An Immunofluorescent Focus Assay for Gross Leukemia Virus (35141)

WILNA A. WOODS, JUDITH MASSICOT, AND MICHAEL A. CHIRIGOS
National Cancer Institute, Bethesda, Maryland 20014

The spontaneous leukemias of AKR mice provide an excellent model for studying human lymphoid leukemias. However, the time-consuming bioassay (1) for determining infectivity has hampered investigations on the Gross leukemia virus (GLV) associated with these leukemias. *In vitro* assay systems have been developed which decrease the time required for assay of infectious virus, but either are indirect measures of infectivity or are cumbersome to perform. The COMUL test of Hartley *et al.* (2) and the XC test of Klement *et al.* (3) provide sensitive systems requiring only 2–3 weeks to complete, but are indirect assays and are somewhat cumbersome. The helper (4) and interference (5) tests utilizing Moloney sarcoma virus pseudotypes provide relatively rapid results but are again indirect measures of infectivity. Demonstration of neutralizing antibody to GLV by bioassay and COMUL test has been reported (2, 6, 7). The development of a rapid, simple assay for infectious GLV and its neutralizing antibody is essential for studies on the virus and its associated neoplasms.

Both fluorescent focus assays for infectivity of several nononcogenic viruses (8–11) and immunofluorescence of tissue cultures infected with murine leukemia viruses (12–14) have been described. The present communication reports the development of a fluorescent focus assay for GLV and GLV-neutralizing antibody in NIH Swiss mouse embryo cell (MEC) tissue cultures.

Methods and Materials. Tissue culture (TC). Monolayers of secondary MEC in rings on microscope slides (15) were employed. Primary NIH Swiss MEC cultures were trypsinized with 0.05% trypsin in Hanks' balanced salt solution (HBSS), and the cells were dispersed in Eagle's minimum essential medium containing 10% fetal calf serum (MEM-10). Each ring was seeded with 10^4 cells in 0.5 ml MEM-10, and the cultures were incubated 16–18 hr at 37° in a humidified 5% CO_2 in air atmosphere. The medium was then removed with suction, 0.05 ml of infectious material was inoculated, and the cultures were returned to the CO_2 incubator for 20–24 hr. The inoculum was then removed with suction, the cultures were washed once with medium, fed with 0.5 ml fresh MEM-10, and returned to the CO_2 incubator for 7–10 days. The medium was changed every 3–4 days.

Culture and characteristics of the AKR-A cell line have been described (16).

Infectious materials. Three sources of wild-type GLV were employed: (1) 10% homogenate of brain tissue, serum, and milk of adult AKR mice obtained from Jackson Laboratories, Bar Harbor, Maine; (2) ascites tumor cells (AKR ascites) originating from a G+ spontaneous tumor of an AKR mouse (16); and (3) cells and tissue culture fluid (TCF) of a G+ lymphoblastic cell line derived from this tumor (AKR-A) (16). Moloney leukemia virus (MLV), Friend leukemia virus (FLV), and Rauscher leukemia virus (RLV) were standard preparations of virus from the plasma of infected BALB/c mice. Three cycles of freezing and thawing were routinely used to disrupt cell suspensions to free intracellular virus; no live cells were seen when these suspensions were examined by the trypan blue dye-exclusion test.

Serum neutralization. All sera were diluted in MEM-10 and inactivated at 56° for 30 min before testing. Viruses were diluted in MEM-10 to contain approximately 50 1 fluorescent focus-forming units (FlFU) or 100 $TCID_{50}$ in 0.05 ml. Equal volumes of serum and virus were mixed and incubated at room temperature for 1 hr. One-tenth milliliter of each serum-virus mixture was inocu-

TABLE I. Antibody Titers of Anti-GLV and Anti-MSV(M) Sera in Neutralization, CF, Membrane-Fluorescence, and Immunodiffusion Tests.

	Titer against GLV and RLV						
	Test						
	Neutralization		Complement fixation		Membrane fluorescence	Immunodiffusion	
Antigen	GLV	RLV	GLV	RLV	GLV	GLV	RLV
Rat anti-MSV(M)	<100	<100	80	80	Neg.	1 line[a]	1 line[a]
Rat anti-GLV no. 1	3200	<100	40	40	20	1 line[a]	1 line[a]
Rat anti-GLV no. 2	1600	<100	160	>80	40	3 lines[b]	1 line[a]
Rat anti-GLV no. 3	1600	<100	80	40	40	1 line[a]	1 line[a]
Normal rat	<100	<100	<10	<10	Neg.	Neg.	Neg.

[a] Line of identity with viral core antigen, determined by standard RLV antigen.
[b] One line of identity with viral core antigen, determined by standard RLV antigen.

lated into the three ring cultures on each slide. Virus titrations and serum controls were included in each test, the inoculum being 0.05 ml. After 24-hr incubation at 37° in the CO_2 incubator, the inoculum was removed, the cultures were washed, and 0.5 ml of fresh medium was added. The cultures were incubated for 9–10 days at 37°, fixed, and stained for fluorescent foci. The neutralization titer is expressed as the reciprocal of the serum dilution reducing the number of FlFU by at least 50% or neutralizing 100 $TCID_{50}$ of virus.

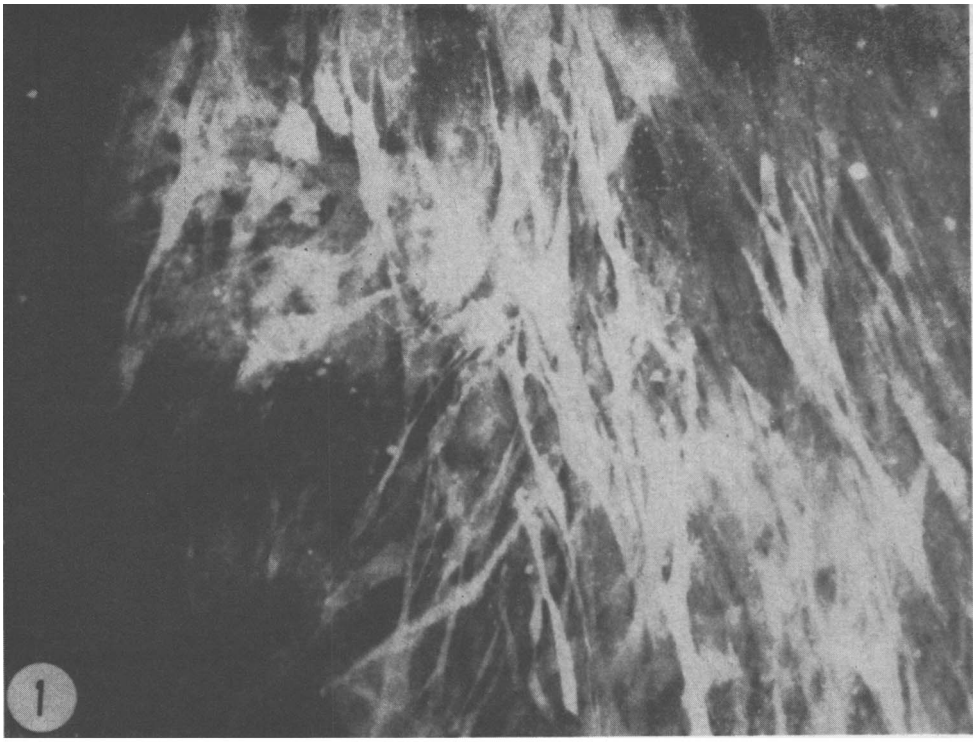
Antisera. Anti-GLV serum was produced in WFuXBNFl hybrid rats by the method of Old *et al.* (17). A serum prepared in Fisher rats bearing MSV(M) virus-induced tumors (Huntingdon Laboratories, Baltimore, Md., NCI Contract 43-69-64) was used as a source of antibody against the group-specific viral core antigen of murine leukemia viruses. The sera were tested for presence of antiviral antibodies by immunodiffusion (7) against a standard Tween–ether-treated GLV antigen[1] and Tween–ether-treated RLV concentrated from the plasma of infected BALB/c mice, and for anticellular G+ antibodies by the membrane fluorescent technique of Aoki *et al.* (18). Complement-fixation (CF) tests (2) were also employed to quantitate antibodies to the GLV complex. Positive and negative controls were included in all tests. Table I summarizes the antibody characteristics of the various serum pools used.

Immunofluorescent staining. The standard procedures used in this laboratory for immunofluorescent staining have been described (16).

Results. Appearance of fluorescent foci in MEC cultures infected with GLV. Diffuse foci of strongly fluorescent cells were observed in MEC cultures infected with GLV after staining with rat anti-GLV or rat anti-MSV(M) serum. The staining with the anti-GLV serum usually appeared uniformly distributed in the cytoplasm of infected cells, with occasional bright granules seen against somewhat less intensely staining cytoplasm. Nuclear fluorescence was never observed. Cultures stained with the anti-MSV(M) serum, which reacted only with the group-specific core antigen, exhibited brightly staining cytoplasmic granules. Control uninfected cultures did not stain, and normal rat serum did not stain infected cultures. The staining first appeared 7 days after inoculation and was maximal 10 days after infection. Localized groups of intensely staining cells were readily counted in cultures inoculated with appropriate dilutions (Fig. 1).

Infectious virus was not demonstrated in TCF from the infected MEC cultures by either the COMUL test or fluorescent staining of MEC cultures inoculated with the

[1] Generously supplied by courtesy of Dr. Lloyd Old, Sloan-Kettering Institute for Cancer Research, New York, N. Y.



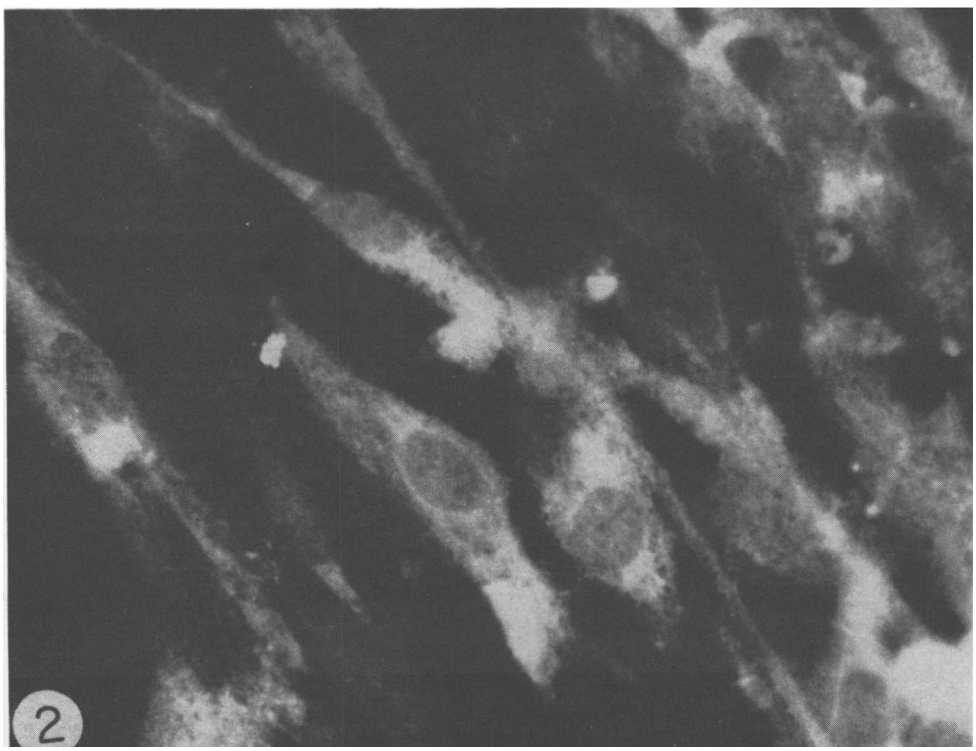


FIG. 1. Swiss mouse embryo cells infected with GLV from AKR-A culture, 10 days after infection. Indirect immunofluorescent stain with rat anti-GLV serum and goat anti-rat conjugate. 100×.

FIG. 2. Swiss mouse embryo cells infected with GLV from AKR-A culture, 10 days after infection. Indirect immunoflourescent stain with rat anti-GLV serum and goat antirat globulin conjugate. Note bright granular cytoplasmic fluorescence of most cells. 400×.

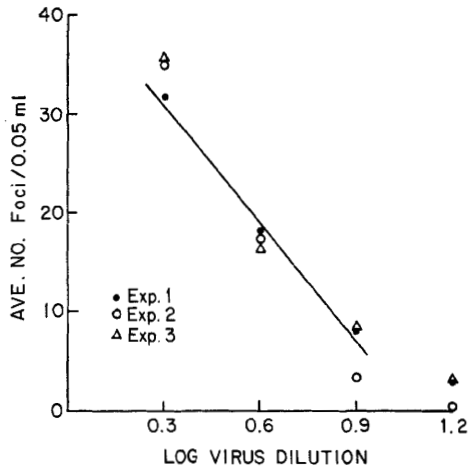


FIG. 3. Titration of GLV from AKR-A tissue culture fluid, 9 days after initiation of culture. Fluorescent foci stained 10 days after inoculation with rat anti-GLV serum and anti-rat globulin conjugate. Results of three titrations of same TCF.

TCF. No cytopathic lesions were observed on either direct observation of unfixed cells or after staining the cultures with May–Grünwald–Giemsa technique.

The specificity of the staining was determined by blocking experiments. The staining was completely blocked by absorption of the serum with either Tween–ether-treated extracts of AKR ascites cells, or with Tween–ether-treated, pelletized GLV. Similar preparations of normal BALB/c spleen, thymus, lymph node cells, or plasma did not diminish the staining. Tween–ether-treated RLV only partially reduced the staining by the anti-GLV serum and completely blocked the staining by the anti-MSV(M) serum.

Staining of MEC infected with MLV, RLV, or FLV with either anti-GLV or anti-MSV(M) serum produced fluorescent foci (MLV) or staining of almost all cells (FLV and RLV); the staining was granular and confined to the cytoplasm. The lack of focal staining in FLV- and RLV-infected cultures may have been due to the long incubation time, since staining in these cultures could be detected on the third day after inoculation. The staining was blocked by all types of murine leukemia viruses which had been Tween–ether-treated, and was, therefore, due to the staining of the group-specific antigen.

C_3H and Swiss 3T3 cells were also tested for susceptibility to infection by GLV. C_3H cells were comparable in sensitivity to the NIH Swiss MEC. However, Swiss 3T3 cells were refractory to infection with GLV.

Titration of GLV by fluorescent focus formation. Twofold dilutions of GLV prepared in MEM-10 were inoculated onto monolayer ring cultures of NIH Swiss MEC. After 10 days' incubation at 37°, the cultures were fixed and stained. Foci were counted under 100 × magnification. Figure 2 shows the results of several titrations of the same preparation, performed several months apart. No significant difference in titer was seen. Table II shows the results of titrations of several different preparations.

Isolation of GLV from tissues and fluids of AKR mice. Fluorescent foci were observed in cultures infected with plasma, milk, and brain suspension derived from adult AKR mice. Cellular extracts of AKR ascites tumor cells also contained infectious GLV (Table II).

Serum neutralization of GLV. Several pools of anti-GLV rat sera were tested for neutralizing antibody to the murine leukemia viruses. These pools were selected on the basis of high titers in the membrane-fluorescent, CF tests, and for the appearance of group-specific and GLV-specific lines in the immunodiffusion tests. The serological characteristics of these pools and their neutralizing titers for murine leukemia viruses are shown in Table I. All pools had high neutralizing

TABLE II. Titration of GLV from Several Tissues of AKR Mice.

Tissue	Titer (FIFU/ml)
AKR-A TCF, d35[a]	4×10^5
AKR-A cells, d30[b]	1×10^3
AKR ascites tumor cells, d7[c]	3×10^2
AKR milk[d]	2×10^1
AKR serum, adult ♀	$>2 \times 10^2$
AKR brain, adult ♂[e]	4×10^1

[a] Day after initiation of primary culture.
[b] Day after initiation of primary culture, 50% (v/v) cell suspension.
[c] Day after tumor inoculation i.p.
[d] Day 16 postpartum, 1:2 in HBSS.
[e] Ten percent homogenate of tissue in HBSS.

TABLE III. Fluorescent Focus Assay of Neutralizing Antibody in Rat Anti-GLV Serum.

Serum	Dilution	No. foci/ring	Average
Anti-GLV	100	0, 0, 0	0
	200	0, 0, 0	0
	400	4, 1, 0	1.7
	800	7, 0, 0	2.3
	1600	16, 11, 10	12.3
	3200	16, 12, 18	15.3
	6400	33, 27, 25	28.3
Normal rat	100	50, 32, 45	42.3
None	—	40, 44, 30	38.0

antibody titers for GLV, but failed to neutralize MLV, FLV, or RLV at a 1:100 dilution. A typical neutralization test is shown in Table III. Normal WFuXBNFl hybrid rat sera failed to neutralize murine leukemia virus. Anti-MSV(M) serum did not neutralize GLV. No correlation was seen between CF- or membrane-fluorescent titer and neutralizing titer.

Serum-neutralizing antibody was also found in the serum of DBA mice inoculated with AKR ascites cells. No CF, cytotoxic, or membrane-fluorescent antibodies were detected in these sera.

Serum blocking by murine leukemia viruses. Only GLV could block the neutralizing activity of the sera (Table IV). Three-tenths milliliter 1:200 serum pool No. 3 was incubated with an equal volume of GLV ($10^{2.4}$FlFU), MLV($10^{4.5}$FlFU), RLV($10^{3.5}$ $TCID_{50}$), or FLV($10^{3.5}TCID_{50}$). After 1-hr incubation at room temperature, any remaining virus was inactivated by heating the mixtures to 56° for 30 min. Twofold dilutions were made in MEM-10, and equal volume of GLV containing 40 FlFU/0.05 ml was added to each dilution. After 1-hr incubation at room temperature, these mixtures were inoculated into MEC cultures to determine residual infectious GLV. Only GLV removed neutralizing activity from the serum, indicating a lack of antigenic relationship between the coat antigens of GLV and other murine leukemia viruses.

TABLE IV. Blocking of Neutralizing Antibody in Rat Anti-GLV Serum by Murine Leukemia Viruses.

Blocking virus	Anti-GLV neutralizing titer
None	1600
Normal spleen	800
MLV	800
FLV	800
RLV	1600
GLV	<400

Discussion. The assay system described in this report provides a rapid, sensitive, reproducible, and direct titration for infectious GLV and its neutralizing antibody. Technical problems are at a minimum in this system. Agar overlay to localize the foci does not appear to be necessary since infectious virus is not demonstrable in the TCF. Further, the use of ring cultures provides a small defined area of infection which can be easily stained by the immunofluorescent technique. The 50% reduction assay for neutralizing antibody is more sensitive than measurement of complete neutralization of infectivity. We found no relationship between the presence of the group-specific antibody or membrane-fluorescent antibody and neutralizing antibody.

Most investigators have grouped the murine leukemia viruses into the FMR and the Gross–AKR-type subgroups (19, 20). Using both cross neutralization and serum-blocking tests, we also found no cross reaction between the wild type AKR–GLV and the FMR viruses. The sensitive 50% serum neutralization assay may allow discrimination between members of the FMR and Gross–AKR subgroups by intratypic serodifferentiation (21).

Summary. An immunofluorescent focus assay for GLV is described. GLV was demonstrated in tissues and fluids of AKR mice, as well as in tumor cells from a spontaneous lymphoma of AKR mice.

A sensitive 50% focus reduction assay for neutralizing antibody to GLV is described.

1. Ioachim, H. L., and Berwick, L., Int. J. Cancer 3, 61 (1968).

2. Hartley, J. W., Rowe, W. P., Capps, W. I., and Huebner, R. J., Proc. Nat. Acad. Sci. U.S.A. 53, 931 (1965).

3. Klement, V., Rowe, W. P., Hartley, J. W., and

Pugh, W. E., Proc. Nat. Acad. Sci. U.S.A. **63**, 753 (1969).

4. Huebner, R. J., Hartley, J. W., Rowe, W. P., Lane, W. T., and Capps, W. I., Proc. Nat. Acad. Sci. U.S.A. **56**, 1164 (1966).

5. Sarma, P. S., Cheong, M. P., Hartley, J. W., and Huebner, R. J., Virology **33**, 180 (1968).

6. Gross, L., Proc. Soc. Exp. Biol. Med. **119**, 420 (1965).

7. Geering, G., Old, L. J., and Boyse, E. A., J. Exp. Med. **124**, 753 (1966).

8. Rapp, F., Seligman, S. J., Jaross, L. B., and Gordon, I., Proc. Soc. Exp. Biol. Med. **101**, 289 (1959).

9. Goodheart, C. R., and Jaross, L. B., Virology **19**, 532 (1963).

10. Hahon, N., J. Infec. Dis. **116**, 33 (1966).

11. Thiel, J. F., and Smith, K. O., Proc. Soc. Exp. Biol. Med. **125**, 892 (1967).

12. Osato, T., Mirand, E. A., and Grace, J. T., Nature (London) **201**, 52 (1964).

13. Osato, T., Mirand, E. A., and Grace, J. T., Proc. Soc. Exp. Biol. Med. **119**, 1187 (1965).

14. Pinkel, D., Yoshida, K., and Smith, K., NCI Monogr. No. **22**, 671, (1966).

15. Woods, W. A., and Robbins, F. C., J. Gen. Virol. **3**, 43 (1968).

16. Woods, W. A., Wivel, N. A., Massicot, J. G., and Chirigos, M. A., Cancer Res. **30**, 2147 (1970).

17. Old, L. J., Stockert, E., Boyse, E. A., and Geering, G., Proc. Soc. Exp. Biol. Med. **124**, 63 (1967).

18. Aoki, T., Boyse, E. A., and Old, L. J., Cancer Res. **26**, 1415 (1966).

19. Old, L. J., Boyse, E. A., and Stockert, E., Nature (London) **201**, 777 (1964).

20. Steeves, R. A., and Axelrad, A., Int. J. Cancer **2**, 235 (1967).

21. Ozaki, Y., Diwen, A. R., McBride, W. D., and Melnick, J. L., J. Immunol. **90**, 288 (1963).

Received June 15, 1970. P.S.E.B.M., 1970, Vol. 135.