Influence of LDH Viruses on Growth of Ehrlich Ascites Tumor in Mice.* (30413)

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The presence of a virus-like agent (LDH agent) in many transplanted mouse tumors, first demonstrated by Riley, is now well established(1-6). The increase in plasma LDH which occurs within 3-4 days following infection is also accompanied by increases of similar magnitude in isocitric dehydrogenase and phosphohexose isomerase(7,8,9). The agent has a number of unusual features. It may be carried in mice in the absence of tumor, and infected mice show no overt symptoms other than the plasma enzyme elevations. The infectivity and elevated plasma enzyme levels persist through the lifetime of the animal. Life span is apparently normal and there are no grossly visible pathological changes or increases in the level of LDH in the tissues(4). Maximum virus titer of from 10^9-10^{11} infectious units per ml of plasma is reached 24 hours following infection, thereafter dropping rapidly to about 10^6 infectious units at 4 days and remaining at about this level throughout the remainder of the lifetime of the animal. Riley reported that the LDH virus accelerated the growth of a virus-free methyl cholanthrene induced tumor(10). Plagemann *et al*(11) failed to find any influence of an LDH virus infection on the growth of hepatoma 129.

In the course of studies on the isolation of a virus-free Ehrlich Mouse Carcinoma (EMC) from tissue culture, we noted that the growth rate of the virus-free tumor as indicated by decreased peritoneal swelling and increased survival time of mice appeared to be slower than that of the stock tumor (which is contaminated with an LDH virus). Since EMC grows as a single cell suspension in the peritoneal cavity its growth rate may be measured quantitatively by counting the harvested cells in hemacytometer chambers.

This paper describes experiments in which the Ehrlich ascites mouse carcinoma was freed of an endogenous LDH agent by passage in tissue culture. The influence of LDH agents from 3 different tumors on growth rate of the virus-free tumor was then measured.

Materials and methods. Mice used in the experiments were Swiss Webster (Dierolf Farms) strain females usually 6-9 weeks old. Ehrlich ascites tumor was a hypotetraploid subline from Dr. Frank Henderson, University of Alberta, and was found to be associated with an endogenous LDH agent. Methods used for bleeding mice and determination of plasma enzyme activity have been described(4). Samples of NCTC 109 tissue culture medium and other tissue culture products used were from Microbiological Associates, Bethesda, Md. Chemicals used for plasma enzyme determinations were from Nutritional Biochemicals, Inc. Mice bearing the CD/5 lymphoma and DBA/59 mammary carcinoma and from which LDH agents were also isolated were kindly supplied by Dr. Morris Barrat, National Cancer Institute.

Tumor inoculation procedure. For routine transfer of tumor, harvesting of cells was performed by aspiration directly from the peritoneal cavity using a 1-ml syringe and #27 needle. The needle was inserted directly into the peritoneum and without opening the cavity. This reduced the possibility of chance contamination with virus. A portion of the aspirated fluid was then diluted with saline and from 1 to 2 million cells were injected into fresh mice. Under these conditions the tumor usually required transfer once weekly. When harvesting ascites tumor cells for quantitative growth rate determination, a different procedure was used (see below).

Measurement of tumor growth rate. Mice from which tumor was to be harvested were first killed either by cervical dislocation or by overanesthesia with diethyl ether. Diluted heparin (1:100) in saline (3 ml) was then injected into the peritoneal cavity using a

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#20 needle. Heparin eliminated the clumping of tumor cells during harvesting. The outer layer of skin was then removed and a small hole was made through the inner fascia sufficiently large to accommodate the bent tip of a Pasteur Pipette. Peritoneal fluid was aspirated carefully into a centrifuge tube, and the cavity was washed 3 times successively with 3 ml portions of saline until no further cells were visible in the aspirated fluid. The harvested cells and fluid were diluted to 15 ml with saline, and the cell population was determined by counting diluted aliquots in Spiers Levy Eosinophil hemacytometer chambers. Results were expressed as millions of tumor cells per mouse. The remaining cells were centrifuged under standard conditions (15 min 2000 $\times$ g) for measurement of total packed cell volume.

Isolation of virus-free tumor from tissue culture. Tumor cells were harvested using sterile procedures from mice which had been inoculated with tumor 5 days previously. At this stage the tumor cell population was usually about 100-200 million and uncontaminated by blood. The cells were centrifuged, washed once with sterile saline, and resuspended in the tissue culture growth medium (80% NCTC 109 medium supplemented with 20% horse serum and containing penicillin (100 units) and streptomycin 50 μg per ml) to give concentrations of from 2-5 million cells per ml. Portions of the suspensions (10 ml) were inoculated into milk dilution bottles and incubated at 37°. Individual bottles of cells were harvested at approximately daily intervals; the cells were washed once with sterile saline before injection into mice. At 0 days (before tumor cell injection) and again at 4-7 days following tumor cell injection mice were bled and the plasma LDH was measured. Presence of virus was indicated by a 5-fold or greater increase in plasma LDH occurring within the 4-7-day period. Tumor cells were harvested from those mice in which tumor growth was accompanied by failure of the plasma LDH to rise. This absence of contaminating virus was then confirmed by transfer of plasma from the tumor-bearing mouse to a second mouse.

Storage of frozen tumor. A portion of the virus-free tumor was used to carry the tumor by serial propagation in confirmed uninfected mice (see below). A second portion was stored frozen at —50°C in a mixture consisting of 70% NCTC 109 medium, 10% horse serum and 20% glycerol. To harvest cells the mixture was thawed slowly at room temperature, the glycerol-containing medium was removed by centrifugation and the cells were resuspended in saline for inoculation into mice. Viable tumors were recovered by this procedure following storage for up to 1 year.

Serial propagation of virus-free tumor. Two lines of the tumor derived from separate tissue culture isolates were carried by serial transfer in virus-free mice. Each was inoculated into 3 mice and at the same time 3 additional mice were inoculated with peritoneal washings from a normal mouse as controls. Plasma LDH was measured in all mice one week later. Harvesting of tumor and peritoneal washings was by aspiration directly from the peritoneal cavity. Tumor for transfer to the next generation was selected from the mouse having the lowest value of plasma LDH. In those cases where tumor-bearing mice had elevated plasma LDH (see below) the tumors were excluded from further transfer.

Results and discussion. In the present study, LDH viruses from 3 different tumors were used. These viruses were isolated from Ehrlich ascites mouse carcinoma grown in Swiss Webster mice, DBA/59 mammary carcinoma and CD/5 lymphoma tumors growing in DBA/2 and BALB/c mice respectively. It was shown that all 3 agents produced an elevation of 5-6-fold in plasma LDH within 4 days of infection, and were capable of being transmitted using filtered plasma alone. The other properties delineating the CD/5 agent as virus-like have been given previously(4).

Plasma LDH levels in mice carrying virus-free Ehrlich mouse carcinoma (EMC). The stock strain of EMC which was contaminated with an LDH virus was maintained in tissue culture and daily transplants of individual cultures were made back into mice. Transplants made after 5 days or less grew tumors which were contaminated with LDH virus,

TABLE I. Plasma LDH Levels in Mice During Serial Transfer of Virus-Free Ehrlich Ascites Cells.

Tumor transplant generation	Series 1 3 mice	Series 2 3 mice	Tumor transplant generation	Series 1A 3 mice	Control 3 mice
1 through 4	N	N	16	N	N
5	E 1/3†	N	17	E 2/3*	N
6	N	N	18	N	N
7	N	E 1/3	19	E 2/3‡	N
8	N	N	20	E 2/3	N
9	N	E 2/3	21	E 1/3	N
10	N	E 1/2	22	N	N
11 through 17	N	N	23	E 2/3†	N
18	N	E 1/3*	24	N	N
19	N	E 1/3*	25	N	N
20	E 1/3†	E 1/3	26	N	N
21	N	E 2/3	27	N	N

* Not infectious. † Infectious virus recovered. ‡ Lethal within 2 days.

N = Normal plasma LDH 150-300 units per ml. E = Elevated plasma LDH > 1000 units per ml.

Two series of virus-free EMC cells isolated after 6 days (Series 1) or 7 days (Series 2) in tissue culture were maintained by serial transfer in groups of 3 mice having normal plasma LDH. A series of 3 "sham" mice as controls was also maintained by transfer of peritoneal washings. LDH was measured in plasma when tumor was harvested. Mouse with lowest plasma LDH was used as donor for next generation. Some cells from each transplant were also stored frozen (see *Methods*). Series 1A was begun from 15th generation series 1 tumor stored frozen for 3 months. Inoculum was usually 10,000,000 cells, tumors harvested weekly. For additional details see *Methods*.

those made from 6 days and older cultures grew tumors which were not contaminated with the virus as judged by normal plasma LDH levels and failure to transmit virus using plasma from the tumor-bearing animals. It was also noted that these virus-free tumors grew more slowly than before tissue culture passage. The virus-free tumor from 2 separate tissue culture isolates was carried for more than 27 generations. With the exceptions noted in Table I, mice bearing the tumor even in the later stages of tumor growth maintained normal plasma LDH levels (Fig. 1). This was in marked contrast to the plasma LDH levels in mice carrying the original strain of EMC or in mice carrying the virus-free tumor intentionally injected into infected mice (Fig. 1). Level of plasma LDH in those animals carrying infected tumors was 10-40 times normal. It is apparent that the high values of plasma LDH which have previously been attributed to the ascites tumor itself are almost entirely due to contamination with the LDH virus.

Incidence of spontaneous elevated plasma LDH in mice bearing virus-free EMC. In preliminary experiments following isolation of a virus-free EMC from tissue culture, the tumor usually became contaminated with an LDH virus by about the 6th tumor transplant

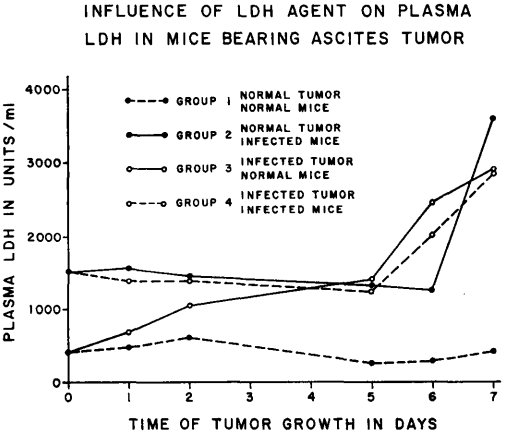

FIG. 1. Plasma levels of LDH measured at intervals following implantation of 5,000,000 EMC cells. Curves refer to conditions outlined in caption to Fig. 2. Group 1 and 2 tumor was virus-free tissue culture derived. Group 2 mice preinfected with CD/5 lymphoma virus. Group 3 and 4 tumor was infected with EMC virus, Group 4 mice also preinfected with CD/5 lymphoma virus. Note: (1) normal LDH levels in absence of virus (Group 1). (2) Increase in LDH over 4 days in normal mice injected with infected tumor (Group 3). (3) High initial LDH level in preinfected mice (Groups 2 and 4). (4) Terminal 10-20-fold increase in LDH in tumor-bearing infected mice (Groups 2, 3, 4).

generation. It was thought that this probably resulted from accidental contamination. In the present series of experiments therefore (Table I) the precaution was taken of carrying 2 lines of cells each in 3 mice at each transplant. Plasma LDH was assayed in all mice both immediately before injecting tumor cells and also immediately before harvesting the cells for the next generation. At the same time as the tumor was being harvested, 3 "sham" mice were treated as controls in identical fashion by transfer of peritoneal fluid. As can be seen in the last column in Table I there was no incidence (0/81) of elevated plasma LDH in any of the controls during the 27 transplant generations. Also in over 90% of the tumor-bearing mice (202/222) plasma LDH was within normal limits. There were, however, 20/222, or about 10%, of transfers in which the tumor-bearing mice had a 5-fold or greater than normal plasma LDH value. In those tumors which were tested, the majority proved to be non-infectious, *i.e.*, the elevation in plasma LDH could not be transferred using plasma from the tumor-bearing mouse. In 3 instances, however, the elevation in plasma LDH of the tumor-bearing mouse was transferable (Table I). It is not certain that this represents accidental contamination since there were no cases in any of the "sham" controls. It is possible that tumor-bearing mice are more susceptible to accidental infection by the LDH agent, or that the tumor activates a latent virus already present. Another possibility is that reappearance of virus may result from a lysogenic relationship between the virus and the tumor cell. A comparison of the antigenic properties of the original EMC LDH agent and of 2 of the "spontaneous" agents is now being made in order to test some of these possibilities.

The above results indicate the difficulty of keeping a tumor line free from LDH agent over prolonged periods, and also offer a possible explanation for the fact that in most laboratories the majority of long transplanted mouse tumors are contaminated with an LDH agent(1,2,4,8,10).

Growth rate of virus-free tumor in normal and infected mice. The tumor inocula used for the comparative growth rate experiments were derived from frozen virus-free EMC cells which had been stored at —50°C for a period of weeks or months. These were regenerated by inoculation into a virus-free mouse and the tumor harvested after 7-8 days was used as inoculum for the growth rate studies. In some experiments the virus-free cells from a single mouse were used to inoculate control mice or mice preinfected with a particular virus. The latter are designated as "normal cells in infected mice" in Fig. 1. In other experiments the virus-free cells were passed for one generation in an infected mouse before inoculation into test mice. These are designated as "infected cells" in Fig. 1.

Groups of 10 control mice and 10 mice preinfected 4 days previously with the chosen virus were injected intraperitoneally with 500,000 EMC cells (for DBA/59 virus experiment Fig. 2) or 5,000,000 cells for the other 3 experiments. (For details see caption to Fig. 2.) Groups of 2, 3 or 4 mice were killed at intervals and the tumor cell population was measured.

Growth of the tumor was accelerated by each of the 3 viruses tested. The acceleration of growth was noted both when infected cells were injected into normal mice and when normal cells were injected into infected mice. In view of the extremely high rate of replication of the LDH agent (virus titer reaches 10^{10} infectious units per ml of plasma 24 hours following infection) these results do not of themselves enable a distinction to be made between effect of the virus on the tumor cell itself or upon the host.

Possibility that LDH agents influence host resistance to tumor. The mode of action of LDH agents in increasing plasma enzyme levels has been shown to involve interference with the normal plasma enzyme clearance mechanisms. Clearance of injected isologous enzymes was decreased to about 50% of the normal rate in mice infected with an LDH agent(12). Similar results were obtained by Notkins(13) and Mahy(14) who also showed that RES function as measured by clearance of colloidal carbon was depressed up to 75% in the first 24 hours following infection. Al-

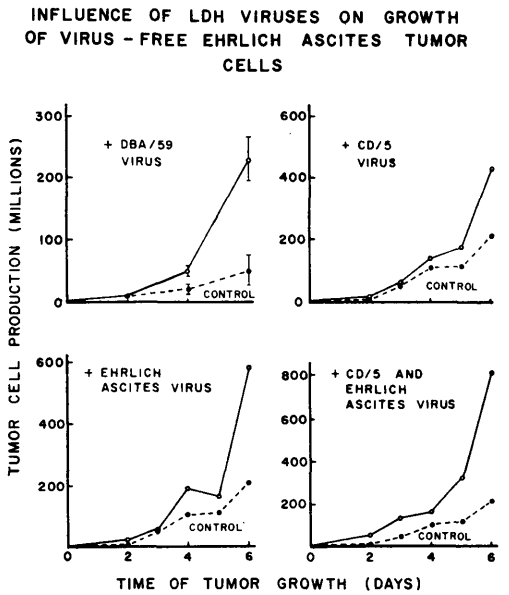

FIG. 2. Influence of LDH agents on growth of Ehrlich mouse ascites tumor. Top left. Cells infected with DBA/59 derived virus injected into mice preinfected 4 days previously with the same virus (upper curve), lower curve uninfected cells in uninfected mice. Inoculum was 500,000 cells. Points represent average tumor cell population in 3 and 4 mice. Top right. Uninfected cells (5,000,000 inoculum) injected into mice infected 4 days previously with CD/5 lymphoma virus. Bottom left. Cells (5,000,000 inoculum) infected with Ehrlich ascites virus injected into uninfected mice. Bottom right. Cells (5,000,000) infected with Ehrlich ascites virus injected into mice preinfected with CD/5 lymphoma virus. Other conditions as described in *Methods* section.

though carbon clearance rapidly returns to normal within 3-4 days, the impairment in plasma enzyme clearance mechanisms persists. It seems possible that the acceleration of tumor growth by LDH viruses described here may be attributable to a similar depression of certain of the host resistance mechanisms.

Summary. 1. Ehrlich Mouse Ascites Carcinoma (EMC) was freed from an endogenous LDH virus by passage in tissue culture, and subsequently carried virus-free for 27 generations in mice. 2. Plasma LDH was from 10- to 40-fold elevated in mice carrying the stock infected tumor. Over 90% of mice carrying the virus-free tumor, however, had normal plasma LDH levels. 3. Growth rate of the virus-free tumor was accelerated by the LDH virus from Ehrlich Mouse Carcinoma itself and also by LDH agents isolated from DBA/59 mammary carcinoma and CD/5 lymphoma. It is suggested that the virus may depress host resistance to the tumor.

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