RESCUE OF THE DEFECTIVE GENOME OF MOLONEY SARCOMA VIRUS FROM A NONINFECTIOUS HAMSTER TUMOR AND THE PRODUCTION OF PSEUDOTYPE SARCOMA VIRUSES WITH VARIOUS MURINE LEUKEMIA VIRUSES*

By R. J. Huebner, J. W. Hartley, W. P. Rowe, W. T. Lane, and W. I. Capps

U.S. PUBLIC HEALTH SERVICE, NATIONAL INSTITUTES OF HEALTH, NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES, LABORATORY OF INFECTIOUS DISEASES, BETHESDA, MARYLAND

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Hartley and Rowe[1] reported that the *in vitro* focus-forming effects of Moloney sarcoma virus (MSV)[2, 3] depended on the presence in the same cells of two virus particles, a defective MSV particle and a fully infectious Moloney leukemia particle. Superinfection with additional Moloney leukemia virus potentiated focus formation by MSV, converting a "two-hit" dose response curve to a one-hit curve; preliminary experiments also suggested a two-hit requirement for sarcoma induction *in vivo* by MSV.[4] These experiments suggested that murine leukemia viruses serve as "helpers" for a defective MSV particle in much the same way that avian leukosis viruses help to complete defective Rous sarcoma virus (RSV) infectious particles, but with the difference that in the MSV system in mouse cells helper virus may be required for cellular alteration as well.[1]

Sarma, Vass, and Huebner[5] described a virus-free sarcoma induced in hamsters by the defective Bryan strain of RSV, the cells from which when propagated in mixed tissue cultures with chicken embryo fibroblasts transferred the noninfectious RSV genome to the latter. When the mixed cultures were superinfected with avian leukosis viruses, fully infectious RSV was released. On the other hand, when uninfected, mixed cultures were implanted in the wing web of leukosis-free chicks, virus-free sarcomas having the avian karyotype were produced. When cells from these sarcomas were grown in tissue cultures, they behaved as typical nonproducer (NP) sarcoma cells. The addition of avian leukosis viruses to these avian cells yielded infectious RSV. In this system, the "nonproducer" hamster and chick cells contain large amounts of complement-fixing (CF) and immunofluorescent-stainable antigens believed to represent the internal protein moiety of the virus,[6, 7] but the genetic information for the outer envelope must be supplied by the helper virus.

In this communication we describe fibrosarcomas induced in hamsters by the Moloney sarcoma virus which carry the defective MSV genome but not infectious MSV or murine leukemia virus nor mouse leukemia group reactive CF antigen. By adding various standard murine leukemia viruses such as the Rauscher, Friend, Moloney, and Gross strains to mixed cultures of MSV-induced hamster tumor cells and normal mouse embryo fibroblasts, we obtained fully infectious pseudotypes of MSV having the immunological characteristics of the helper leukemia viruses. Sarcomas containing the infectious pseudotype viruses were also readily produced when newborn Swiss mice were injected with MSV hamster tumor cells mixed with various murine leukemia viruses.

Materials and Methods.—Viruses: The MSV used for inoculation of hamsters originated from a sixth mouse passage BALB/c tumor preparation (#SV43) obtained from Dr. Moloney. The

virus was passed by us four additional times in BALB/c mice using cell-free tumor extracts. The fourth passage tumors were pooled and processed according to a modification of a procedure described by Moloney.[8] This cell-free 1 gm/ml concentrate had a tumor $dose_{50}$ (TD_{50}) titer of 10^4/0.05 ml when titrated in newborn BALB/c mice and 10^4 focus-forming units/0.4 ml in mouse embryo tissue cultures.

Murine leukemia virus strains used for rescue experiments were the standard laboratory strains passaged in mouse-embryo tissue culture.[9] They have been tested repeatedly for ability to induce foci in tissue culture and sarcomas in newborn mice, with completely negative results.

Virus assays: The procedures used for detection and assay of murine leukemia and sarcoma viruses were those described in previous reports from this laboratory.[1, 9-11] Leukemia viruses were assayed in whole-embryo tissue cultures derived from NIH Swiss mice (MEF) using formation of CF antigen reactive with mouse leukemia group reactive rat serum as the indicator of virus growth.[9] Focus-forming sarcoma viruses were assayed in MEF or the CL-1 line of BALB/c embryo cells[1] without added helper virus. Detection and titrations of sarcoma virus *in vivo* were based on grossly observable tumor formation following subcutaneous and intramuscular injection of suckling NIH or BALB/c mice with 0.05 ml of each dilution of the virus preparations.

Tissue culture cell line of hamster tumor cells: All marker rescue studies were done with a cell line (HT-1) from a first transplant hamster tumor established in culture by trypsinization. In early passages the cells were grown in Eagle's minimal essential medium with glutamine, penicillin, and streptomycin (EMEM) with 10% heated calf serum; later passage cells were grown in EMEM with 10% unheated fetal calf serum. The cultures consisted of small and large round cells which grew both on the plastic surface and in suspension. Transplantation of 10^6 cells of the fourth passage into newborn hamsters resulted in tumor growth within 6 days.

Recovery of MSV pseudotypes in vitro: Mixed cultures of HT-1 and secondary MEF cells were infected with tissue culture passaged mouse leukemia viruses.[9] Plate cultures (50-mm dishes, Falcon Plastics) were seeded with 4×10^4 HT-1 cells and 2×10^5 MEF cells in 4 ml and were infected either at the time of plating or 24 hr later, using 0.4 ml of virus dilution. In early experiments, medium for growth and maintenance was 10% heated calf serum in EMEM; more satisfactory results were later obtained with 10% unheated fetal bovine serum in EMEM, and this medium was used routinely thereafter. Fluids were harvested and replaced with fresh medium at intervals of 2–4 days. Since the HT-1 cells tended to outgrow the MEF cells, it was necessary to add fresh MEF cells to maintain a susceptible cell population for the leukemia viruses; 2×10^5 cells were added per plate, usually between the tenth to thirteenth day. Culture fluids were assayed for focus-inducing virus at various intervals.

Serology: Tests for the presence of the mouse leukemia group reactive CF antigen[1, 9-11] in hamster and mouse tumor extracts were done with serum of a Fischer rat carrying a transplanted MSV-induced sarcoma; this serum reacted in both CF and neutralization tests with all mouse leukemia strains tested, but tended to have highest CF titers against MSV and Moloney virus antigens. Confirmatory CF tests, and focus neutralization tests, were done with sera of rats immunized with mouse leukemia viruses grown in tissue culture.

Animals: All animals were obtained from the NIH animal production colony.

Results.—Induction of MSV tumors in hamsters: Subcutaneous injection of the MSV inoculum (0.1 ml of a 10^{-1} dilution) in two sites produced local sarcomas in newborn hamsters which had gross and microscopic characteristics similar to the spindle cell sarcomas induced in BALB/c mice.[12] Although the same inoculum induced tumors in 12 of 12 newborn BALB/c mice within 10 days, the tumors induced with this dosage in hamsters were much delayed. In 22 newborn hamsters the first tumor was noted at 64 days and the second at 68 days. Subsequent tumors were noted at 170, 196, and 254 days, by which time 9 of the 22 hamsters had developed tumors. When the test was repeated with the same inoculum, 7 tumors were observed by 200 days in 24 hamsters.

Subcutaneous transplantation of minced primary tumor tissue required 70 days or more to produce detectable tumors in newborn hamsters and 100 days in weanlings. However, in both weanlings and newborns, transplanted tumors grew

progressively to large size; they often exceeded 50 mm in diameter and showed no tendency to regress. In contrast to the first transplant passage, second, third, and fourth transplant passages in newborn hamsters often yielded tumors in about half the hamsters in 10 days: however, this response was variable since other groups of hamsters receiving similar transplants sometimes required 30–60 days or more for successful tumor induction. A fifth transplant passage required over 130 days before tumors appeared in three of ten newborn hamsters.

Failure to detect virus or antigen in hamster tumor cells: A variety of techniques were employed in unsuccessful attempts to demonstrate leukemia or sarcoma virus in the hamster tumors and the HT-1 tissue culture cell line. Extracts of primary and transplanted tumors did not induce sarcomata when inoculated into newborn BALB/c, C57BL, and NIH strains of mice or into newborn hamsters; they did not induce foci in MEF cultures when tested with or without Moloney leukemia virus as helper; they did not induce CF antigen in MEF; and they did not react in the CF test with broadly reactive sera of rats immunized with Moloney leukemia virus or the sarcoma virus. Also, hamsters carrying transplants of the tumor never developed CF antibody reactive with mouse leukemia virus antigens or the homologous tumor.

The HT-1 cells were similarly negative. Culture fluids and cell extracts were negative for CF antigen and did not induce foci or CF antigen in mouse embryo cultures. In a preliminary study, electron microscopic examination of thin sections of the cells revealed no particles suggestive of viruses.

Recovery of MSV genome by mixed culture with helper virus: When the hamster tumor cells were grown with NIH mouse embryo cells and the mixed culture inoculated with Moloney leukemia virus, focus-forming MSV was recovered regularly. Table 1 shows results of a representative experiment. Fluids from mixed cultures without helper, or from cultures of hamster cells inoculated with helper virus but without mouse cells, did not induce foci. Thus, all three components—tumor cells, mouse embryo cells, and helper virus—were required for detection of the focus-forming genome.

Strains of mouse leukemia virus other than Moloney were also able to supply the helper virus function. The Rauscher and Friend viruses consistently yielded large numbers of focus-forming units when added to the mixed cultures (Table 1). Smaller numbers of foci were obtained on assay of fluids from mixed cultures infected with Gross Passage A (Table 1) virus or a strain recovered in this laboratory from AKR mouse embryos. No morphologic changes suggestive of transformation were seen

TABLE 1

RECOVERY OF FOCUS-FORMING CAPACITY FROM HAMSTER TUMOR CELL LINE HT-1 BY MIXED CULTURE WITH MOUSE EMBRYO (MEF) CELLS AND MURINE LEUKEMIA VIRUSES AS HELPER

	Helper Virus		No. of Focus-Forming Units/0.4 ml in Fluids Harvested at Various Times (days)				
Cells	Strain	Dose	6	10	12	14	17
HT-1	Rauscher	$10^{6.1}$		0	0	0	0
HT-1 + MEF	None		0	0		0	0
"	Rauscher	$10^{6.1}$	>300	1700	2000	1800	2800
"	"	$10^{4.1}$	190	400		1500	1250
"	"	$10^{2.1}$		68		860	1130
"	"	$10^{1.1}$		0		12	170
"	Moloney	NT*		>200	>200	>200	1050
"	Gross Passage A	$10^{6.6}$		1	2	6	13

* NT = Not titrated.

in the mouse cells of the mixed cultures, but such alterations might well have been obscured by the overgrowth of hamster cells.

MSV rescue experiments in vivo: The ability to retrieve the sarcoma genome by growing the hamster tumor cells with mouse cells and infecting with mouse leukemia virus suggested that a similar phenomenon might be demonstrable *in vivo*. Hamster tumor cells grown in tissue culture were dispersed with trypsin and suspended at a concentration of 10^6 cells per 0.025 ml. Newborn NIH strain Swiss mice were inoculated intramuscularly with 0.05 ml of a mixture of the cells with either control culture fluid or leukemia virus. Of 52 animals receiving transplanted cells with control fluid, none developed tumors. These animals often had transitory swelling of the inoculated leg, presumably due to abortive growth of the hamster cells. Of 55 mice injected with hamster cells plus Moloney virus, 38 (69%) developed progressively growing tumors at the site of inoculation, which eventually killed the animals. Histologically these tumors were typical MSV-induced sarcomata.[12] Similarly, 14 of 18 mice receiving hamster tumor cells plus Rauscher virus developed sarcomas; tumors were also produced by Friend and Gross leukemia viruses plus HT-1 cells.

The *in vivo* rescue of sarcomagenic virus was also successful with cells from two hamster tumor transplants other than those derived from the HT-1 cells.

Properties of the viruses recovered from the in vitro and in vivo rescue experiments: The fluids from the assay plates in the mixed culture experiments and the extracts of tumors developing in infant mice inoculated with HT-1 cells plus Moloney, Rauscher, or Friend virus readily induced foci when passed serially in MEF and CL-1 tissue cultures, and sarcomata when inoculated into infant mice. Table 2 shows some of the virologic characteristics of extracts of mouse tumors induced with viruses rescued *in vitro* or *in vivo* with these helpers. Like the standard MSV tumors, which generally contain CF antigen in titers of 1:8 to 1:16, the tumors induced by the rescued viruses reacted in CF with the MSV rat serum; the CF titers tended to be lower with the Rauscher and Friend rescued viruses, which may be a reflection of lower heterologous reactivity, often encountered with rat antisera to mouse leukemia viruses. The data in the table suggest a rough correlation between CF antigen titer and titer of focus-forming particles; all of those tested readily produced tumors in mice.

TABLE 2

INFECTIOUS VIRUS AND CF ANTIGENS IN EXTRACTS OF MOUSE SARCOMAS INDUCED IN NEWBORN NIH MICE BY PSEUDOTYPE MOLONEY SARCOMA VIRUSES

Pseudotype[a]	Derivation	Mouse passage[b]	Type of extract[c]	Focus-forming titer	CF antigen titer	Tumor Induction[d]	
MSV(MLV)	Mixed TC + MLV[e]	P_0	Crude	$10^{3.1}$	4	NT	NT
"	"	P_1	Conc.	$10^{5.0}$	16	13/13	8 days
"	HT-1 + MLV (*in vivo*)	P_0	Crude	$10^{1.7}$	<2	4/12	12 days
MSV(RLV)	Mixed TC + RLV	P_0	Crude	$10^{3.5}$	4	NT	NT
"	"	P_1	Conc.	$10^{4.7}$	8–16	21/21	8 days
"	HT-1 + RLV (*in vivo*)	P_0	Conc.	$10^{4.4}$	<2	20/20	8 days
MSV(FLV)	Mixed TC + FLV	P_0	Crude	$10^{1.3}$	<2	17/17	9 days
"	"	P_1	Conc.	$10^{5.0}$	2	22/22	8 days
"	"	P_2	Conc.	$10^{5.7}$	4	16/16	7 days

[a] Viruses rescued with various helpers. MLV = Moloney leukemia virus. RLV = Rauscher leukemia virus. FLV = Friend leukemia virus. NT = not tested.
[b] P_0 = Primary inoculation. P_1 = First passage (extract or transplant), etc.
[c] Crude = 10% extract. Conc. = Concentrated virus prepared by Moloney procedure.[8]
[d] No. with tumor/no. mice inoculated with undiluted crude extract or 10^{-1} dilution of concentrate. Days indicate time of first tumor.
[e] Mixed TC = HT-1 hamster tumor cells plus MEF.

TABLE 3

ANTIGENIC SPECIFICITY OF MSV PSEUDOTYPES

Rat antiserum‡	Neutralizing Antibody Titer* vs: Moloney	Rauscher	Friend	Neutralization Index† MSV(MLV)	MSV(RLV)	MSV(FLV)
Moloney	60	0§	0	>25	1	1
Rauscher	10	40	20	1	>22	10
Friend	20	10	40	1	1	39

* Reciprocal of titer as determined in tissue culture neutralization test against standard tissue culture passage leukemia virus lines (nonfocus formers).
† Ratio of number of foci induced by virus plus 1:20 control rat serum (immunized with control tissue culture fluid)/no. induced by virus plus 1:20 immune serum.
‡ Sera of rats immunized with murine leukemia viruses grown in tissue culture.
§ 0 = <1:10.

In preliminary tests with rat antisera selected for having relatively type-specific neutralizing antibody, it appeared that the focus-forming viruses had acquired the serological character of the virus used for retrieval (Table 3), in a manner analogous to that described for the Rous virus "pseudotypes."

Attempts to characterize the agents retrieved with Gross Passage A virus have been hampered by the low titers of focus-forming virus recovered in both the *in vitro* and *in vivo* systems.

Discussion.—The most likely explanation of the results reported here is the same as that proposed for the analogous finding with avian sarcoma virus-induced tumors of hamsters,[5, 13–17] that is, that the defective genome of the sarcoma virus persists in the hamster cells, transfers to the mouse cells both *in vitro* and *in vivo*, and is propagated along with the superinfecting leukemia virus. It has been shown previously[1] that the focus-forming particles require helper virus for their replication in mouse cells. The inability of the leukemia virus to rescue the sarcoma genome directly from the hamster cells is probably due to failure of the leukemia viruses to propagate in hamster cells, though this has not been established beyond the observations that the cells do not form detectable levels of CF antigen following inoculation with various mouse leukemia viruses, and that antibodies to MSV virion preparations were not produced in the hamsters carrying MSV-induced tumors.

The Harvey strain of MSV has been reported to induce tumors in hamsters,[2] but Moloney's isolate has not previously been found to be oncogenic in this species.[18] Whether the tumors induced by the Harvey strain are also noninfectious remains to be determined.

The failure to find the mouse leukemia group CF antigen in the MSV hamster tumor cells represents a marked difference from the otherwise similar avian sarcoma virus–hamster tumor system,[5–7] in which group reactive antigen[19] is consistently present. This apparent discrepancy may be due to differences in the nature of the antigens. The mouse leukemia group antigen is inseparable from the virion[4, 9] and probably represents an antigen in the envelope; the avian leukosis CF antigen in hamster cells is an internal virus protein[6, 7] while envelope antigens, which are type-specific, are not detectable in the noninfectious tumor cells.

It is suggested that the term MSV be retained as the designation of the original sarcoma strains (Moloney's and Harvey's), and that the pseudotypes produced with the Moloney, Rauscher, Friend, or Gross Passage A leukemia viruses be designated MSV(MLV), MSV(RLV), MSV(FLV), and MSV(GLV), respectively.

It will be important to determine whether or not the avian and murine models will provide useful patterns for studies of the etiology of sarcomas and leukemias of other species, including those observed in man.

Summary.—A hamster MSV-induced rhabdomyosarcoma carried *in vivo* and *in vitro* was free of infectious virus and CF antigen. When the hamster tumor cells were grown in contact with mouse cells, either as mixed tissue cultures or by inoculation into newborn mice, in the presence of various murine leukemia virus strains focus-forming and sarcomagenic viruses were readily recovered. Preliminary studies indicate that the focus-forming viruses so recovered have the envelope antigens of the helper virus, and thus represent newly created pseudotypes of MSV.

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First transplant of MSV tumor cells in a newborn hamster: This tumor has the appearance of a pleomorphic fibrosarcoma with some collagen production. There are giant cells similar to those which typified MSV tumors in BALB/c mice and intermixed small round cells in the tumor. There are also scattered larger cells suggestive of a rhabdomyosarcomatous element.

Third transplant in a newborn hamster: This is an undifferentiated tumor composed of sheets of rather uniform round to polygonal cells which have sharp cell margins. Spindle cell differentiation is very poorly developed and rhabdomyosarcoma elements are not seen. There are many mononuclear giant cells within this tumor.

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