JOURNAL OF VIROLOGY, Feb. 1969, p. 126-132
Vol. 3, No. 2

Isolation of Naturally Occurring Viruses of the Murine Leukemia Virus Group in Tissue Culture

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Received for publication 14 November 1968

A tissue culture cell system for isolation and identification of members of the murine leukemia virus group (the complement fixation for murine leukemia test) was modified to permit the isolation of naturally occurring virus from leukemic and normal mice. The important factors for increasing the sensitivity of the test were the use of National Institutes of Health (NIH) strain Webster Swiss embryo cell cultures and the selection of rat-immune sera having complement-fixing antibodies to tissue culture antigens of both the Gross and FMR subgroups. In all, 163 strains of mouse leukemia virus, from 11 inbred mouse strains, have been isolated. Representative virus isolates were shown to possess the properties of the murine leukemia virus group; i.e., they were chloroform-sensitive, noncytopathic agents which replicated in mouse embryo tissue culture and produced group-reactive, complement-fixing antigen and budding C-type particles visible by electron microscopy. These viruses could serve as helpers in the rescue of Moloney sarcoma virus genome from non-producer hamster sarcoma cells, yielding pseudotypes. All of the 19 field isolates tested were neutralized by Gross passage A antiserum but not by potent antisera to the Moloney, Rauscher, and Friend strains. Virus was recovered regularly from embryos and from the plasma and spleen of adult mice of high leukemic strains. In low leukemic mouse strains, different patterns of virus detection were observed. In C3H/He mice, virus was occasionally present in embryos and was found in 40% of adult spleens. BALB/c mice were virus-negative as fetuses or weanlings, but spleens of more than half of the mice over 6 months of age yielded virus. NIH mice have never yielded virus. In reciprocal matings between AKR and BALB/c mice, virus recovery from embryos was maternally determined. The development of tissue culture isolation procedures made possible for the first time the application of classical infectious disease methods to the study of the natural history of murine leukemia virus infection.

It has been known for a number of years that laboratory strains of murine leukemia virus (MuLV) can be propagated in cell cultures (7, 12, 21, 24, 25, 27, 29, 32-36). However, the lack of readily noticeable, consistent alterations in the infected cells severely limited the usefulness of this knowledge, with the result that cell culture procedures did not supply the important diagnostic and quantitative tools that they provide for most of the viruses causing acute infectious disease. In 1965, we described the use of rat antisera in the complement fixation (CF) test to detect antigens shared by all known MuLV strains and the utilization of this reaction as the end point in a cell culture test for detection, titration, and neutralization of laboratory strains of MuLV (14, 30). Mouse embryo tissue cultures (METC) inoculated with established virus strains regularly developed the antigen within 7 to 21 days, depending on the dosage of virus.

A major drawback of the test system described originally was its insensitivity for detection of most naturally occurring (field) strains of MuLV. Two factors have now been identified as being responsible for this insensitivity. First, we found that in contrast to many of the laboratory strains of the FMR subgroup of MuLV, the field strains and the Gross passage A virus (11) have a tissue culture host range restricted to certain strains of mice. Cultures of BALB/c mouse embryos, used in the previous studies, are resistant to most field strains, whereas cultures from National

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Institutes of Health (NIH) Swiss mice have a much broader sensitivity. The second factor was the acquisition of rat antisera having very broad group reactivity in CF. The rat anti-Rauscher virus antisera described previously (14), although capable of detecting group reactive CF antigens produced by viruses of both the FMR and Gross-AKR subgroups in mouse and rat tumor extracts, were found to react well with tissue culture antigens of only FMR subgroup viruses. By selecting rat antisera for reactivity with tissue culture-grown antigens of Gross passage A and an AKR field strain (14), we were able to obtain antisera reactive with antigens of both the FMR and Gross-AKR subgroups in tissue culture as well as in tissue preparations.

This report presents a modified procedure which is sensitive for isolation of MuLV from a wide variety of natural sources. We termed this test for MuLV the "COMUL test" (complement fixation for murine leukemia), by analogy with the similar "COFAL test" (31).

MATERIALS AND METHODS

Animals. Inbred Fischer rats and NIH strain mice were obtained from the Animal Production Section of the National Institutes of Health. The NIH strain mouse is a noninbred Webster Swiss; insofar as is known, the incidence of spontaneous leukemia in these mice is very low. The mice tested for the presence of leukemia virus were obtained from various commerical and institutional breeders; they were sacrificed within 1 day after receipt in our laboratory. Tissues were removed aseptically and made into 10% extracts in Eagle's minimal essential medium with 250 units of penicillin and 250 μg of streptomycin per ml (EMEM), with a Ten Broeck grinder. The extracts were clarified by centrifuging once or twice at 2,500 rev/min for 20 min and were stored at -60 C. Tumor extracts for use as CF antigens were similarly prepared. When not frozen, tissues and extracts were held in an ice bath at all times.

Tissue cultures. Primary mouse embryo tissue cultures (NIH-METC) were prepared as previously described (14) by trypsinization of minced 14- to 17-day NIH Swiss embryos; generally, embryos from several mothers were pooled. Inoculations for virus isolation and assay tests were made in 1-day-old secondary plate cultures (60-mm dishes, Falcon Plastic, Inc., Los Angeles, Calif.) seeded with 350,000 cells. Virus and CF antigen pools were prepared by infecting 32-oz (0.946-liter) bottle cultures seeded the previous day with 4×10^6 cells. All cultures were grown and maintained with twice weekly fluid changes in 10% unheated fetal calf serum in EMEM with 2 mM glutamine, and were held in a humidified atmosphere containing 5% CO_2 at 36 C.

CF tests. CF determinations were done in the microtiter system, with 1.8 units of complement, as previously described (17). Serum pools were routinely used for testing unknown tissue culture antigens at a dilution containing 4 to 8 units of CF antibody.

Production of antisera. Rat antisera used in the CF test were obtained from animals bearing transplanted tumors induced with MuLV or the Moloney strain of murine sarcoma virus (M-MSV; 23). For most of the tests reported here, sera were obtained from inbred Fischer rats carrying transplanted sarcomas induced by M-MSV. Weanling rats were inoculated subcutaneously with small amounts of minced tumor tissue and were bled as late as possible after development of the tumor.

The procedure for evaluating sera for inclusion in pools was as follows. Individual sera were titrated in CF against four to eight units of Moloney and Gross passage A antigens prepared in tissue culture. Those sera titering 1:160 or greater against the latter were further tested for the ability of a 1:40 dilution to detect four units of antigen in tests against a battery of tissue culture-antigen preparations including Rauscher, Moloney, Gross passage A, and AKR viruses; the sera were also screened at a 1:20 dilution against a battery of control antigens, consisting of normal rat thymus, normal NIH mouse spleen, and control NIH-METC. In contrast to the sera from noninbred rats used in earlier studies (14), sera prepared in inbred Fischer rats almost never reacted with control antigens. Sera which showed satisfactory titer, specificity, and sensitivity for detecting antigen were pooled; these pools were referred to here as broadly reactive serum pools.

Several points should be noted concerning the production and testing of antisera. For the most part, obtaining suitable broadly reactive sera has been a difficult and inefficient process. Less than 1% of the rats bearing transplants of lymphomas induced with Gross passage A or Rauscher viruses yielded suitable sera; hyperimmunization with tissue culture virus gave a similarly low frequency of adequate response. However, a higher frequency of broad response was found in recipients of the M-MSV transplanted sarcoma.

An important factor in obtaining sufficiently long survival of the tumor-bearing animals for broad antibody response to occur was the use of tumor lines carried in suckling rats rather than in older animals for inoculation of weanlings for antiserum production.

The broadly reactive serum pools showed group-specific reactivity with both soluble and sedimentable ($15{,}000 \times g$, 1 hr) fractions of MuLV antigens (15). It should be noted that the rat antiserum used by Geering et al. (6) to demonstrate group-reactive antigens in immunodiffusion tests with ether-treated virus showed high-titer broad reactivity in CF tests in our laboratory (this serum was kindly supplied by Lloyd J. Old).

Although we found that CF antibody to MuLV occurs in the sera of many normal, retired breeder rats (30), this did not appear to be a contributory factor in the antibodies studied here, since the naturally occurring antibodies were not broadly reactive and were present chiefly in sera of rats much older than those used for antiserum production.

Virus and CF antigen pools. To produce pools of infectious virus, cultures were harvested 16 to 21 days after inoculation with undiluted tissue culture passage

virus; the pools represented supernatant fluids only or fluids plus sonic-treated cells. For use as antigens in the CF test, the infected cells were suspended in one-twentieth of the supernatant fluid and then were sonic-treated; antigen titers in such preparations were 1:16 or higher. Essentially, no anticomplementary activity was encountered, even when antigens were tested undiluted or at a 1:2 dilution.

Virus isolation procedure. In routine virus isolation attempts, 0.05 or 0.1 ml of the extracts was inoculated into each of two plate cultures. The cultures were harvested at 19 to 21 days by scraping the cells into 0.6 ml of supernatant fluid per plate. One-half of the harvest was frozen and thawed once and sonic-treated for 2 to 3 sec in a plastic tube (12 by 75 mm; Falcon Plastics, Inc.), with a Branson Sonifier with a microtip attachment; sonic-treated materials were screened for CF antigen at a 1:2 dilution, and many of the positives were titered for end point. The untreated portion was stored at −60 C for additional tests or later passage. All negative or partially reactive culture samples were blind-passaged to fresh NIH-METC. Passages were made by inoculating plate cultures with 0.1 ml of crude unclarified tissue culture cell suspension, prepared by disrupting the frozen and thawed cells by gentle sonic-treatment or by aspiration with a syringe and 26-gauge needle. Positive cultures in first or second passage isolation tests ranged in antigen titer from 1:2 to 1:16.

The presence of virus in embryos of various mouse strains was determined by growing the cells from the whole embryos in tissue culture and then testing suspensions of the cultured cells for CF antigen and virus. Primary cultures and secondary cultures (prepared after 6 to 10 days of primary cultivation) were tested for CF antigen at weekly intervals for 3 or 4 weeks; the 3-week cell harvests were tested for antigen-inducing virus in NIH-METC. Routinely, we tried to avoid contamination of the embryos by the mother's blood by using different sets of instruments for opening the uterus and for removing the fetuses. In a few experiments, an electric cauterizing device was used for incising the uterus and detaching the embryo; no difference was found in the ease of detecting virus in cultures prepared from these embryos as compared to those prepared by the routine procedure.

Neutralization tests. Neutralization tests were performed in tissue culture, utilizing as the end point prevention of CF antigen induction (COMUL test) or, in the case of M-MSV pseudotypes, reduction in the number of foci (13). In both tests, virus controls and mixtures of virus and serum were incubated at room temperature for 60 min before inoculation of the cultures. For antigen reduction tests, the test dose of virus used was 10-fold greater than the highest dilution of virus-inducing antigen in 7 days. Standard cell harvests, made 7 days after inoculation, were titrated in the CF test; fourfold or greater suppression of antigen formation was considered positive neutralization. The focus reduction test employed 30 to 100 focus-forming units per plate, and neutralization was defined as 67% or greater reduction in the number of foci.

Preparation of MSV pseudotypes. Mixed cultures of 2×10^5 NIH-METC cells and 8×10^4 MSV hamster tumor cells (HT-1 tissue culture line) were inoculated at the time of planting with tissue culture passage material of various field isolates; the cultures were maintained and harvested as previously described (16). Harvests were assayed for focus-forming virus in NIH-METC and for sarcomagenic virus by combined subcutaneous and intramuscular inoculation of newborn NIH Swiss mice.

RESULTS

Isolation of virus from mice of various strains. Table 1 summarizes our findings when materials from normal and leukemic uninoculated mice were tested. The data represent tests of individual mice and pooled tissues from 2 to 5 animals, the majority of tests being of individual animals. Except for the tests of embryo cells, all isolations are based on recovery of virus in NIH-METC. Virus was recovered regularly from the embryos, plasma, and normal and leukemic tissues of strains of mice with a high incidence of spontaneous leukemia. In the strains with a low leukemia incidence, greater variation in the patterns of virus recovery was seen. In C3H/He mice, virus was detected in embryos and adult tissues, but not as regularly as in high leukemic mice. BALB/c embryos did not yield virus, but the spleens of retired breeders were frequently positive. NIH Swiss mice were consistently negative in all tests. In no instance was a difference in the frequency of virus recovery seen between mice of the same strain raised in different colonies.

The sensitivity of different tests for detecting virus in fetal cells is shown in Table 2. In general, positive CF antigen tests were obtained with primary culture cells after 14 to 21 days and with secondary culture cells at 7 to 14 days; a few embryos were positive only in secondary culture or after passage to NIH-METC. With the exception of NIH Swiss and two of the BALB/c cultures, all results represent the testing of a single litter. Virus was more difficult to detect in extracts prepared from the original embryo mince. In tests of nine embryo pools obtained from high leukemia strains, positive results were obtained with all when the embryo cells themselves were cultivated, but virus was recovered from only four of the nine extracts of embryo tissues.

To investigate further the transfer of virus to the fetus, reciprocal matings were allowed between AKR/N and BALB/cN mice, and the embryos were cultured for virus detection (Table 2, bottom). The embryos consistently showed the

TABLE 1. *Recovery of virus from normal and leukemic mice*

Leukemia incidence	Strain	Colony[a]	Embryo tissue culture	Newborn			Weanling		Adult and retired breeder			Leukemic tissue	Total
				Spleen	Thymus	Pooled viscera[b]	Plasma	Spleen	Plasma	Spleen	Thymus		
High	AKR	L, J, N	7/7[e]	1/1		1/1	5/5	8/8	31/38			7/7	60/67
	C58	L, J	3/3						5/5			3/3	11/11
	C3H/Fg	L	4/5						4/4				8/9
	SJL[c]	L, J								1/1		2/2	3/3
Low or data not available	RF	T, J								2/2		3/4	5/6
	CF-1	C, A	1/2	1/1	1/1				4/9	17/23	5/5		29/41
	CFW	W, C								1/14	0/10		1/24
	DBA/2	N, M		0/1					1/1	4/11	1/1		6/14
	C3H/He	N, H, S	3/10					0/8	2/10	12/30			17/58
	C3Hf/Bi	L, M	0/1						0/1	0/6	1/3		1/11
	BALB/c	N, M, J	0/11		0/4	0/5		0/8	1/25	20/39			21/98
	NIH Swiss	N	0/180[d]							0/16	0/6		0/196[d]
	Wild (rural)									0/11			0/11

[a] Colonies from which mice were obtained: L = L. W. Law, National Cancer Institute, NIH; J = Jackson Memorial Laboratory, Bar Harbor, Me.; N = NIH Animal Production Section; O = Oak Ridge National Laboratory, R. Tyndall; C = Carworth Farms; A = Argonne National Laboratory, M. Finkel; W = A. A. Werder, Univ. of Kansas; M = Microbiological Associates, Inc., Bethesda, Md.; H = W. Heston, National Cancer Institute, NIH; S = A. R. Schmidt Co., Madison, Wis.

[b] Thymus, liver, spleen, kidney.

[c] High incidence of reticulum cell sarcoma.

[d] Approximate number.

[e] No. positive/no. tested.

virus recovery pattern of the maternal strain: all AKR × BALB/c litters were virus-positive, whereas all BALB/c × AKR litters were negative.

Characterization and identification of field isolates. To further establish the virus isolates as belonging to the MuLV family, representative isolates were tested for distinctive properties of this group of agents (Table 3). The field strains were chloroform-sensitive (10%, 10 min at 4 C) and did not react in CF against four to eight units of antibody against other mouse and rat viruses, including lymphocytic choriomeningitis, mouse hepatitis, Sendai, mouse adenovirus, minute virus of mice, polyoma, K, reovirus, Theiler's GDVII, Kilham rat virus, H-1 and SV5.

The field isolates shown in Table 3, as well as nine other strains from AKR, C58, BALB/c, CF-1, and CFW mice, were tested for neutralization by rat antisera to Gross passage A, Moloney, Friend, and Rauscher viruses. All were neutralized by the Gross antiserum, and not by the FMR subgroup antisera.

Examination of infected NIH-METC cells for particles detectable by electron microscopy was kindly carried out by Walter B. Becker, M. David Hoggan, and Jane Dees. Flask cultures were infected with undiluted virus and were maintained for 16 days; a portion of the cell sheet was then harvested in the usual manner to confirm that CF antigen was present, and the remainder was fixed with glutaraldehyde and osmium tetroxide, embedded in Epon, and sectioned. The sections were stained with lead citrate and were observed in a Siemens electron microscope. Numerous C-type particles, budding from plasma membranes and free in intercellular spaces, were observed in all inoculated cultures. Uninoculated cells from the same test were negative for CF antigen and particles; in addition, William T. Hall examined sections of three lots of control NIH-METC, with negative results.

Like the Rauscher, Moloney, Friend, and Gross passage A strains of MuLV (15, 16), all field isolates tested were capable of serving as helper viruses for the rescue of the sarcomagenic marker in nonproducer MSV hamster tumor cells. Fluids from mixed cultures of NIH-METC and HT-1 cells infected with tissue culture passage viruses induced typical foci in NIH-METC. Newborn NIH mice inoculated with such fluids or with harvests from focus-containing NIH-METC cultures developed sarcomata essentially indistinguishable from those induced by M-MSV, and extracts prepared from the tumors induced typical foci in tissue culture.

The various tissue culture isolates were not tested extensively for leukemogenic activity. In a preliminary test in rats, trypsinized NIH-METC

TABLE 2. *Detection of virus in embryo tissue cultures of various mouse strains*

Strain	Development of antigen in embryo cultures		Virus detected on transfer to NIH-METC[a]	Virus detected in fetus extracts[b]
	Primary	Secondary		
AKR	2/6	7/7	7/7	2/5
C58	2/3	3/3	3/3	2/2
C3H/Fg	2/5	4/5	5/5	0/2
CF-1	0/1	1/1	1/1	
C3H/He	1/10	2/10	3/10	0/1
C3Hf/Gr	0/1		0/1	
BALB/c	0/9	0/11	0/8	
NIH Swiss		0/180		
AKR ♀ × BALB/c ♂	2/5	5/5	5/5	
BALB/c ♀ × AKR ♂	0/19	0/19	0/11	

[a] Standard virus isolation test of extracts of primary or secondary tissue culture cells, or both.

[b] NIH-METC tests of 10% extracts of fetuses, all of which were positive on tissue culture cell cultivation.

cells from cultures representing the third tissue culture passage of two isolates from normal AKR mice induced lymphomas in 6 of 9 and 7 of 11 Fischer rats, respectively, in 6 to 15 months after intraperitoneal inoculation as newborns. Similarly, lymphomas developed within 7 to 14 months in 10 of 11 rats receiving second NIH-METC passage of an isolate from an SJL leukemia. No lymphomas developed during 15 months of observation in 16 rats inoculated with control NIH-METC cells.

DISCUSSION

All previous isolations of MuLV have been obtained by animal inoculation, with production of leukemia in mice or rats as the end point. The tissue culture system used here is not only a far more rapid test, but is doubtless capable of detecting viruses which could not be detected in an in vivo system, either because of low titer or lack of pathogenicity. Also, a tissue culture system provides far greater freedom from contamination with related or unrelated viruses.

The data presented here indicate that MuLV are widely prevalent among laboratory mice, not only in inbred strains with a high incidence of leukemia, but also in certain low leukemic strains. Our results confirm the findings of Gross (8, 10) that virus is detectable in fetuses of high leukemic mice, and, in addition, show that the same is true of certain low leukemic strains. The present findings also support the contention that the "C" type particles so frequently seen on electron microscopy of fetal and infant mice (2–4, 18, 19) represent viruses of the murine leukemia group.

Different patterns of virus recovery were seen in high spontaneous leukemia mouse strains as opposed to low leukemic strains, as well as between different low leukemia strains. Virus was almost universally present in AKR, C58, and C3H/Fg mice, regardless of age and the presence or absence of leukemia. C3H/He mice showed a pattern of fetal infection resembling that of the high leukemic strains, but with less regular detection of virus in the fetuses. It will be important to study the relative roles of viral and host genetic makeup and of immunological factors in determining the low susceptibility of this line of C3H mice to both spontaneous and Gross passage A virus-induced leukemia (9).

Although fetal, suckling, and weanling BALB/c mice never yielded virus, older animals of this strain showed progressively increasing frequency of virus detectable in the spleen. In recent studies, over 90% of BALB/c mice over 18 months of age have been virus-positive. These findings suggest that BALB/c mice transmit the MuLV genome vertically, the virus not being detectable in early life, but a change in virus growth pattern resulting in an increasing incidence of detectable virus with age. Horizontal postnatal infection represents another but less likely possibility.

In addition, MuLV with a different tissue culture host range from that found in the normal animals reported here has been recovered from a number of older BALB/c mice, particularly those with primary leukemias and other tumors (*unpublished data*).

Our experience with NIH Swiss mice thus far indicates the absence of infectious virus. However, it should be noted that rare C-type particles have been observed in thymus sections of fetal NIH mice (19), and murine leukemia group CF antigen has been found in a radiation-induced leukemia in an NIH mouse (*unpublished data*). Large numbers of NIH mice have not been observed throughout their life span, and little is known of their neoplastic or virological experience in later life.

To date, all field strains tested for serotype by the neutralization test have been found to be related to the Gross passage A virus, and not to the FMR subgroup. This is in agreement with the findings of Aoki et al. (1), that only the G+ antigen, and not the FMR antigen, is detectable in uninoculated mice. The natural source of the viruses of the FMR subgroup, all representatives

TABLE 3. *Properties relating tissue culture isolates from normal and leukemic mice to the murine leukemia virus group*

Tissue culture passage virus (strain)	MuLV[a] group CF antigen	Other[b] CF antigen	Neutralized by gross passage A antiserum[c]		$CHCl_3$ sensitive	EM C-type particles	MSV rescue	Leukemogenesis in rats
			Parent virus (COMUL test)	Pseudotype (focus reduction)				
AKR leukemia	+	−		+	+	+	+	
Embryo	+	−	+	+	+	+	+	
Adult plasma	+							+
Newborn spleen and liver	+							+
C58 leukemia	+	−		+		+	+	
SJL leukemia	+	−		+	+		+	+
C_3H/Fg embryo	+	−	+				+	
C_3H/HeN embryo	+	−	+		+	+	+	
DBA/2 plasma	+	−		+	+		+	
RFM spleen	+	−		+	+	+	+	
BALB/c spleen	+	−		+	+	+	+	
CF_1 spleen	+		+				+	
Rauscher LV	+	−	−	−	+		+	
Moloney LV	+	−	−	−	+		+	
Friend LV	+	−	−	−			+	
Gross LV	+	−	+	+	+		+	

[a] Development of MuLV group-reactive CF antigen in NIH-METC cells, +.

[b] Tests of 8 to 16 CF antigen units against antisera to various mouse and rat viruses. Blank space = not tested.

[c] Serum obtained from Fischer rats carrying a transplantable lymphoma induced by Gross passage A virus; the serum dilution used contained 2 to 4 units of neutralizing antibody to homologous virus.

of which have been recovered from laboratory passage material (5, 22, 26, 28), remains obscure.

The NIH-METC system reported here has permitted the isolation and laboratory study of a wide variety of strains from naturally infected animals, but is not sensitive to all strains of virus. The most notable example is the virus recovered by Kaplan from C57B1 leukemia (20), which does not induce antigen in NIH-METC, even though the MuLV group antigen is present in the leukemic tissues (30). Also, as mentioned above, we have recently recovered some field strains from tumored BALB/c mice with a distinctly different host range in METC.

Sources of Swiss mice, other than the NIH strain, have not been fully evaluated for their sensitivity for the isolation of naturally occurring viruses of the MuLV group, nor have we evaluated a wide variety of inbred mouse strains. It should be emphasized that in choosing a source of embryos for the tissue culture indicator system, selection must be based not only on sensitivity but on absence of endogenous virus. In view of the frequent presence of leukemia virus in embryo cultures of certain mouse strains reported here, the choice is clearly limited.

The finding that field isolates grown in tissue culture could be employed in the recovery of MSV pseudotypes should facilitate the serological characterization of naturally occurring MuLV, since the antigenic nature of a pseudotype is that of the helper virus (15, 16).

ACKNOWLEDGMENTS

We thank Charles W. Shifler for invaluable technical assistance and Horace C. Turner who supervised the performance of complement fixation tests.

This investigation was supported by the Etiology Area of the National Cancer Institute.

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