

Biological and Serological Properties of Viral Particles from a Nonproducer Rat Neoplasm Induced by Murine Sarcoma Virus (Moloney)

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The viral particles present in a nonproducer rat neoplasm induced by murine sarcoma virus (MSV) Moloney isolate, as detected by electron microscopy, were found to be biologically active on normal kidney cells of random-bred Osborne-Mendel rats. The virus is designated here as MSV (0). MSV (0) differs from other pseudotypes of MSV in its host range, antigenicity, and interference pattern.

An epithelial neoplasm (MSB-1) induced in a strain BN rat by the murine sarcoma virus (MSV) Moloney isolate was found to be free from infectious virus and did not react in the complement-fixing (CF) tests with broadly reactive sera of rats immunized with Moloney lymphoma (10). In a previous report (10), it was shown that MSB-1 neoplastic cells possess virus-specific antigenicity, as demonstrated by transplantation resistance tests; these cells also contain infective MSV-genome, as demonstrated by the rescuing technique described by Huebner et al. (6). More recently, Valentine and Bader (11) examined MSB-1 cells by electron microscopy and detected the presence of type C particles. Subsequently, this finding was confirmed in our laboratory by H. Duc-Nguyen.

This study is primarily concerned with the biological and serological properties of the virus released by MSB-1 cells. The virus reported here has been designated as MSV (0).

MATERIALS AND METHODS

Cell cultures and growth media. All cells were propagated in Eagle's minimal essential medium containing 10% fetal bovine serum, 10% tryptose phosphate broth, and antibiotics (penicillin, streptomycin, and mycostatin). Mouse embryo (ME) cells were prepared from 12-day-old embryos of NIH Swiss mice; rat embryo (RE) cells were prepared from 12-day-old embryos of BN rats; and adult rat kidney (NRK) cells, which were kindly supplied by H. Duc-Nguyen of this laboratory, were prepared from the kidneys of 6-week-old random-bred Osborne-Mendel (OM) rats. Rauscher leukemia virus (RLV)-infected kidney (RRK) cells, obtained from H. Duc-Nguyen, were

derived from a random-bred OM rat infected in vivo with RLV. NRK cells were negative for CF antigen and showed no type C particles (over 100 cells in each of three different preparations were examined), whereas RRK cells were positive for CF antigen and continuously produced RLV (2). MSB-1 cells were established in tissue culture from the third transplant passage in vivo of MSB-1 tumor (10).

Viruses and virus assay. Rauscher pseudotype virus [MSV (RLV)] was originally obtained by superinfection of MSB-1 cells with rat-adapted RLV. MSV [Maloney leukemia virus (MLV)] was obtained from J. Moloney as a tumor extract from BALB/c mice. Both viruses were subsequently grown on ME cells and were used in all experiments. RLV used in the present study is the rat-adapted virus kindly supplied by H. Duc-Nguyen; the properties of this rat-adapted RLV have been reported previously (2). MSV (0) was obtained from MSB-1 cells. All virus suspensions were prepared by scraping infected ME or MSB-1 monolayers off plates; the whole suspension was frozen in a dry ice-alcohol bath and was thawed immediately at 37 C. This procedure was repeated three times. The suspension was then centrifugated at $1,000 \times g$ to remove cell debris, and the supernatant fluid was stored at -70 C. The supernatant fluids constituted the stock viruses. The titer of the virus was determined as focus-forming units (FFU) on mouse embryo cells by the method of Hartley and Rowe (4), and on rat cells by the method of Ting (9), except that the number of rat kidney cells seeded was 3×10^5 per 60-mm plate.

Antiviral sera. All antiviral sera and control sera were obtained from adult BN rats or (Lewis \times BN) F_1 rats. Rats were given three injections of virus-infected rat cells, with an interval of 2 weeks between each injection. Anti-MSV (0) sera were obtained from adult rats which had been immunized by low-dose inoculation (10^5) of MSB-1 cells and had subsequently

rejected a challenge of 5×10^6 MSB-1 cells. All sera were heat-inactivated at 56 C for 30 min, filtered through 0.45- μ membrane filters (Millipore Corp., Bedford, Mass.), and stored at -20 C.

Neutralization test. An 0.2-ml amount of virus was mixed with 0.2 ml of serum diluted 1:5 in medium. The mixture was held at 37 C for 30 min and was then diluted 1:5 or 1:10 in medium. A 0.2-ml amount of the diluted mixture was used to infect monolayers of ME cells or NRK cells in order to determine FFU.

RESULTS

Demonstrations of infectivity of MSV (0) on NRK cells. Previously, it was reported that no infectious virus was recovered from MSB-1 cells, even when MSB-1 cells were co-cultivated with ME cells (10). Therefore, the infectivity of MSV (0) was tested on ME cells which are supposedly very sensitive to MSV. Since MSB-1 tumor was originally induced in BN rats by MSV, BNRE cells were also used to test the infectivity of MSV (0). BNRE cells were not susceptible to infection with MSV (0) (Table 1); however, when NRK cells from OM rats were used as test cells, infectivity of MSV (0) was readily demonstrated. It should be noted here that a membrane (0.45 μ)-filtered (Millipore Corp.) preparation of MSV (0) also induced foci on NRK cells. These foci were principally composed of round cells and were smaller than foci induced by the pseudotype virus MSV (RLV). It is interesting to note that MSV (0) titrates linearly on NRK and RRK cells. This suggested that no helper virus is needed and MSV (0) is self-competent. Evidence for the existence of competent MSV (MLV) has been recently reported by O'Connor and Fischinger (7). One interesting finding is that leukemia virus infection does not seem to interfere with the focus-forming ability of MSV (0) on rat cells. The data in Table 1 also show that RRK cells which have been chronically infected by RLV are sensitive to MSV (0) infection but not to MSV (RLV) infection. On the other hand, Sarma et al. (8) have reported that leukemia viruses interfere with the focus-forming ability of

other pseudotypes of MSV. This suggested that MSV (0) is different from the other pseudotypes of MSV.

Failure to recover MSV (RLV) by mixed infection of ME cells with MSV (0) and RLV. To explore the possibility that infection of ME cells by MSV (0) may require the help of leukemia virus, ME cells were coinfecting with MSV (0) and RLV. Infected cells were passaged twice, and the supernatant fluids of the second passage were tested for infectious MSV on ME and NRK cells. Cells of the second passage were tested for their susceptibility to a challenge of 1,000 FFU of MSV (RLV). Results in Table 2 indicate that (i) no infectious MSV was recovered from ME cells infected with MSV (0) alone or from cells coinfecting with MSV (0) and RLV, and (ii) MSV (0)-treated ME cells did not develop resistance to superinfection with MSV (RLV), whereas RLV-infected ME cells resisted the challenge of MSV (RLV).

Persistence of MSV (0) in infected NRK cells. The smaller size of the foci observed on NRK monolayers infected with MSV (0) suggested that no reinfection occurs on the assay plates. This raised the question as to whether MSV (0)-infected NRK cells will continue to release virus, and, if so, what type of virus. To answer this question, the supernatant fluids of various passages of MSV (0)-infected NRK cells were used to infect ME and NRK cells. Infectivity was observed on NRK cells but not on ME cells (Table 3). This indicated that virus is released by infected NRK cells, and that the virus released has the same host range as MSV (0) obtained from MSB-1 cells. Furthermore, when MSV (0)-infected NRK cells were superinfected with RLV, the virus recovered was found to be capable of infecting ME cells and could be neutralized by anti-RLV serum. This virus also induced tumors in newborn mice, and the tumors had the same histological features as those tumors induced by MSV (RLV) grown on ME cells. These observations eliminated the possibility that MSV (0)

TABLE 1. Tests for infectivity of MSV (RLV) and MSV (0)

Virus	Dilution	Avg FFU ^a per plate on test cells			
		ME	NRK	RRK	BNRE
MSV (0)	Undiluted	0	> 500	> 500	0
	1:5	0	107	118	0
	1:25	0	20	21	0
MSV (RLV)	1:20	184	175	0	31

^a Average value of three plates.

is a passenger virus present in BN rats, and confirmed the notion that MSV (0) belongs to the family of MSV and contains at least part of the genome of the original MSV.

Antigenic specificity of MSV (0). Using rat sera prepared against RLV, MLV, Gross leukemia virus (GLV), and MSV (0), the serological specificity of MSV (0) was studied. Based on the neutralization test, MSV (0) was antigenically distinct from MSV (RLV) (Table 4). Anti-RLV serum did not neutralize MSV (0) and anti-MSV (0) serum did not reduce the focus-forming ability of MSV (RLV). It has been reported that anti-GLV (C58 G⁺ virus) serum, obtained from Lloyd Old, Sloan-Kettering Institute for Cancer Research, neutralized all known pseudotypes of MSV (5), yet did not neutralize MSV (0). This serum is a rat serum prepared against wild-type GLV from C58BL mice and has a very broad spectrum. The same serum has been used by Geering et al. for typing G⁺ antigens, including those in naturally occurring leukemia viruses (3).

TABLE 2. Test of infectivity of supernatant fluid from infected ME cells and susceptibility of infected ME cells to challenge by MSV (RLV)

Culture	Infectivity of supernatant fluid tested on		Test for susceptibility of infected ME cells to superinfection with 10 ³ FFU of MSV (RLV)
	ME cells	NRK cells	
ME cells.....	- ^a	-	4+ ^b
ME cells + MSV (0).....	-	-	4+
ME cells + RLV.....	-	-	0
ME cells + MSV (0) + RLV.....	-	-	0
ME cells + MSV (RLV)...	+	+	NT ^c

^a Infectivity absent, -; infectivity detected, +.
^b Number of foci too numerous to count.
^c Not tested.

TABLE 3. Infectivity of virus released from NRK cells infected with MSV (0)

Passage ^a	Helper virus	FFU per 0.2 ml in fluids tested on	
		NRK cells	ME cells
0	None	6, 9	0, 0
4	None	101, 119	0, 0
7	None	175, 140	0, 0
4 ^b	RLV	Confluent	Confluent

^a Culture used consisted of NRK cells plus MSV (0).
^b Passage 4 cells were superinfected with RLV; two passages later, the fluid was used to test for infectious virus.

TABLE 4. Neutralization of MSV (0), MSV (MLV), and MSV (RLV) by various antisera

Expt. no.	Treatment	Avg FFU ^a /plate	
		MSV (MLV)	MSV (0)
1	Anti-GLV serum ^b	0	113
	Control serum ^b	83	135
	Medium	79	127
2	Anti-RLV serum	27	
	Anti-MSV (0) serum	90	
	Anti-MLV serum	9	
	Medium	118	
		MSV (RLV)	MSV (0)
3	Anti-RLV serum	2.3	66.
	Anti-MSV (0) serum	171	0.66
	Control serum	184	64
	Medium	185	67
4	Anti-RLV serum	0.33	96
	Anti-MLV serum	12	62
	Control serum	90	105
	Medium	97	98

^a Average value of three plates.
^b Rat sera obtained from Lloyd Old. Anti-GLV serum was made against C58 (G⁺) virus.

There was some neutralization of MSV (MLV) by anti-MSV (0) serum; however, the meaning of this slight difference remains to be determined. Anti-MLV serum neutralized MSV (RLV) at a much higher rate than MSV (0). This is not surprising, since MSV was originally isolated from MLV. The possibility of the presence of MSV (0) in MLV stocks cannot be ruled out, especially in those MLV stocks that have not been purified.

DISCUSSION

The finding that avian tumor virus-like particles [RSV (0)] are present in nonproducer Rous cells (1) has recently led to the discovery that these particles are biologically active (12). As discussed in this paper, similar viral particles have now been found in the murine sarcoma-leukemia virus complex.

Thus far, the biological activity of MSV (0) can only be demonstrated with NRK cells of OM rats. Morphological transformation of OM rat embryo cells was also observed; however, the foci were too diffuse for quantitative counting. Valentine and Bader (11) reported that MSV (0) did not infect their RE cell line, and I have also failed to infect mouse kidney cells with MSV (0). The question of whether NRK cells are completely free from naturally occurring leukemia viruses remains. This cell line has been reported

to be negative for CF antigen, with the anti-leukemia virus sera prepared by Hartley (2). No type C particles were found among the 300 cells examined with an electron microscope (H. Duc-Nguyen, *personal communication*). More recently, the presence of CF antigen in NRK cells was tested with the serum prepared by Geering et al. (3). The result was also negative. Therefore, it is concluded that NRK cells are free from known leukemia viruses.

It is not known whether RSV (0) can induce tumors in a chicken strain in which embryo cells are known to be susceptible to infection of RSV (0). An attempt has been made to inoculate, intramuscularly, 52 thymectomized 3-day-old OM rats with MSV (0) and 20 thymectomized 3-day-old OM rats with MSV (RLV). During an observation period of 3 months, none of the rats infected with MSV (0) developed tumors, whereas 13 of the 20 rats infected with MSV (RLV) developed sarcomas at the sites of inoculation. On the other hand, tumor growth was obtained in young OM rats inoculated with NRK cells transformed by MSV (0), and histologically these tumors were identical to those of MSB-1 tumors. Factors such as route of inoculation, gaining entrance to target cells, and immunological response of the hosts often play an important role in viral oncogenesis *in vivo*. Work is now in progress to determine the optimal condition for induction of tumors in OM rats by MSV (0).

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LITERATURE CITED

1. Dougherty, R. M., and H. S. Di Stefano. 1965. Virus particles associated with "non producer" Rous sarcoma cells. *Virology* **27**:351-359.
2. Duc-Nguyen, H., E. N. Rosenblum, and R. F. Zeigel. 1966. Persistent infection of a rat kidney cell line with Rauscher murine leukemia virus. *J. Bacteriol.* **92**:1133-1140.
3. Geering, G., L. J. Old, and E. A. Boyse. 1966. Antigens of leukemia virus: their relation to the antigens of Gross virus and other murine leukemia viruses. *J. Exptl. Med.* **124**:753-772.
4. Hartley, J. V., and W. P. Rowe. 1966. Production of altered cell foci in tissue culture by defective Moloney sarcoma virus particles. *Proc. Natl. Acad. Sci. U.S.A.* **55**:780-786.
5. Huebner, R. J. 1967. The murine leukemia-sarcoma virus complex. *Proc. Natl. Acad. Sci. U.S.A.* **58**:835-842.
6. Huebner, R. J., J. W. Hartley, W. P. Rowe, W. T. Lane, and W. I. Capps. 1966. 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. *Proc. Natl. Acad. Sci. U.S.A.* **56**:1164-1169.
7. O'Connor, T. E., and P. J. Fischinger. 1968. Titration pattern of a murine sarcoma-leukemia virus complex: evidence for existence of competent sarcoma virions. *Science* **159**:325-329.
8. Sarma, P. S., M. Cheong, J. W. Hartley, and R. J. Huebner. 1967. A viral interference test for mouse leukemia viruses. *Virology* **33**:180-184.
9. Ting, R. C. 1966. *In vitro* transformation of rat embryo cells by a murine sarcoma virus. *Virology* **28**:783-785.
10. Ting, R. C. 1967. Tumor induction of thymectomized rats by murine sarcoma virus (Moloney) and properties of the induced virus-free tumor cells. *Proc. Soc. Exptl. Biol. Med.* **126**:778-781.
11. Valentine, A. F., and J. P. Bader. 1968. The production of virus by mammalian cells transformed by Rous sarcoma and murine sarcoma viruses. *J. Virol.* **2**:224-237.
12. Vogt, P. K. 1967. A virus released by non-producing Rous sarcoma cells. *Proc. Natl. Acad. Sci. U.S.A.* **58**:801-808.