Relationship Between the Lactic Dehydrogenase-Elevating Virus and Transplantable Murine Tumors.* (30990)

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More than 50 different transplantable tumors have been found to be associated with the lactic dehydrogenase-elevating virus (LDV, 1) since it was described by Riley *et al*(2). The association of the LDV with so many tumors suggested that it might be causally related to neoplasia(3). Tumors of diverse origin were subsequently found to be free from the virus(3-7) and it was noted that tumors which had been transplanted for many generations were more often contaminated than primary tumors(1). In a few instances it was observed that tumors were free from virus in one laboratory but not in another. These observations indicated that the LDV was probably not involved directly in the production of murine tumors. Riley (8,9) reported that the growth rate of a "virus-free" tumor was increased significantly when transplanted in mice infected with the LDV. This observation suggested that the LDV might play a role in the growth and transplantability of tumors by modification of the physiology of the host.

Studies in this laboratory were undertaken to examine the relationship between the LDV and several transplantable tumors. The results demonstrate that the LDV does not multiply in tumor cells propagated *in vitro*. Several strains of tumor cells were freed from virus by serial propagation *in vitro*. The growth rate of these tumors was not significantly different in normal mice and in mice intentionally infected with the LDV.

Materials and methods. Animals. Female mice of strains C3H and CFW (an inbred line of Swiss Webster mice), 4-6 weeks of age, were used. The LDV is transmissible(10, 11) and to avoid spontaneous infection of normal mice the following precautions were observed. Animals were received from Carworth Farms, New York, at weekly intervals and were housed in isolation quarters prior to use in experiments. Cages were sterilized before use.

Solutions and media. Solution 148 (S148) contained the following in mM: sodium chloride, 116; potassium chloride, 6.5; monosodium phosphate, 1; tris (hydroxymethyl) aminomethane (pH 7.8), 20; and phenol red (sodium salt), 0.02. Solution 77 (S77) had the same composition as S103(12) except that hydroxyproline was omitted. Medium 58 contained 87.5% (V/V) S77, 10% normal horse serum and 2.5% fetal calf serum. Medium 63 was prepared by adding 1.7 g glucose and 1 g Pluronic F68(13)/liter of solution composed of 80% (V/V) S77 and 20% normal horse serum. All media were sterilized by passage through Selas filters of O3 porosity.

Tumors. Tumors were transplanted at intervals of 10-20 days. The ascites form of the Hauschka-Klein tumor was transplanted in CFW mice and hepatomas 129 and 134 in C3H mice by intraperitoneal injection of 0.2 ml of ascites fluid containing 5-10 $\times$ 10^6 cells. Sarcoma 180 (S180) was maintained as a solid tumor and transplanted subcutaneously. Tumor cells grown *in vitro* were transplanted in mice by injection of 0.2 ml of suspension containing 1-5 $\times$ 10^6 cells. Tumor cells grown *in vivo* or *in vitro* were enumerated as follows. The cells were suspended in S148 containing 0.005% trypsin (Nutritional Biochemicals Corp., Cleveland, Ohio; 2 $\times$ crystalline, 50% $MgSO_4$) and incubated at 37°C for 10 minutes. Clumps of cells were disrupted by vigorous and repeated pipetting and then enumerated in a Coulter counter (14).

Tumor cells in the ascites form were harvested aseptically, diluted in medium 58 to a concentration of 2-5 $\times$ 10^5/ml and 5-ml aliquots were dispensed into T-30 flasks(15). Stationary cultures of S180 were prepared in

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the same manner as those from normal mouse tissues(16). Stationary cultures were maintained as described previously(17). In instances where some cells did not attach to the glass they were recovered by centrifugation at 500 *g* for 10 minutes when the medium was replaced. Shake cultures were prepared by suspending cells in medium 63 at a concentration of 1-2 $\times$ 10^5/ml and incubating in Florence flasks on a rotary shaker as described earlier(13). Subcultures were prepared at intervals of 3-4 days by diluting the suspension with medium 63 to a concentration of 1-2 $\times$ 10^5 cells/ml.

Lactic dehydrogenase-elevating virus. The LDV was isolated from a CFW mouse bearing S180 and was propagated in cultures of mouse spleen or lung(16) and assayed by infectivity titrations in CFW mice(10). Viral stocks were stored at —17°C.

Additional methods. Plasma was obtained by an orbital bleeding technique(18). Samples of heparinized blood were centrifuged at 1200 *g* for 10 minutes and the lactic dehydrogenase (LDH) activity of the supernatant plasma was determined spectrophotometrically as described previously(19,20). The hematocrit was also determined in some instances. Hemolyzed specimens were discarded.

Results. Serial propagation of murine tumors in vitro. Primary cultures of S180 proliferated in medium 58 with a generation time of approximately 24 hours and continued to grow at this rate for at least 10 passages. When S180 cells from either stationary cultures or from animals were placed in shake cultures in medium 63 they multiplied at approximately the same rate and serial propagation was readily achieved. The initial behavior of the Hauschka-Klein tumor and hepatomas 129 and 134 in culture was markedly different from that observed with S180. When all 3 strains of tumor were placed in stationary cultures in medium 58 only about one-fifth of the cells attached to the glass. The cells, both in the medium and on the glass, promptly began to degenerate. Replacement of the medium was continued at intervals of 3-4 days and there was little indication of cell proliferation during the first 2 weeks in culture. After this time a slow increase in the number of surviving cells became evident. Most of the newly-formed cells were not attached to the glass and continued to proliferate in suspension in stationary flasks. When these free-floating cells were placed in shake-flasks in medium 63 the rate of multiplication increased significantly. After 2-3 subcultures the generation time was 20-24 hours and maximum concentrations of 0.5-1.0 $\times$ 10^6 cells/ml were obtained. All 3 ascites tumor lines behaved essentialy the same and have been serially propagated through 18 subcultures over a period of 3 months. During this period all strains maintained their capacity to produce tumors in mice which were grossly indistinguishable from those produced by the parental lines maintained by serial transplantation in animals.

Lack of association between LDV and tumor cells in vitro. The Hauschka-Klein tumor, hepatomas 129 and 134, and S180 were previously found to be associated with LDV *in vivo*(10). Suspensions of freshly excised S180 tissue and intraperitoneal fluids from mice bearing the ascites tumors contain virus in concentrations of 10^7-10^8 ID_{50}/g wet weight(10). Upon cultivation *in vitro*, however, the virus disappeared rapidly from the culture fluids of all 4 tumors. No virus could be detected either in the medium or in the cells after 2-3 weeks in culture. A serially propagated culture of L5178Y lymphosarcoma was also found to be free of the virus although the same tumor maintained by *in vivo* transplantations was associated with it(10). All of the tumors remained free from LDV on continued serial propagation either *in vitro* or *in vivo*. The results of a typical experiment in mice are presented in Table I. In this instance "virus-free" S180 was transplanted serially in mice seven times without evidence of infection with the LDV as judged by the absence of an elevation of LDH in the plasma of recipient animals 4 days after receiving the tumor implant. It will be noted that when mice were injected with S180 and the LDV simultaneously, the virus remained associated with the tumor throughout the 6 succeeding transplantations as indicated by the high levels of plasma-LDH of the recipient mice 4 days after injection. At this time the elevated plasma-LDH was due solely to in-

TABLE I. Serial Transplantation of Sarcoma 180 in CFW Mice in the Absence and Presence of LDV.

Transplant No.	Sarcoma 180*		Sarcoma 180 + LDV†	
	Units LDH/ml of plasma, 4 days after transplantation‡	Tumor-take§	Units LDH/ml of plasma, 4 days after transplantation‡	Tumor-take§
1	900	3/3	6,500	3/3
2	850	2/3	5,200	3/3
3	1,300	3/3	5,500	3/3
4	950	3/3	6,000	2/3
5	870	3/3	6,100	3/3
6	930	3/3	6,700	3/3
7	850	3/3	6,100	3/3

* Initially (transplant No. 1) mice were injected subcutaneously with 10^6 S180 cells which had been serially propagated *in vitro* for 10 weeks. Succeeding transplants were made at intervals of 12-15 days. At this time the diameters of the tumors varied from 1.5-2.0 cm.

† Mice were injected with 10^6 S180 cells and 10^5 ID_{50} of LDV/mouse. Succeeding transplants were made as in 1 without additional injection of virus.

‡ Mean values of 3 mice.

§ Numerator = number of mice developing tumors; denominator = total number of mice injected. Mean survival times were approximately 3 weeks for both virus-infected and uninfected mice.

fection with LDV since the tumor had not grown sufficiently to contribute to this value.

Studies of tumor growth in LDV-infected and uninfected mice. The results of a number of experiments with S180 in addition to those summarized in Table I indicate that infection with LDV had no effect on the transplantability or growth rate of the tumor. For example, of 100 normal and 100 infected mice receiving 5-500 $\times$ 10^4 S180 cells from either stationary or shake cultures, 93 normal and 87 virus-infected mice developed tumors (Table II). The mean time of death of mice infected with LDV was not significantly different from that of the uninfected controls. Similar results were obtained from the Hauschka-Klein tumor and hepatomas 129 and 134 (Table II) by experiments analogous to those described for S180. In addition the results presented in Table II demonstrate that the growth of the Hauschka-Klein tumor and hepatomas 129 and 134 in uninfected mice resulted in only a slight increase in plasma-LDH levels, whereas a more substantial elevation was caused by S180. Mice injected simultaneously with LDV and tumor showed an initial increase in plasma-LDH typical of viral infection. The maximum LDH activity of plasma of these mice during the terminal stage of tumor growth was appreciably higher than expected from the results with either tumor or virus alone. This synergistic effect of virus and tumor was somewhat smaller in the case of the ascites tumors than with S180. The data presented in Table II also include the hematocrit of tumor-bearing mice in the presence and absence of LDV measured either after 4 days or during the terminal stages of tumor development. No relationship was found between the LDH activity of plasma and that of the ascitic fluid. In "virus-free" mice bearing a tumor, the LDH activity of the ascitic fluid was 7-10 times higher than that of the plasma (Table II). On the other hand, the level of LDH was essentially the same in plasma and ascitic fluid of infected mice. The LDH activity of ascitic fluid of mice injected with both virus and tumor, however, was slightly higher than that of uninfected mice bearing a tumor. Ascites tumor cells from virus-infected and uninfected mice contained LDH activity from 1000-4000 units/10^6 cells (30-100 $\times$ 10^6 tumor cells/ml ascitic fluid).

Discussion. The results of these studies indicate that the original populations of cells from the Hauschka-Klein tumor and from hepatomas 129 and 134 were heterogeneous with respect to their ability to proliferate in media 58 and 63. Only a very small proportion of the original cells was capable of growth under these conditions. It is of interest, however, that the cells which were selected on a nutritional basis retained their capacity to

TABLE II. Effect of Growth of Various Tumors on LDH, Hematocrit and Mean Death Time of Mice in the Presence and Absence of LDV.

		LDH activity, units/ml ± SD, $\times 10^{-2}$			Hematocrit, % ± SD			
		Plasma‡		Ascitic fluid				
Tumor*	LDV†	4 days	T.S.	T.S.	4 days	T.S.§	Tumor-take‖	Mean death time, days ± SD
Hauschka-Klein	Absent	10 ± 1	18 ± 10	250 ± 100	52 ± 2	35 ± 11	26/30	20 ± 7
	Present	76 ± 25	143 ± 130	400 ± 270	50 ± 6	27 ± 11	28/30	19 ± 5
Hepatoma 129	Absent	9 ± 2	13 ± 10	150 ± 80	53 ± 5	53 ± 6	33/33	20 ± 5
	Present	84 ± 11	203 ± 121	300 ± 100	49 ± 5	53 ± 6	36/36	21 ± 8
" 134	Absent	10 ± 6	21 ± 11	130 ± 40	48 ± 9	40 ± 10	19/21	13 ± 3
	Present	68 ± 14	158 ± 87	160 ± 60	49 ± 8	41 ± 8	12/21	15 ± 4
Sarcoma 180	Absent	9 ± 1	81 ± 12				93/100	20 ± 3
	Present	70 ± 5	390 ± 62				87/100	19 ± 5

* Each mouse was injected intraperitoneally with 1-3 × 10^6 tumor cells (Hauschka-Klein, Hepatoma 129, Hepatoma 131) or subcutaneously with 5-500 × 10^4 S180 cells. All tumors had been serially propagated *in vitro* for at least 6 weeks.

† Each mouse was injected with 10^4 ID_{50} of LDV simultaneously with tumor cells where indicated.

‡ Plasma-LDH activity of uninfected mice is 7.7 ± 2.2 × 10^{-2} and of viral-infected mice is 54 ± 13 × 10^{-2}(20).

§ T.S. = terminal stage of tumor growth (10-20 days after injection).

‖ Numerator = number of mice with tumor; denominator = total number of mice injected.

produce tumors after prolonged serial propagation *in vitro*. Nutritional heterogeneity is not as evident with S180 and cells from this tumor proliferate readily in a variety of media(21).

The data indicate that the LDV does not multiply in mouse tumors and that the virus is lost when cells are serially propagated *in vitro*. When a tumor which has been freed from virus in this manner is transplanted to an infected animal it remains associated with the virus in subsequent passages (Table I). This continued association between the LDV and tumor is facilitated by the fact that virus is readily transmitted from one animal to another(10,11) and that it persists in relatively high titer in the plasma and tissues of infected animals indefinitely without causing overt disease(10,22). Yaffe has also reported that a mouse hepatoma which had been grown *in vitro* was free from the LDV (6). Other investigators have reported that many spontaneous and some transplantable tumors were not associated with the LDV (3-7). Collectively the data demonstrate that the LDV is frequently carried by transplantable tumors as a contaminant.

The LDV employed in these studies did not affect the rate of growth or transplantability of the tumors employed. Other investigators have obtained similar results with additional tumors(3,23). Clough *et al*(24) and Riley(8,9), on the other hand, found that the rate of growth of tumors free from virus was greater in mice infected with LDV than in uninfected animals. Gregory *et al* (25) reported recently that one strain of LDV accelerated the growth of Ehrlich ascites carcinoma whereas another strain of virus delayed the growth of the same tumor. The latter findings suggest that some of the observed differences in the effect of the LDV on the growth of tumors may reflect variations in the virus employed in different laboratories. The extent to which differences among strains of virus account for the overall data remains to be determined.

The results presented in Table II demonstrate that the degree of anemia which develops in tumor-bearing mice is a function of the tumor and that this is not influenced by infection with the LDV. The severe anemia originally attributed to infection with LDV(7) has recently been shown to be due to *Eperythrozoon coccoides*(26).

The data presented in Table II demonstrate that the LDH activity of plasma from infected mice bearing large tumors is several-fold greater than that expected from the values observed with either virus or tumor alone. The results of other studies indicate that infection of mice with LDV results in

impaired clearance of enzymes from the peripheral circulation(27-29). The synergistic effect of tumor and virus on the level of LDH in the plasma, therefore, appears to be due to impaired clearance coupled with an increase in enzyme by release from tumor cells.

Summary. The following murine tumors were propagated serially *in vitro*: Sarcoma 180, Hauschka-Klein ascites tumor, and hepatomas 129 and 134. The lactic dehydrogenase-elevating virus initially associated with all of these tumors *in vivo* did not multiply in cell cultures and by serial propagation *in vitro* the tumors were freed from the virus. The results indicate that the relationship between the lactic dehydrogenase-elevating virus and transplantable mouse tumors is that of a common contaminant. The "virus-free" tumor cells produced tumors when implanted into mice. The growth rate and transplantability of these tumors was the same in normal mice and in mice infected intentionally with the virus.

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