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Properties of Noninfectious and Transforming Viruses Released by Murine Sarcoma Virus-Induced Hamster Tumor Cells

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The cell culture lines HTG2 and HTG3 were established from a transplantable hamster tumor induced by a murine sarcoma virus (strain Gz-MSV) after 17 and 60 in vivo passages, respectively. The viruses released by these two cell lines markedly differ in morphology, antigenic composition, infectivity, transforming ability, and enzymatic activity. HTG2 virions contained the sarcoma genome but were noninfectious for mouse and hamster cells (S+H-virus). HTG3 virions transformed hamster but not mouse cells. Whereas HTG2 cells and its virus contained murine type C virus gs-1 antigen, all HTG3 clonal lines expressed both murine and hamster type C virus gs-1 antigens. The RNA-dependent DNA polymerase activity of HTG2 virus was very low, whereas that of HTG3 virus was relatively high. HTG2 virions contained electron-lucent centers only. HTG3 virus consisted of the expected mixture of virions with electron-dense and electron-lucent centers. Many broken or incomplete virions were present in both viruses. HTG2 virus is a noninfectious "defective" sarcoma virus without detectable helper virus. Data obtained in these experiments suggest that HTG3 virus is a hamster type C virus pseudotype of Gz-MSV (Gz-MSV [HaLV]). The genome of Gz-MSV is capable of antigenic expression in heterologous cells and in the presence of heterologous viruses. Attempts to chemically activate hamster type C virus (HaLV) from HTG2 cells were unsuccessful. The HTG1 cell culture line, established from another Gz-MSV-induced hamster tumor, initially released a virus indistinguishable from the HTG2 virus. After in vitro passage, spontaneous activation of HaLV occurred in HTG1 cells, and the resultant Gz-MSV (HaLV) had properties similar to those of the HTG3 virus.

We have described the unique properties of hamster tumors induced by the Gz-MSV-induced strain of murine sarcoma virus (4, 5, 17). The tumors, and cell culture lines derived from them, release a type C virus expressing murine but not hamster gs-1 antigen. The virus is noninfectious for hamster, rat, or mouse cells, and has a deficiency of RNA-dependent DNA polymerase (15). The sarcoma genome can be demonstrated in the virions by cosedimenting the particles with murine leukemia helper virus (MuLV) (5). The noninfectious virus was named S+H- to indicate the presence of the murine sarcoma genome but the absence of detectable helper virus of hamster or murine origin (5). After prolonged in vivo or in vitro passage, certain Gz-MSV-induced hamster tumors expressed both hamster and mouse gs-1 antigens, suggesting spontaneous activation of a covert, endogenous hamster type C virus (HaLV). This investigation characterizes the resultant pseudotype virus (Gz-MSV [HaLV]), and compares its properties with those of the S+H- virus.

MATERIALS AND METHODS

Cell cultures. A Gz-MSV-induced hamster sarcoma (BW1-Tr8) has been passaged in vivo for more than 80 passages. The HTG2 and HTG3 cell culture lines were established from BW1-Tr8 at in vivo passages 17 and 60, respectively. Both in vitro lines are highly tumorigenic, and have generation times of approximately 18 h. They consist of rounded and plump spindle-shaped refractile cells, which adhere poorly to the substratum, and they grow to high saturation density without evidence of contact inhibition. The HTG1 cell culture line was established from

another Gz-MSV-induced hamster tumor, and its morphologic features and tumorigenicity are similar to those of the HTG2 and HTG3 lines. Cell lines were cloned in microtiter dishes (Falcon Plastics, Oxnard, Calif.) by seeding 0.4 cells/well. Clonal and subclonal line numbers are indicated by hyphens. 3T3FL cells, a derivative of the original 3T3 cell line (21), were obtained from Robert Bassin. The R1 line, a clonal, contact-inhibited diploid fibroblast hamster cell line was established by us. The BW1M line was established from a Gz-MSV-induced BALB/c mouse tumor. Cell cultures were maintained in McCoy's 5A medium (GIBCO, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal calf serum and antibiotics.

Virus preparations. For infectivity and enzyme studies, supernatant fluids were concentrated 50- to 100-fold by differential centrifugation. Virus preparations purified by double sucrose density-gradient centrifugation were obtained from Electro-Nucleonics Laboratories, Bethesda, Md., and were used for enzyme and antigenic studies.

CF tests. Complement-fixation (CF) tests were performed by a standard micro-procedure (8, 20). Viral preparations tested for mouse and hamster type C virus group-specific (gs) antigens were prepared as 20 to 50% cell pack antigens, 10% tumor extracts, or virus concentrates containing approximately 10^{11} virus particles/ml. Broadly reacting murine gs antiserum (pool 30) was prepared in Fischer rats bearing serially transplanted M-MSV-induced tumors (7). Highly species-specific guinea pig antisera to electrofocussed purified gs-1 antigens were kindly provided by R. V. Gilden (10, 14).

Virus activation. Attempts to chemically activate HaLV from HTG2 cells were performed by seeding 3×10^5 cells/flask and 24 h later exposing them to 200 μg/ml of iododeoxyuridine (IUdR) or iododeoxycytidine (IDC) for 24 h, or 20 μg/ml for 48 h (1). Supernatant fluids were collected 3, 7, and 14 days after treatment, concentrated 100 times by pelleting through a 10% glycerol solution, and assayed for RNA-dependent DNA polymerase activity. Cell pack antigens were prepared 7, 14, and 21 days after treatment.

Infectivity studies. 3T3FL and R1 cells were seeded into flasks (75 cm^2) (3×10^5 cells/flask). Secondary hamster embryo cells were seeded into 60-mm dishes at the same density. After 24 h they were infected with 1 ml of virus preparation for 1 h at 37 C. In some experiments, duplicate flasks or dishes were pretreated with DEAE-dextran (25 μg/ml, 1 h) (6). The cells were observed for evidence of transformation and, after subculture, cell pack antigens were prepared for CF testing. Virus concentrates ($\times 50$) prepared from tissue-culture supernatant fluids were injected into newborn mice and hamsters by the intramuscular-subcutaneous route.

Ultrastructural studies. For electron microscope studies, cell and virus pellets were fixed in 3% glutaraldehyde and postfixed in osmium tetroxide or Dalton's chrome osmium. After embedding, tissues were sectioned on an LKB Ultratome III with a diamond knife, stained with uranyl acetate and lead citrate, and examined with a Hitachi II E microscope.

RNA-dependent DNA polymerase studies. Assays for RNA-dependent DNA polymerase were performed by previously published methods (16, 19). The assays were carried out by incubating the reaction mixture at 37 C for 1 h. The standard reaction mixture (0.05 ml) contained 1.5×10^9 virions (2–7 μg of protein), 0.03% Triton X-100, 50 mM Tris-hydrochloride (pH 8), 30 mM NaCl, 5 mM dithiothreitol, 1 mM $MnCl_2$, 80 μM dATP, and 5.6 μM ^{3}H-dTTP (8,100 counts per min per pmol). For the endogenous (viral RNA directed) reaction, the standard reaction mixture contained, in addition, 80 μM each of dCTP and dGTP. The synthetic templates $dT_{(12-18)}$· poly rA and $dT_{(12-18)}$· poly dT were obtained from P-L Biochemicals, Milwaukee, Wis. Protein measurements were performed by Lowry's method (13).

RESULTS

CF tests. The results of the CF tests are summarized in Table 1. The Gz-MSV-induced hamster tumor transplant line BW1-Tr8 was positive for mouse but not hamster gs antigen at in vivo passage 15. However, when tested at passage 56, the line was positive for both mouse and hamster gs antigens. The HTG2 line, and two clonal lines derived from it also were positive for mouse but not for hamster gs antigens. The HTG3 cell line, eight clonal and two subclonal lines derived from it were all positive for both mouse and hamster gs antigens. Virus concentrates prepared from the supernatant fluids of the HTG2 and HTG3-1-3 lines were positive for mouse gs-1 antigen only and mouse and hamster gs-1 antigens, respectively. The CF antigens of the purified virus concentrates were ether soluble and non-sedimentable.

Virus induction experiments. Attempts to activate an endogenous hamster type C virus from HTG2 cultures by treatment with IUdR and IDC were unsuccessful. Cell pack antigens prepared from treated and control cultures were positive for mouse gs-1 but were negative for hamster gs-1 antigen. Virus pellets prepared from treated and untreated cultures showed comparably low levels of RNA-dependent DNA polymerase activity (up to 500 counts/min, using the template $dT_{(12-18)}$· poly rA).

Infectivity. Morphological changes were not observed in mouse and hamster cells infected with HTG2 virus concentrates and mouse cells infected with HTG3-1-3 virus preparations, and cell pack antigens prepared from the infected cultures were negative for mouse and hamster gs antigens. Occasional hamster cultures infected with HTG3-1-3 virus concentrates showed a few large foci of transformed cells 6 to 8 days after infection (Fig. 1). The cells in the foci were plump, spindle-shaped, and highly

TABLE 1. *Murine and hamster type C virus antigens in hamster tumors, cultured hamster tumor cells, and density-gradient purified virus preparations*

Line	Type of preparation	CF antigen titer versus antisera		
		Mouse (pool 30)[a]	Mouse gs-1 (guinea pig)	Hamster gs-1 (guinea pig)
BW1-Tr8 Passage 15	Tumor homogenate	>32	>4	<2
BW1-Tr8 Passage 56	Tumor homogenate	>32	ND[b]	>4
HTG2	Cell pack	>32	8	<2
HTG2-3	Cell pack	>32	ND	<2
HTG2-5	Cell pack	>32	ND	<2
HTG2	Purified virus	32	>4	<2
HTG3	Cell pack	16–32	>4	>4
HTG3-1	Cell pack	ND	>4	>4
HTG3-3	Cell pack	ND	>4	>4
HTG3-4	Cell pack	ND	>4	>4
HTG3-5	Cell pack	ND	>4	>4
HTG3-6	Cell pack	ND	>4	>4
HTG3-7	Cell pack	ND	>4	>4
HTG3-8	Cell pack	ND	>4	>4
HTG3-9	Cell pack	ND	>4	>4
HTG3-1-1	Cell pack	ND	>4	>4
HTG3-1-3	Cell pack	ND	>4	>4
HTG3-1-3	Purified virus	>32	>32	16

[a] MSV pool 30 was a pool of sera from rats carrying tumor transplants of Moloney MSV-induced rat tumors.
[b] ND, not done.

refractile. They showed loss of contact-inhibition and were poorly adherent to the substratum. Some of the foci of transformed cells were selected, pooled, and grown to mass culture. Inoculation of 10^5 to 10^6 cells into weanling hamsters resulted in the rapid appearance of progressively growing tumors at the inoculation sites. Cell pack antigens prepared from this culture were positive for hamster gs-1 antigen.

Ultrastructure. Electron microscope studies of sucrose density gradient purified virus pellets and cell packs revealed that the virus from HTG2 cells morphologically appeared to be type C particles, but possessed atypical features. Examination of 2,000 virions revealed no dense nucleoids, and all the particles retained their electron-lucent or "immature" form (Fig. 2). In contrast, virions from the HTG3-1-3 and BW1M cultures consisted of type C particles with both electron-dense and electron-lucent nucleoids, and some of these had visible tails (Fig. 3). Two thousand virions of HTG3 virus were examined, and 8% had electron-dense nucleoids.

In addition, several electron-lucent particles present in all three cultures appeared to be incomplete, with a segment of the outer membrane and nucleoid shells absent (Fig. 2, 3).

Polymerase activity. The data from the RNA-dependent polymerase assays are presented in Table 2. The results obtained with

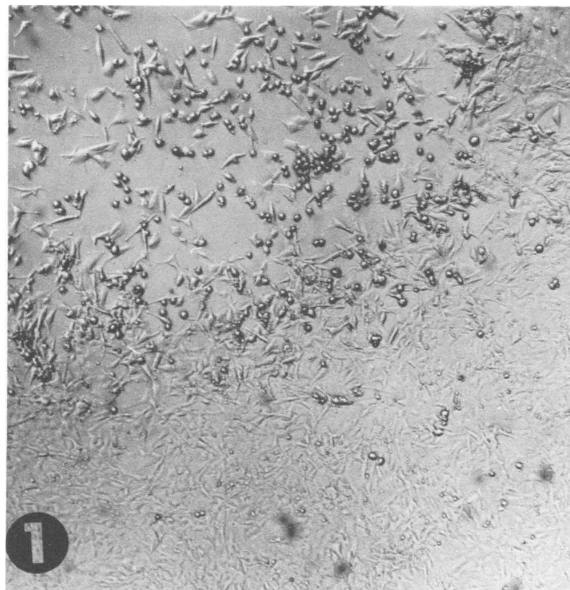

FIG. 1. *Part of transformed focus of R1 cells 8 days after infection with HTG3 virus. Magnification ×50.*

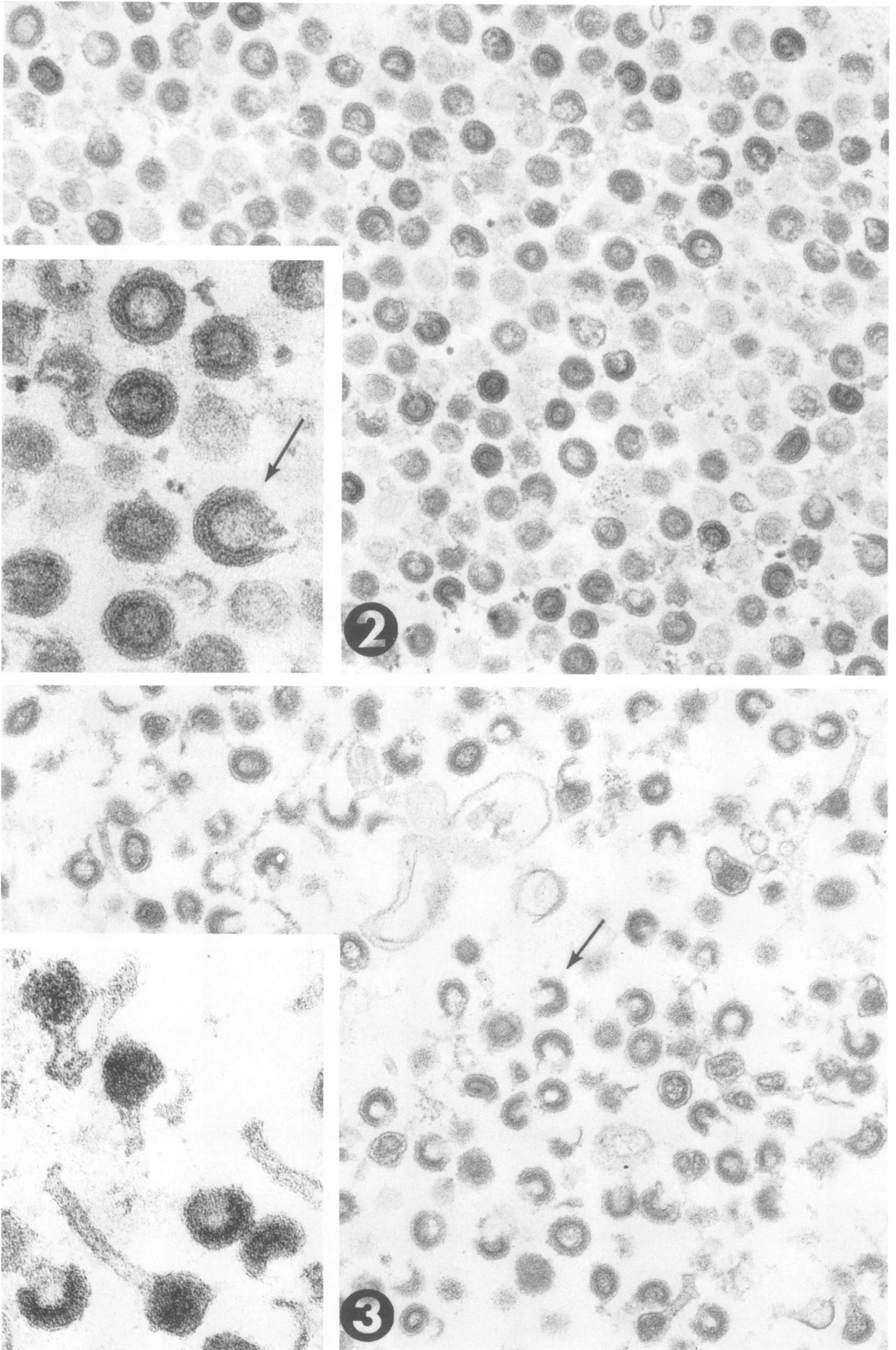

FIG. 2, 3. *Electron micrographs of HTG2 (Fig. 2 and inset) and HTG3 (Fig. 3 and inset) viral pellets. Many broken or incomplete particles are present in both viruses (arrows). All HTG2 virions have electron-lucent nucleoids, whereas HTG3 pellet has virions with electron-lucent as well as electron-dense nucleoids. Some of the virions with electron-dense nucleoids also have clearly visible tails. Magnification ×57,000 (insets ×114,000).*

MuLV-M, BW1M and HTG3-1-3 viruses were similar: an easily detected endogenous reaction, greatly stimulated by the use of the synthetic template $dT_{(12-18)}$·poly rA. The polymerases of these three viruses showed a marked template preference for $dT_{(12-18)}$·poly rA than $dT_{(12-18)}$·poly dA, having 53- to 184-fold greater activity with the use of the former template. By contrast, the endogenous reaction of the HTG2 viral polymerase was only minimally detectable under our assay conditions, and the use of $dT_{(12-18)}$·poly rA as a template resulted in only a sixfold increase in activity. The HTG2 viral polymerase only showed a slight synthetic template preference (1.5-fold) for $dT_{(12-18)}$·poly rA.

HTG1 virus. Virus concentrates prepared from the HTG1 cell line between passages 10 and 12 ("early" passage) contained murine gs-1 but not hamster gs-1 antigen, low polymerase activity (500–1,000 counts/min, using the template $dT_{(12-18)}$·poly rA) and were noninfectious for hamster and mouse cells. Electron microscope studies revealed only electron-lucent particles. After approximately 30 passages ("late" passage), HTG1 cell-pack antigens were positive for both mouse and hamster gs antigen. Virus concentrates (×50) prepared from it transformed hamster embryo cells (titer = $10^{2.7}$ focus forming units/ml), but not mouse cells. Two of six newborn hamsters inoculated with the virus concentrate developed local sarcomas 21 and 36 days after inoculation. The polymerase activity of concentrated supernatant fluids was relatively high (>500,000 counts/min, by using $dT_{(12-18)}$·poly rA as a template), and electron microscope studies revealed both electron-lucent and electron-dense virions.

TABLE 2. *Endogenous and template-primer dependent DNA polymerase activity of different viral preparations*

Virus	^{3}H-TMP incorporation (pMol/μg of protein)			
	Endogenous	$dT_{(12-18)}$·poly rA	$dT_{(12-18)}$·poly dA	$dT_{(12-18)}$·poly rA/ $dT_{(12-18)}$·poly dA
MuLV-M[a]	0.141	138.4	0.75	184
BW1M[b]	0.06	1.7	0.032	53
HTG-2	0.002	0.012	0.008	1.5
HTG3-1-3	0.02	7.23	0.04	181

[a] Moloney leukemia virus.

[b] The BW1M line was established from a Gz-MSV induced mouse tumor and releases sarcomagenic and leukemogenic viruses.

DISCUSSION

The HTG2 and HTG3 viruses, even though derived from the transplantable hamster tumor, differ in many of their properties. The "noninfectious" HTG2 virus expresses mouse gs antigen, whereas HTG3 virus expresses mouse and hamster gs antigens. The HTG3 virus is a hamster tropic sarcomagenic virus and, presumably, a hamster type C virus (HaLV) pseudotype of Gz-MSV. Its designation, according to established convention (9) is Gz-MSV (HaLV). Hamster cells transformed by other MSV strains are either "nonproducer" cells without demonstrable virus release and antigen expression or they release the respective HaLV pseudotype of MSV which contain hamster gs antigen only (11, 12). Additional evidence that the defective Gz-MSV genome controls the synthesis of the mouse gs-1 antigenic marker is the ability of cat cells transformed by the feline pseudotype of Gz-MSV to express murine and feline gs antigens (18).

The RNA-dependent DNA polymerase activity of HTG2 virus is relatively low. Its endogenous (viral RNA directed) reaction was just detectable by our assay system. The use of $dT_{(12-18)}$·poly rA as a template primer resulted in only a sixfold increase in activity. The polymerases of Gz-MSV of mouse origin and HTG3 virus had 3- to 10-fold greater endogenous activities, and they showed a marked template primer preference for $dT_{(12-18)}$·poly rA. In general, viral polymerases show a template preference for $dT_{(12-18)}$·poly rA, whereas cellular polymerases prefer $dT_{(12-18)}$·poly dT (16). The lack of infectivity of HTG2 may, at least in part, be related to its deficiency of RNA-dependent DNA polymerase (15).

Even though Gz-MSV(MuLV), HTG3, and HTG1 ("late" passage) viruses consist of the expected mixture of particles with electron-lucent and electron-dense particles, all HTG2 and HTG1 ("early" passage) virions appeared electron lucent (Fig. 2, 3). With few exceptions the virions of almost all type C viruses contain a preponderance of dense nucleoids (3). Budding and recently released type C virions have electron-lucent centers, each surrounded by two electron-dense shells (3). At some time after their release, the extracellular virions develop poorly defined electron-dense nucleoids without discernible organization or symmetry. Particles with electron-dense nucleoids may form long artifactual protrusions or "tails" (2). The significance of our ultrastructural findings and their relationship, if any, to the lack of infectivity of the HTG1 ("early" passage) and HTG2 viruses remains to be determined.

Spontaneous in vitro activation of HaLV occurred in the HTG1 cell line, suggesting that the presence of HaLV in HTG3 cells may be the result of spontaneous activation during animal passage rather than due to viral contamination.

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