Syncytial Giant Cell Focus Assay for Viruses Derived from Feline Leukemia and a Simian Sarcoma[1] (37180)

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(Introduced by F. B. Bang)
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Recently, we described a mixed culture cytopathogenic (MC) test that was useful for the detection and quantitation of a feline leukemia virus (FeLV) and of a virus associated with preparations of simian sarcoma virus (SSV-1) (1, 2). Briefly, this technique involves amplification of the test virus in fibroblasts cells of feline or canine origin or a canine tumor cell line, M.132-1 and then co-cultivation with XC cells, a rat tumor cell line (3). Infection is indicated by the formation of multinucleated giant cells in the mixed cultures. This procedure has advantages over serological tests for the detection of viral antigens but it requires replicate cultures and their frequent subpassage.

This report describes the successful modification of the MC test for a more rapid and quantitative assay of FeLV and SSV-1. In this modified technique, each infectious unit of the test virus causes a focus of syncytial giant cells (SGC). These foci can be enumerated by microscopic scanning of stained cultures.

Materials and Methods. Cell cultures. A canine tumor cell line, M.132-1, and a rat tumor cell line XC, were used as initiator of infection and detector of the test viruses, respectively. The origin and procedures for culturing these cells have been described previously (1).

Viruses. A stock of the Theilen stain of FeLV was prepared from chronically infected M.132-1 cell cultures. The virus was concentrated 100-fold by centrifugation and designated FeLV 461. The origin of this virus and the procedures for its preparation have been described previously (4).

A stock of SSV-1 was prepared from Raji cell cultures, chronically infected with the virus. The origin and preparative procedures of this virus stock, designated 814, have been described in detail elsewhere (2).

Endpoint dilution assay. The M.132-1 monolayer cultures were infected with decimally diluted virus stocks and maintained with subpassages at intervals of 4–5 days. The cultures were tested by the MC method 21 days postinfection and the titers of the virus stocks were determined as described previously (1, 2).

Syncytial giant cell focus assay. Test virus adsorption was carried out in suspension in Falcon[3] 2027, 13 × 100 mm, sterile, screw-capped plastic tubes previously incubated for about 30 min in a humidified 5% CO_2 atmosphere. Cells did not attach to the plastic surface of these tubes.

Appropriate concentrations (see below) of freshly trypsinized M.132-1 cells in 0.1 ml growth medium and 0.1 ml of virus dilutions were mixed at the bottom of the tubes and incubated at 37° for 1 hr in a stationary horizontal position to provide maximum random adsorption of virus to the cells in a thin fluid layer. Following adsorption, the content of each tube was suspended in 10% heated fetal bovine serum (FBS) supplemented McCoy's 5A (Grand Island Biological Co., New York) growth medium. Aliquots were transferred quantitatively to four or more 60 mm diameter tissue culture dishes as outlined under the different experiments. Identical samples of M.132-1 cells incubated with 0.1 ml of Hanks' balanced salt solution (HBSS) were used as controls. The cultures were incubated at 37° in a humidified 5% CO_2 atmosphere. Between 96 and 120 hr superna-

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tant fluid from each culture was replaced with 5 ml 10% FBS supplemented Eagle's minimum essential medium (EMEM) containing 1.5×10^6 freshly trypsinized XC cells. Following 48 hr of incubation, the cultures were washed in HBSS, fixed and stained simultaneously for 10 min in a solution containing 3 parts of methylene blue, 1 part basic fuchsin and 1 part methanol. The fixative–stain solution was freshly mixed from 1% stock stain solutions in methanol. The plates were washed in tap water, air dried and scanned at a magnification of about 35 with an inverted microscope.

Results. Determination of optimal concentration of M.132-1 cells. The optimal cell plating density that would yield cultures with localized and well isolated colonies of M.132-1 cells was investigated. This was necessary because M.132-1 cells grow rapidly to heavy densities and have a tendency to form floating cell clumps (1). A starting concentration of 1×10^4 cells/60 mm plate yielding heavily overgrown cultures with numerous floating cell clumps on the fifth day after initiation, thus rendering the cultures of little value in quantitative virus assays. Use of semisolid overlays of agar or methocel failed to prevent cell detachment or movement and caused the cells to exhibit abnormal morphology and poor growth characteristics.

The M.132-1 cultures that were initiated with a starting density of 8×10^3 cells/plate had evenly scattered individual cells on the culture surface during the first day of growth. These grew to macroscopic colonies consisting of single cells and clumps in 4 days. Microscopic patches of cells in monolayer were also present. No difference was noted between control and virus-infected cultures. Very few floating cells or cell clumps were present in the culture medium of these sparsely plated cultures. Thus a starting density of 8×10^3 cells/plate was considered optimal.

Syncytial giant cell (SGC) focus formation. In an initial SGC focus assay experiment, duplicate samples of 3.2×10^4 freshly trypsinized M.132-1 cells were inoculated with serial twofold dilutions of FeLV 461. Following incubation for viral adsorption cells from each sample were suspended in 4 ml of growth medium. Aliquots were distributed quantitatively to 4 plates containing 3 ml growth medium each. The tubes were rinsed with an additional 4 ml medium which was again distributed equally to the plates. The final volume was 5 ml/plate. After 4.5 days incubation the cultures were overlaid with XC cells as described above.

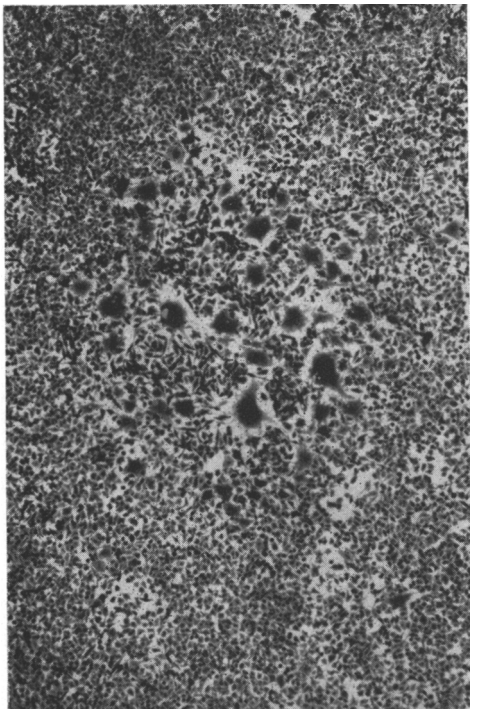

FIG. 1. A focus of large and abnormally shaped multinucleated giant cells in a monolayer lawn of XC cells. Sparsely plated FeLV infected M.132-1 culture co-cultivated with XC cells 4.5 days after virus infection for 48 hr. 180×.

When the lawn of XC cells developed, localized discrete foci of syncytial giant cells were observed only in the virus-infected M.132-1 cultures (Fig. 1). The giant cells were distinguished by their larger size, abnormal shape and multiple nuclei. Some cells contained a dozen or more nuclei. In addition to the giant cell foci, the XC cell lawn in the infected cultures also contained numerous M.132-1 colonies readily distinguishable by the presence of clumps of M.132-1 cells as well as occasional large mononucleated cells (Fig. 2). These uninfected M.132-1 colonies

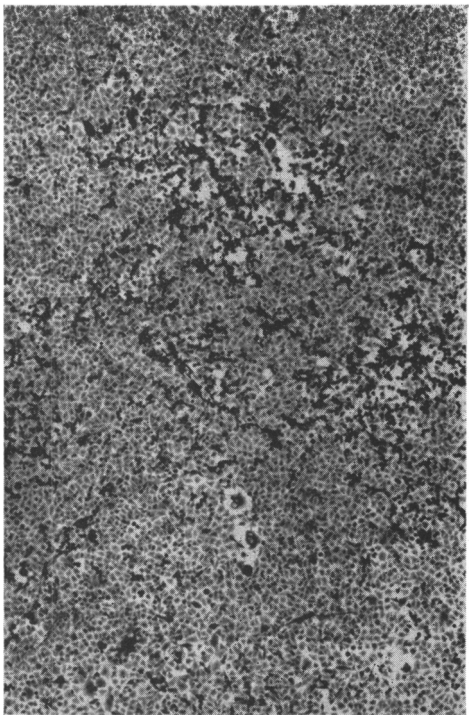

FIG. 2. Uninfected M.132-1 colonies in the same culture described in Fig. 1. Darkly stained M.132-1 cells are seen in a monolayer lawn of XC cells. 180×.

were identical to the M.132-1 colonies that developed on the XC cell lawn in control cultures. Infrequently, some of the uninfected M.132-1 colonies, both in the control and virus inoculated cultures, contained small round cells with four to six nuclei. Small multinucleated cells were routinely encountered in control XC cell cultures and were easily differentiated from the syncytial giant cells that develop following virus infection.

Enumeration of SGC foci. SGC foci were identified and counted when they had either (a) a cluster of abnormally shaped large multinucleated cells with four or more of the cells having six or more nuclei, or (b) large multinucleated cells bordering the periphery of a dense M.132-1 colony that had many smaller multinucleated cells distributed in all parts of it. Occasional foci of three or less large multinucleated giant cells were excluded from the enumeration since these were considered as probable satellite foci.

A direct correlation between virus dilution and the number of SGC foci was repeatedly obtained. Table I illustrates the results of one such experiment.

Under similar experimental conditions, two other isolates[4] of FeLV also formed readily countable SGC foci in M.132-1 cultures. However, in a few preliminary attempts, a preparation of FeLV (Rickard strain)[5] failed to produce SGC foci. This was consistent with our and others (Axler, Ohio State University, Columbus, OH, personal communications) inability to detect the MC reaction in M.132-1 or feline embryo fibroblast cultures inoculated with the Rickard strain of FeLV.

Virus neutralization. In a neutralization ex-

[4] One isolate was provided by Dr. P. S. Sarma, NCI, National Institutes of Health, Bethesda, and the other was found in a feline sarcoma virus preparation obtained from Dr. L. Bustead, University of California (1).

[5] FeLV (Rickard strain) was prepared as a 10X concentrate from C-type virus shedding feline thymic cells obtained from University Laboratories, Highland Park, NJ.

TABLE I. Correlation Between FeLV Dilutions and Syncytial Giant Cell (SGC) Foci.[a]

Virus dilution	No. of SGC foci/plate								SGC foci		
	A		B		C		D		Total		
	1[b]	2[b]	1	2	1	2	1	2	1	2	Mean
1:80	22	20	13	22	35	27	27	32	97	101	99
1:160	11	18	19	13	8	14	12	5	50	50	50
1:320	6	3	3	5	6	8	5	9	20	25	23
1:640	0	3	4	0	4	1	0	5	8	9	9

[a] M.132-1 cultures were co-cultivated 4.5 days after infection with XC cells for 48 hr.

[b] Duplicate samples; 3.2×10^4 M.132-1 cells in 0.1 ml adsorbed in suspension with 0.1 ml of FeLV 461 and distributed into 4 plates each.

TABLE II. Neutralization of FeLV Induced SGC Foci by Anti-FeLV Serum.

Virus dilution	Replicate	Total no. of syncytial giant cell foci[a] Virus[b]	Virus + [c] HBSS	Virus + [c] Antiserum
1:80	1	641	477	1
	2	486	458	0
1:160	1	329	224	0
	2	284	228	1

[a] M.132-1 cells (4×10^4) in 0.1 ml adsorbed in suspension with 0.1 ml virus samples and distributed into five plates each. The cultures were cocultivated 4.5 days after infection with XC cells for 48 hr.

[b] FeLV 461 titered prior to incubation at 37° for 1 hr.

[c] 0.1 ml of 1:40 and 1:80 dilutions of FeLV incubated with 0.1 ml of Hanks' balanced salt solution or antiFeLV (Theilen) serum diluted 1:2.

periment 0.1 ml of different dilutions of FeLV 461 were incubated with 0.1 ml of 1:2 diluted anti-FeLV (Theilen strain) serum at 37° for 1 hr. The antiserum was prepared in guinea pigs against band-purified virus.[6] Tubes containing 4×10^4 M.132-1 cells in 0.1 ml medium were inoculated with 0.1 ml of the virus-serum mixtures, adsorbed for 1 hr and distributed equally into five plates. Cultures were subsequently treated as described above for titration of virus by the SGC technique. A reduction of SGC foci indicated that FeLV was neutralized by the antiserum (Table II). That the neutralization of FeLV 461 was specifically due to the presence of FeLV (Theilen strain) antibodies in the serum was inferred from our earlier observations (2, 4).

Comparison of SGC focus assay and endpoint dilution assay. In order to compare the sensitivity of the enumeration assay with the endpoint dilution method of titrating FeLV, parallel experiments were carried out. Monolayer cultures initiated with 3×10^5 M.132-1 cells 24 hr earlier were inoculated with 0.2 ml of serial tenfold dilutions of FeLV 461 and were handled as described previously for the MC reaction (1). For ease of calculating the 50% endpoint four replicate cultures were used for each virus dilution. For the SGC focus assay, tubes containing 3.2×10^4 M.132-1 cells each in 0.1 ml medium were inoculated with 0.1 ml of 1:80, 1:160 and 1:320 dilutions of virus sample used for the endpoint dilution assay.

Results of these experiments showed the stock virus titer to be 1×10^6 tissue culture infectious dose ($TCID_{50}$)/ml by the endpoint dilution method and 2.75×10^5 focus forming units/ml by the SGC focus assay procedure. Assuming a Poisson distribution, one $TCID_{50}$ would be expected to contain 0.69 infectious units of the virus, and thus the virus stock was calculated to contain 6.9×10^5 infectious units/ml by the endpoint dilution method. From these results it appears that the infectivity titer determined by the SGC focus assay method is about 2.5 times less sensitive than the endpoint dilution procedure.

A comparison of the results above with those in Table II suggests that the same virus stock yielded a higher titer by the SGC focus assay when the initial number of M.132-1 cells used for adsorption of the virus inoculum was increased. Therefore, experiments were carried out in which the same concentration of virus inoculum was adsorbed onto varying concentrations of M.132-1 cells. The virus cell mixtures in each tube were suspended in growth medium and plated equally into the requisite number of plates to give a starting density of 8×10^3 cells/plate. In two such experiments (Table III), the virus titers increased with increasing numbers of cells before reaching a plateau at approximately 6.4×10^4 cells. At this concentration of cells for virus adsorption, the virus titer determined by the SGC focus assay approximated to that obtained by the endpoint dilution technique.

Syncytial giant cell focus assay for simian sarcoma virus preparations. Since it has been shown that M.132-1 cultures can be infected with SSV-1 preparations and the virus infections detected by the MC reaction (2), the applicability of SGC focus assay for the quantitation of SSV-1 stocks was determined in a number of experiments.

In general the experimental conditions for the SGC focus assay were as described above for FeLV titrations. In one experiment, dilu-

[6] Antiserum was obtained from Electronucleonics Laboratories, Bethesda, MD.

TABLE III. Correlation Between M.132-1 Cell Concentration During Adsorption and FeLV Titer.

Expt 1[a]			Expt 2[b]		
Cell concn $\times 10^4$	No. of plates	Virus titer[c] $\times 10^6$/ml	Cell concn $\times 10^4$	No. of plates	Virus titer[c] $\times 10^6$/ml
3.2	4	0.54	3.2	4	0.42
4.8	6	0.94	4.8	6	0.54
6.4	8	1.03	6.4	8	0.84
8.0	10	1.37	9.6	12	0.80

[a] 0.1 ml of a 1:160 dilution of FeLV 461 was adsorbed with the indicated number of cells in 0.1 ml suspension at 37° for 1 hr. The virus–cell mixtures were distributed equally into the indicated number of plates to obtain a concentration of 8×10^3 cells/plate.

[b] A dilution of 1:320 of the virus was used.

[c] Titers were computed from the number of syncytial giant cell foci that appeared after co-cultivation with XC cells.

tions of SSV-1 preparation 814 was adsorbed onto 4×10^4 M.132-1 cells and the cells distributed into 5 petri plates. The multinucleated giant cell foci that appeared in mixed cultures were counted following the earlier described criteria for identifying SGC foci. Results of this experiment (Table IV) clearly show the applicability of SGC focus assay for SSV-1 and/or an associated viral agent (SSAV-1) (5) in the SSV-1 preparations.

Discussion. The need for a quantitative focal lesion method in the study of feline and simian tumor viruses is obvious. The SGC focus method is reliable for the viruses tested and relatively simple. Results are obtained in a shorter time than the previously described MC reaction (1). The SGC focus assay method may be adapted to evaluate the relative potency of different antisera. In addition, SGC focus assay has potential application for clonal isolation of FeLV and SSV-1 or its associated agent.

We have not yet been able to develop a macroscopic assay procedure similar to that for murine leukemia virus (6), nor have we been able to adapt the SGC focus assay utilizing feline or canine embryo fibroblasts. However, microscopic enumeration of the SGC foci by itself is not a serious drawback. Microscopic methods have been used with considerable success in the study of other virus infections (7, 8).

Summary. A syncytial giant cell (SGC) focus assay for feline leukemia and simian sarcoma virus preparations has been developed. This assay is based on the cytopathogenic reaction that appears in cultures where XC cells are overlayed on virus infected M.132-1 cells. Results of titrations of virus stocks were determined in a period of 1 wk

TABLE IV. Correlation Between SSV-1 Dilutions and Syncytial Giant Cell (SGC) Foci.[a]

Virus dilution	No. of SGC foci/plate										SGC foci		
	A		B		C		D		E		Total		
	1[b]	2[b]	1	2	1	2	1	2	1	2	1	2	Mean
1:20	54	61	55	28	48	71	65	23	66	53	288	236	262
1:40	26	22	17	24	32	21	12	9	17	59	104	135	120
1:80	69[c]	9	2	12	0	5	2	22	12	3	85	51	68

[a] M.132-1 cultures were co-cultivated 4.5 days after infection with XC cells for 48 hr.

[b] Duplicate samples; 4×10^4 M.132-1 cells in 0.1 ml adsorbed in suspension with 0.1 ml of SSV-1 814 and distributed into 5 plates each.

[c] Actual number of SGC foci observed in this petri dish. The relative high count in this plate compared to its four other replicates is due to an error in uneven suspension and nonuniform distribution of the virus adsorbed cells into the 5 petri plates.

by the use of the SGC focus method. SGC focus formation was prevented when the test virus was reacted with specific antisera.

1. Rangan, S. R. S., Wong, M. C., Moyer, P. P., and Jensen, E. M., Appl. Microbiol. **23**, 628 (1972).

2. Rangan, S. R. S., Wong, M. C., Ueberhorst, P. J., and Ablashi, D. V., J. Nat. Cancer Inst. **49**, 571 (1972).

3. Svoboda, J., Nature (London) **186**, 980 (1960).

4. Rangan, S. R. S., Moyer, P. P., Cheong, M. P., and Jensen, E. M., Virology **47**, 247 (1972).

5. Deinhardt, E., Wolfe, L., Massay, R., Hoekstra, J., and McDonald, R., Int. Symp. Comparative Leukemia Res., 5th, Padova, Sept. 1971, in press.

6. Rowe, W. P., Pugh, W. E., and Hartley, J. W., Virology **42**, 1136 (1970).

7. Hartley, J. W., and Rowe, W. P., Proc. Nat. Acad. Sci. USA **55**, 780 (1966).

8. Sarma, P. S., Log, T., and Theilen, G. H., Proc. Soc. Exp. Biol. Med. **137**, 1444 (1971).

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