LDH Levels in Blood and Tissues of Mice Infected with an LDH Agent.* (28610)

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Riley(1) reported that a number of transplantable mouse tumors were associated with a transmissible, viral-like agent. This agent can be propagated independently of the tumor by injection of plasma from infected mice. Riley observed that infection with the agent brought about an increase of from 5- to 10-fold in the plasma lactic dehydrogenase (LDH) levels in otherwise normal mice. Since tumor growth rate and LDH levels in tumor-bearing mice were also increased, it was proposed by Riley(2) that a synergism existed between the tumor and the agent. Notkins *et al*, however, showed that the agent was associated only with established transplantable laboratory tumors(3), and was not found in a number of spontaneous or chemically-induced neoplasms of recent origin. It was therefore reasoned that the agent was an accidental contaminant of transplantable tumors and was not directly associated with tumor growth. It was apparent, however, from the results of both Riley and Notkins *et al* that some interaction between the agent and the tumor did occur, since tumor-bearing mice with the agent also showed levels of LDH which were very much higher than those in mice which carried the tumor alone, and the effects were not additive(4). The plasma LDH level in animals bearing the tumor alone was in turn much higher than that of normal mice either before or after infection with the agent.

The amount of LDH enzyme involved in the increase in serum following infection of mice with the agent is considerable. It was of interest, therefore, to obtain some measure of the LDH content of the cellular elements of the blood and also that of different mouse tissues as possible sources of the increased enzyme in plasma. This paper describes the isolation of an agent from the CD/5 lymphoma and the DBA/2 mammary carcinoma, which has properties similar to those described by Riley(2,1). Measurements of the LDH content of a number of mouse tissues and of blood cells, both in normal animals and in animals infected with the agent, were made. Some additional observations on the replication of the agent and of hematocrit values in mice carrying the agent are also reported.

Materials and methods. Mice bearing the CD/5 lymphoma and the DBA/2 carcinoma were kindly supplied by Dr. Morris Barrett of National Cancer Institute. The tumors were maintained by serial transfer in BALB/c and DBA/2 mice, respectively, by use of a modification of the cytosieve method of Snell(5). Blood was obtained from mice by the intraorbital technique of Riley(2), following light anesthesia with diethyl ether. Determinations of LDH enzyme were made by the method of Wacker *et al*(7), using a modification adapted to the use of the Beckman model DB recording spectrophotometer. One unit of enzyme defined by this method is about twice as large as that defined by the method of Wroblewski(8). Day-to-day variations in absolute enzyme units which were brought about by variations in temperature, quality of reagents, etc., were compensated for by direct daily comparison with a standard enzyme source. (Small samples of pooled horse serum, individually frozen and stored until use.)

Measurement of LDH Levels in Tissue Extracts. Mice were sacrificed by cervical dislocation and 25 to 50 mg slices of brain, heart, kidney, liver, lung, pancreas, skeletal muscle, spleen, submaxillary gland and thymus were removed. The tissue samples were homogenized in ice-cold distilled water (5 mg organ/ml distilled water) using a 50 ml Tissue Grinder Tenbrock for 3 minutes, followed by 3 minutes' treatment in Model MSE 20

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TABLE I. Plasma LDH Levels in Mice Injected with CD/5 Lymphoma and DBA/59 Mammary Carcinoma.

Mouse strain	No. of mice	Type of tumor inj*	Plasma LDH levels (units/ml) 0 days	1 wk	Before sacrifice†
DBA/2	24	DBA/59	280	1604	2993 (range 1294-8400)
BALB/c	9	CD/5	280	1900	4735 (range 2486-12,000)
BALB/c	7	DBA/59‡	462	1512	1505

* Animals were inj subcutaneously in left groin with between 1 and 2.5 million cells.
† Animals were sacrificed usually at 6-8 wk, depending upon size of tumor.
‡ The BALB/c mouse is completely resistant towards DBA/59 tumor.

kc sonicator at 200 watts while cooling in an ice bath. After clarifying by centrifugation at 1500 × *g* for 10 minutes, the supernatant was withdrawn for enzyme determinations.

Measurement of LDH in Red and White Blood Cells. The small heparinized capillary tubes available commercially† which were used for bleeding the mice from the orbital cavity were calibrated for volume per unit length. These tubes were of reasonably uniform bore, with an average volume of .00255 ml/mm length ± .00010 ml/mm. After drawing blood, the tube was plugged at one end with modeling clay and centrifuged under standard conditions (1500 × *g*, 25 min.) to pack down the cells. The volume of packed red blood cells and supernatant plasma were measured using a millimeter ruler. The volume of buffy coat (white blood cells and platelets) sitting above the red blood cells was determined by measuring the length of the segment, using a calibrated microscope stage. The capillary tube was then broken into 3 measured lengths using a glass cutter. The first portion contained plasma alone; the second contained plasma, red blood cells and buffy coat; and the third, red blood cells alone. LDH determinations were made on the plasma. The other 2 portions were diluted in 100 volumes of distilled water, causing lysis of the formed elements. This was followed by 3 minutes sonication as for the tissue extracts. Hemoglobin content was determined by differential readings of optical density at 580 and 650 mu. From the measurements made, the LDH content of plasma and red blood cells was calculated directly and that of the white blood cell and platelets by difference (see sample calculation in Table III). Measurements of total white blood cells per mm^3 were made by standard methods, using 1% acetic acid as the diluent(9).

Results and discussion. Identification and Proof of Viral Nature of LDH-Agent From CD/5 Lymphoma and DBA/59 Carcinoma. Four days following the injection of one million cells of either of the above tumors into BALB/c or DBA/2 strain mice, there was an increase of 5- to 7-fold in the plasma LDH level. In a further 10 to 15 days the BALB/c mice injected with the CD/5 cells developed a palpable subcutaneous tumer at site of injection; at this time the plasma LDH levels increased still further to as great at 45 times normal (Table I) before the large size of the tumors necessitated sacrifice of the animals. In DBA/2 mice injected with the DBA/59 tumor a similar secondary rise in plasma LDH occurred following development of the tumor (Table I). Variations in the secondary rise of plasma LDH appeared to correlate with the size of the tumors, in agreement with Riley(1). In BALB/c mice injected with the DBA/59 tumor, there was no tumor growth and no secondary rise in the plasma LDH above the 5- to 7-fold initial increase after injection. The initial elevation in plasma LDH could also be produced by intraperitoneal injection of a cell-free frozen and thawed tumor homogenate, or by plasma from a tumor-bearing mouse (Table IA). It is apparent from these results that the initial rise in plasma LDH is not dependent upon tumor growth.

† Micro Blood Collecting Tubes (heparinized with ammonium heparinate), #B3095-2, Scientific Products, Evanston, Ill.

TABLE IA. Transmission of LDH Agent by Cell-Free Extracts from Infected Mice.

Infecting material	No. and strain of mouse	Mean plasma LDH (units/ml) 0 time	5 days
2800 × *g* supernatant from frozen and thawed CD/5 tumor cells (.5 ml)	3 BALB/c	280	1593
2800 × *g* supernatant from frozen and thawed and homogenized* CD/5 tumor cells (.5 ml)	3 "	280	1492
.01 ml plasma from BALB/c mouse with CD/5 tumor	4 " †	518	1100
.01 ml plasma from infected BALB/c mouse	39 "	358	1956
Idem	6 DBA/2	446	1920
.01 ml plasma from normal BALB/c mouse (control)	5 BALB/c	319	288
.2 ml physiological saline (control)	6 "	274	311

Mice were bled both before and 5 days following intraperitoneal injection of infecting material. Plasma LDH values were then determined as described in *Methods*.

* Homogenized for 5 min in a Tenbrock tissue grinder and centrifuged for 30 min at 2800 × *g*. Filtrability of the agent is demonstrated by results in Table II.

† Plasma from these infected mice was the original source for serial transfer of the agent through 9 more generations as depicted in Fig. 2.

The plasma LDH factor was subsequently transferred through 9 more generations of tumor-free mice by intraperitoneal injection of 0.01 ml of plasma alone, without any indication of diminution in effectiveness (Fig. 2). Following this 9th generation transfer, 1 ml of plasma was found to contain between 10^4 and 10^5 infectious units. The LDH factor clearly has the ability to replicate in mice in the absence of tumor tissue. Filtration of a 1:10 dilution of the plasma through a millipore filter, average pore size 0.45 μ, did not reduce the infectivity, although filtration of a 1:1000 dilution did (Table II).

The agent was stable to heating at 50°C,

TABLE II. Viral-Like Properties of LDH Agent.

Treatment of infected plasma before injection	Plasma LDH levels in injected mice 0 days	7 days
None	445	1748
Heated 50° 1 hr	462	1918
" 57° "	168	238
Filtered 1:10 dilution†	146	1347
" 1:1000 " †	408	426
Treatment of mice following inj*		
1 ml saline daily	146	1350
1 ml antibiotic‡ daily	126	1425

* With untreated infectious plasma (.01 ml).

† Filtered through MA Millipore Filter, avg pore size .45 μ.

‡ Containing: Penicillin 100 units, streptomycin 50 μg, achromycin 50 μg, and mycostatin 50 μg. Administered intraperitoneally.

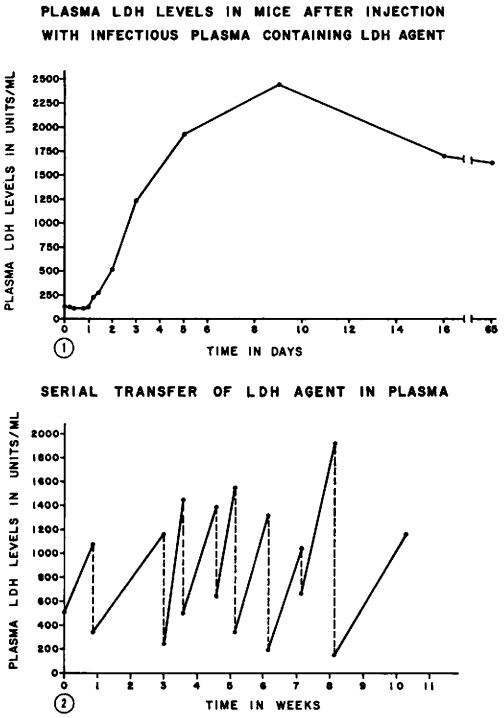

FIG. 2. Source of original injection of LDH agent at 0 wk was 0.01 ml of plasma from a BALB/c mouse bearing the CD/5 lymphoma (Table IA). This plasma was injected intraperitoneally into 3 normal BALB/c animals. After one wk, plasma from these animals was injected into 3 more normal BALB/c animals. This procedure was followed for 9 transfer generations. Diagonal solid lines represent mean plasma LDH rise for each generation, and vertical dotted lines, the transfer into fresh mice.

but not at 57°C (Table II). The LDH enzyme activity of plasma showed a similar thermal inactivation spectrum. The agent was antibiotic insensitive; daily injection of 1 ml of an antibiotic mixture (penicillin 100 units; streptomycin, 50 mg; achromycin 50 mg; and mycostatin 50 mg for 5 days following infection) failed to prevent the usual 5- to 7-fold rise in plasma LDH levels of injected mice (Table II).

Time-Course of Rise in Plasma LDH Levels. A group of 4 mice was injected intraperitoneally with 0.01 cc of plasma from an infected mouse. The plasma used was from the 9th passage of the agent in tumor-free mice and represented about 1000 infectious units. The mice were bled in pairs at intervals of 6 hours following injection. The sharp rise in plasma LDH began 29 hours following injection and reached a maximum of 18.7 times normal after 9 days (Fig. 1). At 70 days the enzyme level was still 14 times normal. In mice which were followed for a longer period the enzyme levels were still elevated 9 months following the original infection, although the levels had fallen below the maximum. In mice in which the LDH levels had fallen close to that observed for high normals, a second infection with the agent did not in general lead to a second rise in the plasma LDH.

Tissue and Blood-cell Levels of LDH in Normal and Infected Mice. The average plasma value of LDH in normal mice is 280 units per ml. The total plasma enzyme in a 25 g mouse (assuming blood volume = 8% of body wt.) is therefore about 280 units. The average plasma value of LDH in mice infected with the LDH-agent is about 1700 units per ml. Total plasma LDH in mice bearing a tumor, in addition to the LDH factor, ranges from about 2500 to 12,600 units per ml (Table I). This wide range of enzyme in the plasma of tumor-bearing mice is a function of tumor size, and is probably also influenced by factors such as tumor cell necrosis.

The amount of LDH in 1 ml of packed red blood cells is 44,800 units (Table III), *i.e.*, about 45,000 units per 25 g mouse. The white blood cells and platelets have more LDH per unit volume, about 174,000 per ml (Table II), but the total blood pool present in this fraction is only 3500 units. (A sample calculation for white blood cell and platelet LDH is shown as an appendix to Table III.)

TABLE III. LDH Levels in Plasma, Red Blood Cells and Buffy Coat in Normal and Infected Mice.

	LDH units per ml of plasma or packed cells		
	Plasma	Red blood cells	Buffy coat
	Normal mice		
No. mice	8	7	6
Mean LDH	396	44,800	174,440
S.E.M.*	± 112	± 4,350	± 32,200
	Infected mice		
No. mice	7	6	6
Mean LDH	2,142	42,560	162,680
S.E.M.*	± 97	± 2,970	± 19,500

* Standard error of mean.

Sample calculation of LDH value for buffy coat: Microblood collecting pipette = .00269 ml/mm. Length occupied by buffy coat 1.1 mm. The micropipette was broken carefully into 3 portions of measured length. The center portion (10 mm) contained red blood cells, buffy coat and plasma. Measured plasma LDH from upper segment was 2321 units/ml. Measured red blood cell LDH from lower segment was 39,480 units/ml. Measured LDH in center segment was 574 units in 10 mm. Correction for red blood cells (9% of center segment) and plasma = 96 units. Total LDH in center segment due to buffy coat = 478 units in 1.1 × .00269 ml = 161,541 units/ml of buffy coat.

Tissue levels of LDH enzyme in normal and LDH agent-infected mice are shown in Table IV. Skeletal muscle has the greatest amount of LDH enzyme (135,000 units per g). Total skeletal muscle LDH is thus approximately 1,500,000 units per 25 g mouse. Liver has the next highest level of LDH (126,000 units/gram), with a total liver pool of LDH of approximately 180,000 units. Spleen and heart are next with about 68,000 and 59,000 units per gram, respectively; their contribution to the body pool of LDH is about 7,500 units each. The other tissues contain considerably smaller amounts of enzyme.

For none of the tissues or cells tested were the LDH levels in mice infected with the LDH factor significantly higher than in the normal animal (Table VIII). The elevated plasma enzyme in infected mice is therefore not reflected in a corresponding elevation in

TABLE IV. Tissue LDH Levels in Normal and LDH Factor-Infected Mice.

Tissue	Normal mice No.	LDH, units/mg†	Total body LDH pool	LDH factor-infected* No.	LDH, units/mg†	Total body LDH pool
Brain	10	35.3 ± 5.2	13,800	7	29.7 ± 2.5	11,540
Heart	7	59.1 ± 7.0	7,400	6	59.6 ± 4.3	7,460
Kidney	7	44.8 ± 4.6	7,340	6	36.4 ± 3.7	5,800
Liver	10	126 ±13.8	176,000	7	107 ±13.6	150,000
Lung	7	34.2 ± 6.5	14,500	6	34.4 ± 5.6	14,600
Thymus	3	23.2 ±10.0	—	6	36.4 ± 4.3	—
Pancreas	6	16.8 ± 6.0	—	6	20.2 ± 2.6	—
Skeletal muscle	7	135 ±28.7	1,520,000	6	121 ±23.8	1,360,000
Spleen	10	65.8 ± 6.4	7,800	7	66.4 ± 5.0	7,850
Submaxillary gland	6	33.6 ± 8.3	—	6	40.0 ± 1.6	—

* In all infected mice plasma LDH levels were at least 5 times greater than in normal mice.
† Mean and standard error of mean.
Total body pool of LDH enzyme present in the particular tissue is obtained by multiplying mean tissue weight by mean LDH content.

any of the tissue tested. It can be calculated from the data given in Tables III and IV that the total additional enzyme in the plasma of infected mice (approximately 1000 units) could be provided by as little as 0.5% of total liver tissue or by 2.2% of the total red blood cells. However, without data on the clearance rate of LDH enzyme from the plasma, it is difficult to predict what degree of tissue necrosis or hemolysis might be required to maintain this elevated level. Liver tissue in infected mice shows no overt necrosis, but there is a small and highly significant difference in the hematocrits of normal and infected mice (Table V). White blood cell counts were not significantly different in normal and infected mice (Table V), and because of the small contribution these cells make to the total body pool of LDH, it seems safe to eliminate them as a possible source of the increased enzyme in plasma.

Despite the difference in hematocrit values, evidence that red blood cell hemolysis is not the source of increased plasma LDH has been obtained. Red blood cells contain high levels of certain other enzymes, particularly glutamic-oxalacetic transaminase (GOT) and malic dehydrogenase (MDH). Preliminary results indicate that the plasma levels of these enzymes are not evaluated in mice infected with the LDH factor. Unless the clearance rates for these 2 enzymes from plasma are very much greater than for LDH, therefore, it seems unlikely that hemolysis can account for the effects observed. Studies on clearance rates of plasma enzymes are in progress.

TABLE V. Hematocrits and White Blood Cell Counts in Normal and Infected Mice.

Type of mouse	No.	Mean hematocrit (%)*	No.	Mean WBC/mm³*
Normal	90	50.17 ±.52	16	8543 ± 773
Infected	80	47.14 ±.47	13	9430 ±1139
		$p << .001$		$p > .1$

* ± standard error of mean.

Although the thermal inactivation spectrum of the LDH agent has similar characteristics to the thermal inactivation of the enzyme itself, results obtained by Notkins *et al.*(4) indicate that it is unlikely that the enzyme activity is associated with the agent *per se*. Thus, these workers showed that the infectious titer in non-tumor-bearing mice was about 10,000 units for an increase in plasma of 1500 units of LDH enzyme per ml. The infectious titer in tumor-bearing mice carrying the factor was about the same, although the additional enzyme in plasma was about 40,000 units per ml. It is also unlikely that the agent acts directly to increase the activity of the plasma enzyme, or indirectly to activate a proenzyme in plasma, since we found no increase in plasma LDH enzyme when normal plasma was incubated with plasma bearing a high titer of the infectious factor. Attempts by us to duplicate

the action of the agent by releasing enzyme from cells in tissue culture (MBIII strain mouse lymphoblast) have also given negative results.

Summary. 1. An infectious agent similar to that described by Riley(1) has been demonstrated in 2 transplantable mouse tumors (CD/5 lymphoma and DBA/59 mammary carcinoma). The presence of the agent is detected only by a rapid rise in the plasma lactic dehydrogenase beginning about 29 hours following infection, reaching maximum levels in 9 days and persisting for up to 9 months. 2. Levels of the enzyme in brain, heart, kidney, liver, lung, pancreas, skeletal muscle, spleen, submaxillary gland, thymus and red and white blood cells were determined. No significant differences in any of these tissues were noted between normal and infected mice. 3. Hematocrit values were slightly but significantly lower than normal in mice bearing the LDH agent. These results are discussed in terms of possible sources for the increased level of plasma lactic dehydrogenase which results from infection with the agent.

1. Riley, Vernon, Lilly, F., Huerto, E., Bardell, D., Science, 1960, v132, 545.

2. Riley, Vernon, *ibid.*, 1961, v134, 666.

3. Notkins, A. L., Berry, R. T., Moloney, J. B., Greenfield, R. E., *Nature,* 1962, v193, 79.

4. Notkins, A. L., Greenfield, R. E., PROC. SOC. EXP. BIOL. AND MED., 1962, v109, 988.

5. Snell, George D., *J. Nat. Cancer Inst.,* 1953, v13, 1511.

6. Riley, Vernon, PROC. SOC. EXP. BIOL. AND MED., 1960, v104, 751.

7. Wacker, W. E. C., *New Eng. J. Med.,* 1956, v255, 449.

8. Wroblewski, F., LaDu, F. S., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 210.

9. Maile, John B., *Laboratory Medicine, Hematology,* 2nd Ed., C. V. Mosby Co., St. Louis, Mo., 1962, p804.

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Non-Enzymatic Dissolution of Fibrin "s" by Acidic Dyes, Surfactants, and Naphthalene Sulfonates.* (28611)

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We have found that many synthetic organic compounds induce marked fibrinolytic activity when dissolved in human plasma(1, 2,3). This activity is dependent upon the chemical structure of the compound(1,2). In addition, a factor is required which is present in human plasma or serum, but not in bovine plasma. The active compounds, however, do not induce dissolution of fibrin "s," the urea-soluble fibrin which is formed when, for example bovine fraction I is clotted by thrombin in the absence of calcium. Calcium is required for the function of the enzymatic fibrin stabilizing factor which converts fibrin "s" into urea-insoluble fibrin "i"(4). Preliminary observations indicated that minor molecular modifications, such as the introduction of a second hydrophylic group, deprived compounds of their ability to generate enzymatic fibrinolytic activity in human plasma, but endowed them with the new capacity to dissolve fibrin "s." This finding prompted an investigation of the relationship between the presence of strong hydrophylic groups in an organic molecule and its capacity to dissolve fibrin "s."

Material and methods. Preformed standard clots(5) were made from thrombin-clotted bovine fibrin, and the compound to be tested was dissolved either in human citrated plasma (variety III) or in buffered saline (variety IV)(6). The solutions were

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