Potentiation of Murine Sarcoma Virus (Harvey) (Moloney) Oncogenicity in Lactic Dehydrogenase-Elevating Virus-Infected Mice (35482)

W. TURNER, P. S. EBERT, R. BASSIN, G. SPAHN, AND M. A. CHIRIGOS

National Cancer Institute, and Microbiological Associates, Inc., Bethesda, Maryland 20014

Lactate dehydrogenase-elevating virus (LDV), a transmissible agent capable of permanently elevating plasma lactate dehydrogenase levels in mice, was first described by Riley *et al.* (1). The finding that animals and very often patients with advanced cancer have abnormally high plasma lactic dehydrogenase activity (2–4) and the association of LDV with a large variety of murine tumors (1), suggested a possible etiological relationship between LDV and murine tumors. However, Yaffe showed that LDV was more frequently associated with tumors with a long transplantation history in mice (5). This finding and the observation that tumor cells free of detectable LDV have been shown to maintain their oncogenicity in mice led to the belief that LDV is not associated with the malignant process induced in mice by transplantable tumor cells or oncogenic viruses, but that LDV is most likely a passenger virus transmitted to tumor cells and oncogenic virus pools by propagation in mice naturally infected with LDV (5). However, Steeves *et al.* (6) reported markedly enhanced spleen focus formation by a member of the Friend virus complex [spleen focus-forming virus (SFFV)] in mice pre-infected with LDV.

The Moloney strain of murine sarcoma virus (M-MSV) induces tumors in adult mice which regress (7), while infection of adult mice with the Harvey strain of murine sarcoma virus (H-MSV) results in a low incidence of small progressively growing tumors (8). Chirigos *et al.* (9) and more recently Turner *et al.* (10) demonstrated enhanced M-MSV infection in adult Balb/c mice co-infected with murine leukemia viruses. The parameters employed by these investigators as indicators of enhanced M-MSV infection in mice were: (i) absence of tumor regression; and (ii) increased incidence of death with tumor in dually infected mice as compared to M-MSV controls.

This report concerns the enhancement of M-MSV and H-MSV oncogenicity in mice infected with LDV.

Materials and Methods. Mice. Conventional and specific pathogen-free (SPF) adult male Balb/c mice, 8–12 weeks old, used in these studies were obtained from Charles River Breeding Laboratories, Wilmington, Mass. and Microbiological Associates, Walkersville, Md., respectively.

Viruses. LDV. Stock virus was prepared from infected mouse plasma as described by Mahy *et al.* (11) and stored at −70°. The titer of the LDV pool was determined by the method of Notkins and Shochat (12) and expressed as the dose which infected 50% of the mice (ID_{50}). The stock LDV had a titer of $10^{8.5}$ ID_{50}/ml and was diluted appropriately in phosphate-buffered saline (pH 7.0).

H-MSV. Stock H-MSV consisting of a 1-g equivalent cell-free concentrate of sarcoma tissue was obtained from Dr. Jennifer J. Harvey, National Institute for Medical Research, Mill Hill, London, England. A virus pool was prepared from this stock virus by infecting newborn Balb/c mice and preparing a 10% cell-free extract of sarcoma tissue (10). The titer of this pool was $10^{3.0}$ LD_{50}/ml when titrated intramuscularly (im) in new born mice and exhibited a "two-hit" titration pattern when assayed by focus formation of

3T3 cells (13).

M-MSV. The M-MSV pool employed in these studies was prepared from monolayer cultures of 3T3 mouse cells 96 hr after infection with M-MSV derived from Balb/c mouse tumor concentrate. Virus was harvested by gently suspending the infected 3T3 cells in culture fluid and subjecting the suspension to 3 cycles of freezing (—70°) followed by low speed centrifugation to remove cellular debris. Aliquots of the clarified virus suspension were distributed in plastic tubes and stored at —70° until used. This preparation gave a "one-hit" titration pattern when assayed by focus formation in 3T3 cells (13) and contained approximately $10^{5.7}$ focus-forming units (FFU)/ml of M-MSV. In addition, this virus preparation was shown to be free of LDV.

The one-hit titration pattern obtained with the M-MSV pool indicate that this pool contained a full complement of Moloney leukemia virus (MLV) (13). The two-hit titration pattern obtained with the H-MSV indicates that this pool contained less than a full complement of MLV (14).

Cell cultures. 3T3 FL, a subline derived from the original 3T3/mouse cell line isolated by Todaro and Green (15) was obtained from Flow Laboratories, Ltd., Irvine, Scotland. Growth medium for these cells consisted of McCoy's 5A medium supplemented with 10% unheated fetal calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml).

The 3T3 cells were shown to be equal in sensitivity to focus formation by competent and defective stocks of MSV as well as to "helper" activity of added murine leukemia virus (Bassin, R. H., Tuttle, N., and Fischinger, R. J., Int. J. Cancer, in press).

Focus assay. Focus assays of MSV preparations in 3T3 FL cells carried out using a modification of the assay procedure described by Hartley and Row (16). Freshly trypsinized 3T3 FL cells were plated in 60-mm plastic plates at a concentration of 1×10^5 cells/plate. Following 24-hr incubation at 37° in an atmosphere consisting of 5% CO_2 in air, culture fluids were removed and 0.2 ml of MSV was added. The virus was allowed to absorb for approximately 60 min at 37°, after which 4 ml of growth medium was added to each plate. Plates were scored for foci 5 days after infection. Optimum concentrations of murine leukemia "helper" virus were added to alternate MSV assay plates in order to express the focus-forming potential of any defective MSV present in the preparation (13).

Statistical methods. The p values were obtained by comparing each treated group with control group by the X^2 test (17) and the Fisher exact test (18).

Results. Since Steeves *et al.* (6) demonstrated enhanced Friend leukemia virus (FLV) induced spleen focus formation and replication in mice previously infected with LDV, it was of interest to determine the course of M-MSV and H-MSV infections in LDV-infected mice.

Adult Balb/c mice were inoculated im with $10^{7.8}$ ID_{50} of LDV 3 days prior to im inoculation of groups of these mice with $10^{4.0}$ FFU of M-MSV or $10^{2.3}$ LD_{50} of H-MSV. Results in Table I show that LDV infected mice challenged with M-MSV resulted in (i) a 49% reduction in the incidence of tumor regression as compared to control; (ii) a 49% increase in the incidence of death with tumor over M-MSV controls; and (iii) a 61-day reduction in the median survival time (MST) of dually infected mice as compared to M-MSV controls.

Similarly, H-MSV challenge of LDV infected mice resulted in (i) a 75% increase in tumor incidence over H-MSV controls; (ii) a complete absence of tumor regression as compared to 100% tumor regression in H-MSV controls; (iii) a 50% increase in the incidence of death with tumor over control; and (iv) a 22-day reduction in MST over controls. Statistical analysis of the increased incidence of death with tumor in LDV infected mice challenge with H-MSV ($p<.01$) or M-MSV ($p<.001$) as compared to MSV controls was shown to be significant. Thus, the data clearly show enhanced MSV-induced oncogenicity and infection in mice pre-infected with LDV.

The time of LDV infection in mice in relation to MSV challenge appears to be of considerable importance since simultaneous challenge of mice with LDV and MSV did not

result in enhancement of MSV infection in dually infected mice.

The increased tumorigenicity of H-MSV in LDV infected adult mice (Table I, Group 4) prompted investigations on the nature of H-MSV recovered from tumors of mice dually infected with H-MSV and LDV. Data exhibited in Table II show that challenge of LDV-infected mice with H-MSV resulted in a 90% increase in the incidence of tumor induction in the dually infected mice as compared to mice inoculated with H-MSV alone (H-MSV controls). A 10% cell-free extract of tumors from mice dually infected with H-MSV and LDV contained $10^{2.3}$ FFU/ml of H-MSV when assayed on 3T3 LF cells without "helper" virus and $10^{3.0}$ FFU/ml of H-MSV when assayed in the presence of "helper" virus. Only one mouse developed a tumor (10%) in the group of mice inoculated with H-MSV alone. Prior to complete regression of the tumor, it was removed to prepare a 10% cell-free extract. The tumor extract was shown to be devoid of focus-forming activity on 3T3 FL cells when assayed in the presence or absence of "helper" virus. This result is not surprising, since the tumor induced by the inoculation of H-MSV alone was quite small and probably contained a small amount of virus which was eliminated or inactivated by the extraction procedure. This would also explain the lack of tumor induction when the tumor extract was bioassayed in normal or LDV-infected adult mice.

Inoculation of mice with a 10% extract of tumors from mice dually infected with H-MSV and LDV resulted in a 10% incidence of tumor while LDV infected mice inoculated with the same preparation showed a 60% incidence of tumor formation. These data clearly demonstrate the presence of increased titers of H-MSV in tumors of mice dually infected with H-MSV and LDV as compared to H-MSV controls and the ability of LDV to enhance the oncogenicity of H-MSV in adult mice.

Discussion. The results of the present study clearly show the enhancing effects of LDV infection on MSV oncogenicity and infection in adult mice. Adult mice previously infected with LDV and subsequently infected with M-MSV or H-MSV showed (i) increased incidence of death with tumor; (ii) reduction of the incidence of tumor regression; and (iii) reduction of the median survival time as compared to mice infected with MSV alone. In addition, mice dually infected with LDV and H-MSV showed an increase in the incidence of tumor induction over control. Enhancement of MSV infection in LDV-infected adult mice appears to be dependent upon the time of LDV infection. Mice infected with LDV 3 days prior to MSV infection showed a significant enhancement of MSV infection. However, simultaneous infection of adult mice with LDV and M-MSV did not result in an enhancement of MSV infection.

Berman and Allison reported that H-MSV induced a low incidence of progressively growing tumors in adult mice (8). In our experiments mice pre-infected with LDV and H-MSV showed a 75–90% increase in tumor incidence over control (Tables I and II). Thus it was of interest to examine the *in vitro* and *in vivo* activity of H-MSV extracted from tumors of mice dually infected with LDV and H-MSV. A 10% extract of tumor from the dually infected mice when assayed on 3T3 FL cells was shown to contain $10^{2.3}$ FFU/ml of H-MSV in the absence of "helper" virus and $10^{3.0}$ FFU/ml of H-MSV in the presence of "helper" virus. In contrast, extract of tumor from mice infected with H-MSV alone was devoid of focus-forming activity on 3T3 cells in the absence or presence of "helper" virus. This observation is of interest since it has been shown that H-MSV recovered directly from tumor material does not, in general, produce foci in mouse cells (19) (Bassin and Harvey, unpublished observation). The focus-forming activity of H-MSV recovered from tumors of mice dually infected with LDV and H-MSV is probably due to the presence of higher titer of H-MSV in these tumors as compared to H-MSV controls rather than a change in the genome of this virus. This hypothesis is supported by the observation that H-MSV extracted from tumors of mice dually infected with H-MSV and LDV produced only a 10% incidence produced in adult mice by classical H-MSV (8). The fivefold difference in titer of H-MSV focus-forming virus extracted from tumors of dually infected mice

TABLE I. Results Showing Potentiating Effects of LDV on Infection and Tumorigenicity in Adult Mice Induced by MSV(M) and MSV(H).

Group	No. of mice/group[a]	Viruses inoculated[b]	Response to M-MSV in 100 days and H-MSV in 57 days: Tumor incidence (%)	Tumor regression (%)	Death with tumor (%)	Survival/ total	Median survival time (days)	p values
1	35	M-MSV	100	66	34	23/35	100	—
2	35	LDV, M-MSV 3 days later	100	17	83	6/35	39	$<.001$[c]
3	20	H-MSV	25	100	0	20/20	>57	—
4	20	LDV, H-MSV 3 days later	100	0	50	10/20	35	$<.01$[d]
5	35	LDV	—	—	—	20/20	>100	—

[a] 6–8-week-old SPF Balb/c mice were employed for M-MSV experiment, while conventional 6–8-week-old Balb/c mice were employed for H-MSV studies.

[b] Mice were pre-infected (D_{-3}) intramuscularly (im) with $10^{7.8}$ ID_{50} of LDV in a 0.2-ml volume, and challenged im 3 days later (D_0) with 10^4 FFU of M-MSV or $10^{2.3}$ LD_{50} of H-MSV in 0.2-ml volume.

[c] The p value was obtained using the X^2 test.

[d] The p value was obtained using the Fisher exact test.

assayed in the presence and absence of helper virus suggests the presence of defective H-MSV virions in the extract.

The possibility that LDV potentiation of MSV oncogenicity in mice is probably due to LDV-induced modifications of the host is further supported by results showing a 50% increase in the incidence of tumor induction in LDV-infected mice inoculated with H-MSV recovered from tumors of mice dually infected with H-MSV and LDV over tumor induction by the same virus preparation assayed in saline-injected adult mice.

The mechanism by which LDV enhances

TABLE II. The Recovery, *in Vitro* and *in Vivo*, of MSV(H) from Tumors Induced in Mice Dually Infected with H-MSV and LDV.

Group	No. of mice/group	Virus inoculated	Tumor incidence (%) (14 days)	Focus-forming units[d] (FFU)/ml of 10% tumor extract: "Helper" virus (—)[e]	"Helper" virus (+)	Tumor response induced by inoculating 10% tumor extract in adult mice inoculated with: Saline	LDV[g]
1	10	H-MSV[a]	10	0	0	0/10[f]	0/10
2	10	LDV, H-MSV[b] 3 days later	100	$10^{2.3}$	$10^{3.0}$	1/10	6/10
	10	LDV[c]	—	0	0	—	—

[a] Mice inoculated im with 0.2 ml of 10% extract of H-MSV tumor (D_0).

[b] Mice inoculated im with 0.2 ml of 10% extract of H-MSV tumor 3 days after (D_0) im inoculation of $10^{7.8}$ ID_{50} of LDV (D_{-3}) in 0.2 ml.

[c] Mice inoculated im with $10^{7.8}$ ID_{50} of LDV in 0.2-ml volume (D_{-3}).

[d] Tumors were excised 14 days after H-MSV infection and 10% tumor extract was prepared.

[e] Helper virus (—) = assayed without "helper" virus; "helper" virus (+) = assayed with optimal amounts of "helper" virus.

[f] Number of mice with tumor/number of mice inoculated.

[g] Mice pre-infected with LDV 3 days prior (D_{-3}) to H-MSV infection (D_0).

H-MSV and M-MSV infection in mice is not known. However, Howard *et al.* (20) reported the enhancement of humoral antibody formation and inhibition of cellular immunity in mice infected with LDV. More recently, Law *et al.* (21) and Hellström and Hellström (22) have demonstrated the importance of cellular immunity in the regression of M-MSV-induced tumors in mice. While other reports have clearly shown that immunosuppressive agents such as cortisone (23), murine leukemia virus (9, 10), and heterologous antilymphocyte serum (24) markedly enhanced M-MSV infection in mice. Thus, in consideration of the importance of cellular immunity on the course of M-MSV infection in mice and the ability of immunosuppressive agents to enhance M-MSV infection in mice, it is possible that LDV may enhance MSV infection in dually infected mice by suppressing their cellular immune response. Investigations to elucidate the mechanism by which LDV enhances MSV infection in mice is now under way in this laboratory.

Summary. Adult Balb/c mice previously infected with LDV and subsequently challenged with H-MSV or M-MSV showed (i) increased tumor incidence, (ii) reduction in the incidence of tumor regression, (iii) increased incidence of death with tumor, and (iv) reduction of the median survival compared to H-MSV- or M-MSV-infected controls.

Extracts of tumor from mice inoculated with H-MSV alone were devoid of focus-forming virus when assayed on 3T3 FL cells while extracts of tumors from mice dually infected with LDV and H-MSV contained $10^{2.3}$ FFU/ml of virus in the absence of "helper" virus and $10^{3.0}$ FFU/ml in the presence of "helper" virus.

The mechanism by which LDV potentiate H-MSV and M-MSV oncogenicity in adult mice is discussed.

1. Riley, V., Lilly, F., Huerto, E., Bardell, D., Science **132**, 545 (1960).

2. Hill, B. R., and Levi, C., Cancer Res. **14**, 513 (1954).

3. Hsieh, K. M., Suntzeff, V., and Cowdry, E. V., Cancer Res. **16**, 237 (1956).

4. Wroblewski, F., Cancer **12**, 27 (1959).

5. Yaffe, D., Proc. Amer. Ass. Cancer Res. **3**, 280 (1961).

6. Steeves, R. A., Mirand, E. A., Thomson, S., and Avila, L., Cancer Res. **29**, 1111 (1969).

7. Fefer, A., McCoy, J. L., and Glynn, J. P., Cancer Res. **27**, 1626 (1967).

8. Berman, L. D., and Allison, A. C., Int. J. Cancer **4**, 820 (1969).

9. Chirigos, M. A., Perk, K., Turner, W., Burka, B., and Gomez, M., Cancer Res. **28**, 1055 (1968).

10. Turner, W., and Chirigos, M. A., Cancer Res. **29**, 1956 (1969).

11. Mahy, B. W. J., Rowson, K. E. K., Parr, C. W., and Salaman, M. H., J. Exp. Med. **122**, 967 (1965).

12. Notkins, A. L., and Shochat, S. J., J. Exp. Med. **117**, 735 (1963).

13. Fischinger, P. J., and O'Connor, T. E., J. Nat. Cancer Inst. **40**, 1199 (1968).

14. Bassin, R. H., Simons, P. J., Chesterman, F. C., and Hawley, J. J., Int. J. Cancer **3**, 265 (1968).

15. Todaro, G. J., and Green, H. J., Cell Biol. **17**, 299 (1963).

16. Hartley, J. W., and Rose, W. P., Proc. Nat. Acad. Sci. U.S.A. **55**, 780 (1966).

17. Sendecan, G. W., and Chochran, W. G., "Statistical Method," 6th ed., p. 217. Iowa State Univ. Press, Ames (1967).

18. Finney, D. J., "Table for Testing Significance in 2 × 2 Table." Cambridge Univ. Press, London/New York (1963).

19. Chesterman, F. C., Harvey, J. J., Rourmarkin, R. R., and Salaman, M. H., Cancer Res. **26**, 1759 (1966).

20. Howard, R. J., Notkin, A. L., and Mergenhagen, S. E., Nature (London) **221**, 873 (1969).

21. Law, L. W., Ting, R. C., and Stanton, M. F., J. Nat. Cancer Inst. **40**, 1101 (1968).

22. Hellström, I., and Hellström, K. E., Inst. J. Cancer **4**, 587 (1969).

23. Schochat, D. K., Fefer, A., and Moloney, J. B., Cancer Res. **28**, 517 (1968).

24. Hook, W. A., Chirigos, M. A., and Chan, S. P., Cancer Res. **29**, 1008 (1969).

Received Sept. 2, 1970. P.S.E.B.M., 1971, Vol. 136.