaction. Under these cultural conditions, many syncytial giant cells were detected as early as 3 to 4 hr. The number of giant cells and the number of nuclei in them increased during the next few days (Fig. 2). Some of the multinucleated cells contained many dozens of nuclei. Also, it was not unusual to find one or more large vacuoles in some of the syncytial cells. Continued incubation beyond 48 to 72 hr resulted in the depletion of cells, including the giant cells in the cultures. This could be due to lysis of the large giant cells and sloughing of cells, since, in heavy cultures, both infected and control M-132-1 cells tend to form spherical clumps and to float off the culture substrate. FeLV assay results on M-132-1 cells were, therefore, scored within 48 hr of addition of XC cells. Control M-132-1 cultures cocultivated with XC cells were devoid of multinucleated giant cells (Fig. 3).

For the detection and assay of FeLV infections, FEF and DEF virus-treated and control cultures were passaged in a manner to obtain a monolayer nearly 70% confluent by 24 hr at which time $10^4$ XC cells were added. Syncytial giant cells were observed within a day of mixed-cell cultivation; however, they became prominent 4 or 5 days later. The multinucleated giant cells were distributed along with normal XC cells as localized islands bordered by a network of elongated cells apparently of fibroblastic origin (Fig. 4 and 5). Unlike the virus-infected M-132-1 cultures, FeLV-inoculated FEF and DEF cultures could be carried for longer periods without deterioration of the cytopathic reaction; hence, end points for virus assays were determined on day 6 or 7 after XC cell addition.

Mixed cultures of normal FEF or DEF and XC cells under identical conditions were devoid of the cytopathic reaction described above for FeLV-infected cultures (Fig. 6 and 7). Both types of cells continued to grow as a homoge-
results obtained by the MC reaction and by the COCAL method are clearly comparable in a majority of instances. As an exception, DEF cells inoculated with virus stock 184 were negative for virus infection by the MC test when performed 21 days postinoculation, whereas a parallel COCAL test was positive. This discrepancy could be explained by the fact that in these experiments the DEF cultures were not in the optimal condition for registration and detection of multinucleated giant cells, since the cultures were confluent, multilayered, and not subpassaged regularly. The same population of virus-inoculated DEF cultures, however, registered a positive MC reaction when tested under optimal conditions at a later time, whereas similar cultures treated with control preparation 186 were still negative. Virus stock 184 assayed on FEF cells titered one dilution higher by COCAL than by MC test. Whether this discrepancy is a true reflection of the relative sensitivity of the two tests

![Fig. 3. Control M-132-1 cells cocultivated with XC cells showing the presence of the two types of cells. x200.](image)

nous population. Depending on the concentration of embryo fibroblasts at the time of XC cell addition, the cultures either were completely overgrown by XC cells or contained few networks of elongated cells along with XC-type cells. Other control cultures, such as FeLV-infected FEF and DEF or XC cell cultures alone, had no or very infrequent syncytial cells (Fig. 8, 9, and 10).

XC cell cultures inoculated with serial 10-fold dilutions of the virus stocks did contain some multinucleated giant cells when monitored over a period of a week. However, their frequency of occurrence was not similar to those observed in the MC tests. Nor was there a direct correlation between virus dilutions and the number of giant cells observed in these cultures.

The MC reaction served as a convenient visual indicator for an assay of FeLV in FEF, DEF, and M-132-1 cell cultures. The results obtained from these tests and those determined in parallel by the COCAL method are compared in Table 1. It is evident that the

![Fig. 4. Feline leukemia virus-infected feline embryo fibroblasts cocultivated with XC cells. Island of multinucleated giant cells and XC cells with elongated fibroblastic cells are seen at the peripheral regions. x500.](image)
tested for virus neutralization on M-132-1 cells (Table 2). It is clear that FeLV was selectively neutralized by the anti-FeLV serum as determined by CF and MC tests.

An additional indirect support for the validity of the MC tests for FeLV infections was obtained by testing the susceptibility of M-132-1 cells to a preparation of tissue culture derived MuLV (R). Cultures of mouse embryo fibroblasts and M-132-1 cells were inoculated with serial 10-fold dilutions of the MuLV stock, and the cultures were carried with serial subpassage at 3- to 4-day intervals. After 21 days, the cultures were tested by MC test. Syncytial giant cells were observed only in mouse embryo fibroblast cultures infected with MuLV (2). The MuLV-inoculated M-132-1 cultures which were negative for virus infection by the MC test were also negative for the presence of MuLV-specific CF antigens.

The applicability of the MC test for other isolates of FeLV was determined by titrating a

is difficult to evaluate since the FEF cultures inoculated with a dilution of $10^{-4}$ virus stock were not available for an additional MC test at a later time. Also, the nonoptimal conditions of these cultures for the registration of MC test cannot be excluded.

Even though the virus stock 184, derived from canine cells, infects M-132-1, FEF, and DEF cultures, it has a higher end point titer in FEF than in M-132-1 or DEF cultures, as determined by the CF test. Whether this reflects the different concentrations of FeLV-specific CF antigens induced in cultures of canine origin is a question for further investigation.

Virus neutralization studies. To confirm the validity of the MC assay for the FeLV preparations, equal volumes of serial 10-fold dilutions of virus stocks 184 and 461 were incubated with either a 1:4 dilution of anti-FeLV guinea pig serum, anti-MuLV rabbit serum, or growth medium at 37 C for 1 hr with agitation. Samples of the above mixtures were

Fig. 5. Feline leukemia virus-infected dog embryo fibroblasts cocultivated with XC cells. Number of multinucleated giant cells and XC cells are seen as islands bordered by elongated fibroblastic cells. x200.

Fig. 6. Normal feline embryo fibroblasts cocultivated with XC cells. Island of XC cells bordered by feline embryo fibroblasts is seen. An occasional large cell with a single or a few nuclei is also seen. x200.
cytopathogenic test for FeLV infections
cally visible quantitative plaque assay for FeLV have not been successful. However, multinucleated giant cells developed with the same rapidity in mixed cultures of XC and M-132-1 (FeLV) cells when either one of the cell types was exposed to ultraviolet irradiation.

**DISCUSSION**

The MC test offers an additional indicator system for the detection and assay of FeLV infections in cell cultures. This method, as has been pointed out by Klement et al. (2) for MuLV, has the distinct advantage of not requiring specific, difficult to produce serological reagents needed in CF and immunofluorescence tests. Rowe et al. (6) have developed a plaque assay technique based on this reaction for the in vitro quantitation of MuLV. Although our studies in this direction using FeLV have been unsuccessful, the MC test has the inherent potential to detect focal areas of FeLV infections in an appropriate host cell system.

The detection of FeLV infection by the MC test in a majority of the titration experiments

tissue culture-derived FeLV stock obtained from P. S. Sarma, National Institutes of Health, Bethesda, Md., on M-132-1 and FEF cells. Comparable titers were obtained for this stock on the two cell types as determined by both COCAL and MC tests. In addition, FEF cultures chronically infected with two other isolates of FeLV obtained from C. Rickard, Cornell University, Ithaca, N.Y., gave positive MC reaction upon the addition of XC cells. Also, serially subpassaged FEF cultures inoculated with a feline sarcoma virus preparation (virus stock used was prepared from a sarcoma induced in a newborn kitten by a feline sarcoma virus stock obtained from L. Bustead, University of California, Davis) is approximately a month before developed multinucleated giant cells in the MC test. Parallel cultures tested by the CF method indicated the presence of FeLV-specific antigens in these cultures.

Preliminary studies similar to those of Rowe et al. (6) for the development of a macroscopi-

**FIG. 7.** Normal dog embryo fibroblasts cocultivated with XC cells showing island of XC cells bordered by fibroblastic cells. A few larger cells are also seen among the XC cells. x200.
Fig. 9. Feline leukemia virus-infected dog embryo fibroblasts. ×200.

Fig. 10. XC cells with a few large cells, some of which contain more than a single nucleus. ×200.

Table 1. Comparison of complement fixation (CF) and mixed culture cytopathogenic assay of feline leukemia virus in different cell types

| Cell type | Days post-inoculation | Virus 41 | Virus 184 | Virus 461 | Control 186 |
|-----------|-----------------------|----------|-----------|-----------|-------------|
| FEF       | 14                    | 10⁻²     | 10⁻²      | 10⁻⁵      | 10⁻⁵        |
|           | 21                    | 10⁻²     | 10⁻⁴      | 10⁻⁵      | 10⁻⁵        |
|           | 19                    | 10⁻²     | 10⁻⁴      | 10⁻⁵      | 10⁻⁵        |
|           | 20                    |          | 10⁻⁴      |           |             |
|           | 22                    | 10⁻²     | 10⁻⁴      | 10⁻⁵      | 10⁻⁵        |
|           | 25                    |          | 10⁻³      |           |             |
|           | 24                    |          | 10⁻⁴      |           |             |
| M-132-1   | 16                    |          | 10⁻³      |           |             |
|           | 19                    |          | 10⁻⁴      |           |             |
|           | 20                    |          | 10⁻⁴      |           |             |
|           | 22                    |          | 10⁻⁴      |           |             |
|           | 25                    |          | 10⁻⁴      |           |             |
| DEF       | 14                    | 10⁻²     | 10⁻⁴      | 10⁻⁵      | 10⁻⁵        |
|           | 21                    | 10⁻²     | 10⁻⁴      | 10⁻⁵      | 10⁻⁵        |
|           | 22                    | 10⁻²     | 10⁻⁴      | 10⁻⁵      | 10⁻⁵        |
|           | 23                    | 10⁻²     | 10⁻⁴      | 10⁻⁵      | 10⁻⁵        |

* Stock samples were assayed by serial 10-fold dilutions. Dilutions used were: virus, 10⁴ to 10⁻⁵; control, 10⁰ to 10⁻⁵.

* Abbreviations: FEF, feline embryo fibroblast; DEF, dog embryo fibroblast.

* Results expressed as highest dilution of virus inocula at which infections were detected by either test.

* Represents failure to detect virus infection.

* DEF cultures were not regularly subpassaged, and some of the MC test plates were confluent and multilayered.
was done 21 days after virus inoculation. However, in a number of instances, FeLV-inoculated cultures were found to be positive for virus replication by the MC method when tested as early as 8 to 14 days (Tables 1 and 2). It appears that, under appropriate experimental conditions, the MC test could be adapted successfully to assay FeLV preparations in a relatively short period of time.

In the murine system, it has recently been suggested that specific components of the viral particles act as activators for the fusion process and that these components may be present in the membranes of the infected cells (1). The presence of a similar fusion-inducing factor in FeLV preparations and virus-infected cells can be postulated since a few multinucleated giant cells were observed in some of the XC cells treated with FeLV. However, the virus inoculum added may have been insufficient for extensive fusion of the XC cells, since the above workers have shown a requirement of greater than 10⁵ virus particles for this reaction.

Johnson et al. (1) recorded a graded response based on the number of multinucleated cells and the number of nuclei per cell in XC cell cultures treated with the fusion factor from MuLV-infected cultures and found this to be directly correlated to the amount of fusion factor added. On a similar index system, it would appear that the amount of fusion factor produced in FeLV-infected M-132-1 cells is considerably higher than that in virus-infected FEF or DEF cells, since the MC effect in M-132-1 cells is rapid and more striking. If the virus particles are the sole responsible agent for the fusion of XC cells, as postulated by Johnson et al. (1) for the murine system, by analogy it is likely that FeLV-infected M-132-1 cultures contain more virus particles than similarly infected feline or canine embryo cells. A quantitative determination of the amount of virus particles in identically infected cultures of the different susceptible cell types would help elucidate this point.

In the MuLV-XC cell system, syncytiat giant cells were produced by using homogenates of virus-infected cells as well as density band-purified virus preparations (1). Also syncytiat were observed immediately adjacent to fluorescing foci of infected mouse cells (2). These observations strongly point to the XC cells as the main source of syncytiat giant cells. Preliminary immunofluorescence studies using FeLV-specific antisera showed intensely fluorescing cytoplasmic areas in some of the giant cells that developed in M-132-1 (FeLV) cultures after the addition of XC cells. This suggests that some of the syncytiat cells could have incorporated M-132-1 (FeLV) cell components in addition to XC cells. On the other hand, it is possible that XC cells have a relatively low susceptibility to FeLV and that a few of the viral antigen-synthesizing XC cells contribute to syncytiat cells, which are exclusively formed by the fusion of XC cells. That a small percentage of syncytiat giant cells do indeed incorporate M-132-1 (FeLV) cells or cell components was indicated in a preliminary autoradiographic experiment in which "H-thymidine-labeled M-132-1 (FeLV) cells were cocultivated with XC cells. In these giant cells, along with a number of unlabeled nuclei, one or sometimes two well labeled nuclei of M-132-1 (FeLV) cells were clearly identifiable.

### Table 2. Neutralization of feline leukemia virus (FeLV) infection of M-132-1 cells

| Days post-inoculation | Virus | 184 | 461 |
|-----------------------|-------|-----|-----|
|                       |       | No serum* | Anti-FeLV* | No serum | Anti-MuLV | No serum | Anti-MuLV |
|                       | XC | CF | XC | CF | No serum | XC | No serum | Anti-MuLV |
| 8                     | 10⁻² | ND* | — | — | 10⁻³ | 10⁻³ | 10⁻³ | 10⁻³ |
| 11                    | 10⁻³ | 10⁻² | — | — | 10⁻³ | 10⁻³ | 10⁻³ | 10⁻³ |
| 14                    | 10⁻⁴ | 10⁻⁴ | — | — | 10⁻⁴ | 10⁻⁴ | 10⁻⁴ | 10⁻⁴ |
| 16                    | 10⁻⁵ | 10⁻⁵ | — | — | 10⁻⁵ | 10⁻⁵ | 10⁻⁵ | 10⁻⁵ |
| 19                    | 10⁻⁶ | 10⁻⁶ | — | — | 10⁻⁶ | 10⁻⁶ | 10⁻⁶ | 10⁻⁶ |

*Values expressed as highest dilution of virus inocula at which infections were detected. For this experiment, serial dilutions from 10⁻¹ to 10⁻⁷ of the virus were used.
*Viruses diluted from 10⁻¹ to 10⁻³ were used.
*Viruses diluted from 10⁻¹ to 10⁻⁴ were used.
*Not done.
*Represents failure to detect virus infection.
ACKNOWLEDGMENTS

This investigation was supported by Public Health Service contract no. NIH-69-2079 from the National Cancer Institute.

We are thankful to Janet M. Ditmore, Patricia J. Ueberhorst, and Smith Sae Hoo for assistance in certain phases of this study.

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