The Distribution and in Vitro Propagation of an Agent Causing High Plasma Lactic Dehydrogenase Activity*

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SUMMARY

Experiments were carried out to investigate the extent of occurrence of a lactic dehydrogenase-augmenting agent in various types of tumors and its association with some known tumor-producing agents. It has been found that the agent was commonly associated with tumors which had been transplanted many times. Primary and transplanted tumors which were not exposed to exogenous sources of contamination did not show the presence of the agent. No association has been found between the agent and a variety of tumor-producing viruses. Lymphomas induced by Moloney virus showed the presence of the agent. However, tumors induced by the same virus, obtained from tissue culture, were free of the lactic dehydrogenase agent. The agent can be propagated and maintained apparently indefinitely by serial passages on primary mouse embryo tissue cultures.

Animals, and very often patients, with advanced cancer have abnormally high plasma lactic dehydrogenase (LDH) activity (11, 12, 14, 22). It has been reported recently that a transmissible factor has been detected in the blood of all animals examined bearing transplanted or spontaneous tumors. The factor which induced a sustained elevation of plasma LDH within 48 hours after injection into normal animals had many of the features of a virus and could be transmitted apparently indefinitely by serial plasma passage in normal adult mice. The association of this LDH-augmenting agent (abbreviated in this paper as LA) with a large variety of tumors, and its absence in the plasma of normal control mice, raise the question of the generality of this phenomenon and the possible etiological relation between the agent and the tumor (20).

In the experiments reported most of the tumors tested have been transplanted many times and could possibly be carriers of a "passenger" virus, unrelated to carcinogenesis. It is also possible that a virus endemic to the author's stock has been repeatedly activated or introduced into the experiments by technical procedures. Experiments were therefore undertaken to test the prevalence of such an agent in tumors of different origin, and the possible relation of LA to known carcinogenic viruses. In a preliminary study (24) a similar or identical agent was found in association with a few tumors with a long transplantation history. Primary tumors as well as transplanted tumors of recent origin, however, did not usually contain a detectable LDH agent. The extension of this study is reported here.

MATERIALS AND METHODS

Plasma LDH activity was determined by the procedure of Wroblewski and La Due (23).

Tissue culture technic.—Unless otherwise noted, cultures were prepared by trypsinization and plating of 18- to 19-day-old (C57BL × BALB/c)F1 embryos in 50-mm. Petri dishes. Eagle's Essential Medium (3) containing 10 per cent calf serum was used as the culture medium throughout the experiments. The cultures were kept in a 37°C water-jacketed incubator, in an atmosphere of air containing 5 per cent CO₂.

The presence of LA in the cultures was determined by injection of 0.05 ml. of medium into mice which were bled for LDH assay 48 hours later. In
some experiments, semi-quantitative assays were performed by injecting mice with serial dilutions of the collected medium. LDH levels induced by the agent were, without exception, far above the normal range—more than 1500 units, as compared with 150–600 units in untreated mice and in animals given injections of noninfective (LA-) material.

Leukemia was induced by Moloney leukemogenic virus obtained from Dr. H. Ginsburg and Dr. L. Sachs, to whom the author is grateful. Details concerning the tumors induced by this agent and methods of in vitro culture of the virus are described in references 6 and 7.

RESULTS

The occurrence of an LDH-augmenting agent.—In the first study, various tumors available in one of the laboratories at the Stanford Medical Center were tested for the presence of LA (24). The Ehrlich ascites tumor and Hepatoma 134 (1), which had been transplanted for some years before they were introduced into the laboratory, and a group of transplanted and primary tumors which originated and were kept in the same laboratory, were studied.

In each experiment plasma from one animal with an advanced tumor (or from an animal treated as indicated) was injected into a group of young, healthy mice. The LDH level of the recipients’ plasma was determined several times, beginning 2–3 days following the plasma injection. Whenever a significant rise was noted, plasma from the recipient was taken for determination of further transmissibility. Tumors containing a transmissible agent were called LA-positive. The results are summarized in Table 1. It can be seen that the two tumors showing a clear-cut association with an agent similar or identical to that described by Riley et al. were those introduced into the laboratory only after a very long transplantation history. On the other hand, with a single exception, LA was not detected in any of the several types of primary tumors or transplanted tumors originating in the same laboratory. The exception was a doubtful positive result obtained in one of the twelve experiments in which donors with primary radiation-induced leukemia

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Transplant generation</th>
<th>Host</th>
<th>Presence of LA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ehrlich ascites</td>
<td>&gt;100</td>
<td>C57BL</td>
<td>2/2</td>
</tr>
<tr>
<td>Hepatoma 134</td>
<td>&gt;100</td>
<td>C3H</td>
<td>3/3</td>
</tr>
<tr>
<td>SBZ Spontaneous sarcoma originated in an old (C57BL×C3H)F1 mouse</td>
<td>9</td>
<td>(C57BL×C3H)F1</td>
<td>0/2</td>
</tr>
<tr>
<td>Spontaneous sarcoma originated in an old (C57BL×BALB/c)F1 mouse</td>
<td>12</td>
<td>(C57BL×BALB/c)F1</td>
<td>0/3</td>
</tr>
<tr>
<td>Spontaneous lymphoma originated in old BALB/c breeder</td>
<td>11</td>
<td>BALB/c</td>
<td>0/3</td>
</tr>
<tr>
<td>LF Thymic lymphoma induced by C57BL leukemia virus in a (C57BL×BALB/c)F1</td>
<td>5</td>
<td>(C57BL×BALB/c)F1</td>
<td>0/2</td>
</tr>
<tr>
<td>Thymic lymphoma induced by C57BL leukemia virus in a (C57BL×BALB/c)F1</td>
<td>Primary</td>
<td>(C57BL×BALB/c)F1</td>
<td>0/3</td>
</tr>
<tr>
<td>AK leukemia</td>
<td>Primary</td>
<td>AK</td>
<td>0/2</td>
</tr>
<tr>
<td>Spontaneous mammary carcinoma</td>
<td></td>
<td>An old BALB/c breeder</td>
<td>0/1</td>
</tr>
<tr>
<td>Radiation-induced lymphoma</td>
<td>Primary</td>
<td>C57BL</td>
<td>1/3</td>
</tr>
</tbody>
</table>
were used: three out of nine mice given injections of plasma from a single donor developed high plasma LDH activity. In contrast to the other positive results in which all the recipients developed elevated plasma LDH activity within 48 hours, these three mice developed a high LDH level about 2 weeks after the plasma injection and two previous bleedings. There was, therefore, a possibility that these mice were infected through the experimental procedure. Plasma taken from these three animals exhibited full transmissibility and induced high LDH levels ($2 \times 10^3$) in all the mice given injections.

No association has been found between the LA and tumors induced by the AK leukemogenic agent (9) or C57BL leukemogenic agent (13), nor has it been found in animals given injections of active preparations of these viruses.

TABLE 2

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Transplant generation</th>
<th>Host</th>
<th>Presence of LA</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBL1/p. sarcoma induced by benzpyrene (BP)</td>
<td>&gt;100</td>
<td>C57BL</td>
<td>3/3</td>
</tr>
<tr>
<td>SBL4 sarcoma induced by BP</td>
<td>60</td>
<td>C57BL</td>
<td>0/2</td>
</tr>
<tr>
<td>B1</td>
<td>22</td>
<td>C57BL</td>
<td>0/2</td>
</tr>
<tr>
<td>CB</td>
<td>12</td>
<td>C3H</td>
<td>0/2</td>
</tr>
<tr>
<td>CB1</td>
<td>9</td>
<td>C3H</td>
<td>0/2</td>
</tr>
<tr>
<td>H1</td>
<td>4</td>
<td>C3H</td>
<td>0/2</td>
</tr>
<tr>
<td>Untreated C57BL</td>
<td>0/0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated C3H</td>
<td>0/0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL injected with normal plasma</td>
<td>0/0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* See footnote to Table 1.

Similar results were obtained when this study was repeated with tumors available in two laboratories at the Weizmann Institute of Science. The results obtained in one of these laboratories (Laboratory No. 2) are summarized in Table 2. This laboratory is engaged mainly in immunogenetic studies. All the tumors maintained now in this laboratory, except one, originated and were kept in the same laboratory. It was found that these tumors, some of which had been transplanted for more than 2 years, were LA-negative. The only LA-positive tumor, SBL1/p, was a subline of a tumor which had been induced by benzpyrene in this laboratory a few years ago (4), but has been reintroduced after it was kept for the last 3 years in another laboratory where some universal, long transplanted tumors (e.g., MC1A and Sa37) were also maintained (18).

The results obtained with tumors from the Moloney leukemogenic virus which had been maintained for some time in tissue culture, were LA-negative. It appears that the LA has been lost during the in vitro passage. It will be shown later that, though LA can be grown on primary tissue cultures, under certain conditions the agent disappears from infected tissue cultures as well as from LA-positive tumors grown in vitro.

The LDH elevation associated with tumor growth. —It has been reported (21) that following tumor grafting, plasma LDH increases rapidly, even before tumor growth is apparent. It has been suggested (20) that this elevation is due to the presence of LA. Since many transplanted tumors did not show the presence of such a transmissible agent, it was of interest to investigate the influence of such tumors on their host plasma LDH. Animals were grafted with various tumors, and their plasma was studied at different intervals. It
was found that animals with advanced tumors of both LA-positive and LA-negative types had high LDH levels, but the kinetics of elevation were different in the two groups (Chart 1). When LA-positive tumors were grafted there was a very rapid elevation within 48 hours, which reached 5–10 times the normal value. This elevation was identical to that described by Riley et al. as phases 2 and 3 (21). The same type of curve was also obtained when the mice were given injections of plasma of animals carrying LA-positive tumors (see Hepatoma 134 cells and Hepatoma 134 plasma, Chart 1). However, a completely different curve was obtained when animals were grafted with tumors of the LA-negative type (lymphoma cells, Chart 1). In this case, phases 3 and 4 were missing, and LDH activity was almost uninfluenced and remained for some time in the normal range. The elevation followed rather than preceded tumor progression. The LDH increase and the level depended greatly on the type of tumor (usually moderate with sarcomas and high with lymphomas). Similar results were obtained when plasma LDH elevation following a leukemogenic dose of radiation was studied.\footnote{H. S. Kaplan, K. Smith, and D. Yaffe, unpublished data.}

It appears that two independent processes are responsible for the LDH elevation associated with tumor transplantation:

1. A slow elevation caused by some as yet undetermined physiological changes associated with tumor growth, probably nonspecific cell destruction and tissue damage (23).

2. A sharp elevation caused by the transmissible agent (when present).

In vitro propagation of LA.—In agreement with the finding of Riley (20), potency of active plasma could be maintained for many serial passages in mice without detectable loss of activity. This activity could be transmitted by injection of doses as small as .05 ml. of $10^{-4}$–$10^{-5}$ dilution in saline. It could withstand 2 months of freezing at $-20^\circ$ C., lyophilization, and filtration through UF sintered glass filter, but was inactivated by heating for 30 minutes at $65^\circ$ C.

In order to obtain further evidence on the viral nature of the agent, an attempt was made to grow it in tissue culture. It was found that activity can be maintained and serially passaged in vitro. An illustration of a representative experiment is given in Chart 2. In this experiment a litter of embryos was trypsinized and plated, and after 1 day each of these cultures was inoculated with 0.05 ml. of

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Transplant generation</th>
<th>Host</th>
<th>Presence of LA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mol 10 /47. Lymphoma induced by MLV</td>
<td>47</td>
<td>C57BL</td>
<td>4/4</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>Primary</td>
<td>Swiss mice injected at birth with active preparations\footnote{Three different tissue culture lines.} of MLV which had been maintained for several weeks in tissue culture.</td>
<td>1/1</td>
</tr>
<tr>
<td>Mol 85H47. Lymphoma induced by MLV, maintained in tissue culture</td>
<td>1</td>
<td>8d-generation offspring of Swiss mice given injections of MLV derived from tissue culture (high incidence of leukemias). Swiss mice given injections at birth of normal mouse plasma.</td>
<td>0/3</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>Primary</td>
<td>DBA</td>
<td>0/2</td>
</tr>
<tr>
<td>MT1 sarcoma induced by polyoma virus</td>
<td>25</td>
<td>Injected with active preparation of polyoma virus obtained from tissue culture.</td>
<td>0/6</td>
</tr>
<tr>
<td>Closed line of a tumor induced by polyoma virus</td>
<td>7</td>
<td>Swiss</td>
<td></td>
</tr>
</tbody>
</table>

* See footnote to Table 1.

\footnote{Three different tissue culture lines.}

TABLE 3

<table>
<thead>
<tr>
<th>Presence of a Transmissible LDH-augmenting Agent in Plasma of Tumor-bearing Mice (Laboratory No. 3)</th>
<th>Tumor</th>
<th>Transplant generation</th>
<th>Host</th>
<th>Presence of LA*</th>
</tr>
</thead>
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<td>Primary</td>
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</tr>
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<td>Mol 85H47. Lymphoma induced by MLV, maintained in tissue culture</td>
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<td>7</td>
<td>Swiss</td>
<td></td>
</tr>
</tbody>
</table>
heparinized plasma taken from the heart of infected animals. The medium was changed twice a week, and cell layers were washed with saline before new medium was added. Four of these cultures (Experiment 613) were assayed at intervals until disintegration of the cultures due to overcrowding and shedding from the surface terminated the experiment. All the cultures were found to contain the active agent, and activity was maintained throughout the life of the cultures. It can be seen that, whereas the activity of the medium in the blank dish rapidly decreased, the activity of the medium from the culture remained high despite the twice-weekly medium changes. Injection into mice of medium collected from control uninoculated normal tissue culture did not cause LDH elevation.

In Experiment 614 one of the infected cultures was trypsinized 2 days after plasma inoculation, and the cells were washed twice and then replated. As shown in Part B of Chart 2, this treatment did not destroy the LA activity; the cultures continued to liberate the agent, in some instances with an increase in titer. However, after an additional trypsinization and cell passage, infectivity decreased and was eventually lost. That this loss of LA activity reflects some change in the culture rather than an inability of the infective agent to multiply continuously in vitro is shown in Part C of Chart 2 (Experiment 618). Here the infective agent was maintained by serial passages of medium. Every few days a new primary tissue culture was prepared and inoculated with 0.1–0.2 ml. of medium collected from the preceding passage. Under these conditions it has been possible to maintain LA activity more than 80 days till the termination of the experiment.

It was of interest that the infected cell cultures appeared morphologically entirely normal and were indistinguishable from control cultures which had either not been treated or inoculated with normal mouse plasma.

That the loss of LA activity in the cell passage experiment was due to change in the culture and not in the agent is further shown in Chart 3. In this experiment one litter of embryos was trypsinized, plated, and divided into four groups (a, b, c, d). Groups a and b were given inoculations of plasma containing LA. On the 7th day cultures of Groups b (infected) and c, d (noninfected) were trypsinized and replated. As can be seen in Chart 3, Group a, which has not been replated, preserved its LA activity till the disintegration of the cell layer, whereas Group b, which has been replated, lost the LA activity after being replated to secondary cultures.

Once the cultures lost their LA activity, further inoculations with LA-containing plasma did not resume LA activity of the cultures.

However, the fact that these cultures could not be reinfected with active plasma cannot be attributed to immunity due to previous contact with the agent, since the same type of apparent immunity to LA inoculation was manifested when groups c and d were inoculated as secondary (d) and tertiary (c) cultures. The nature of the loss of LA and the refractory stage which is developed just by replating the cultured cells awaits further investigation.

The disappearance of LA under certain tissue culture conditions may explain why some of the leukemias induced by Moloney leukemogenic virus obtained from tissue culture did not show...
Chart 2.—Multiplication of LDH-augmenting agent in tissue culture after inoculation with 0.03 ml. of plasma from mice carrying the agent. Titer of LA in medium was determined by injection of mice with serial tenfold dilutions of medium collected from tissue cultures.

Solid vertical bars, LA titer of tissue culture medium.
Open bars, titer of medium inoculated with active plasma without added cells. The medium in this Petri dish was not changed.

a. Inoculated primary tissue culture.
b. LA activity of medium collected during successive cell passages.
c. Maintenance of LA activity by serial medium passage in primary tissue cultures.

d. Medium passage (618)

Time in days

Chart 3.—Recoverability of LDH-augmenting agent from different tissue culture lines.
Presence of LA was determined by injection with medium collected from tissue cultures.

a, b, c, d = cultures derived from the same embryo tissue pool. Time of inoculation with infective plasma (LA+) and assay results as indicated.
LA activity (Table 3). It seems that a similar loss of LA occurred when Moloney leukemogenic virus was maintained by serial passage in tissue culture. In another experiment, an attempt was made to intentionally free an LA-positive tumor from the agent. A tumor with a long transplantation history—Hepatoma 134—was utilized. This tumor had been induced by carbon tetrachloride administration several years ago in the laboratory of Dr. H. B. Andervont (1) and has been maintained in the Laboratory No. 1 for the past 3 years. After it had been found that plasma of animals grafted with this tumor contain LA (Table 1), the tumor was excised from its host, trypsinsized, and plated in the same way as the embryo cultures. Infectivity of the tissue culture medium persisted for a few days and then disappeared. This loss of LDH-augmenting activity was not due to disappearance of malignant cells from the culture, as was indicated by the fact that, when a culture was trypsinsized on the 32d day and the cells were injected into 2-day-old C3H mice, all animals developed tumors within 15 days.

Three lines of tumors were independently established in this way. No LA activity could be found in plasma of mice grafted with tumors belonging to two out of the three lines. (These tumors are now in their fourth passage.) The third line was also initially noninfected (LA-negative) but regained LA activity in its second passage. No histological differences between the “cured” (i.e., without LA activity), and the original LA-positive tumor have been detected. The same loss of LA activity has also been observed with another LA+ tumor—SBL1/p (Table 2): after this tumor has been maintained in tissue culture for 25 days and then reinoculated into mice, an LA-negative subline of the tumor has been obtained. This subline is now on its fourth passage, and no LA activity could be found in its host’s plasma.

When the plasma LDH levels of mice grafted with the “cured” tumors were studied, it was found that the typical sharp phase of rapid elevation which was characteristic of the original Hepatoma 134 was absent. The elevation of LDH activity in these tumors was slow, followed tumor growth, and resembled the curve obtained with LA-negative tumors (see Chart 1, “cured” Hep. 134).

These results were expected in accordance with the assumption discussed previously concerning the two processes responsible for the observed LDH elevation curve.

DISCUSSION
The assumption of the viral nature of LDH-augmenting agent described by Riley et al. is further supported by the fact that it can be maintained and propagated in tissue culture. Despite the extreme dilution expected from serial in vitro passage, combined with twice-weekly washing and medium changing, for eight serial passages activity not only did not decrease but increased. The minimal effective dilution caused by the serial passage (not including medium changing) is more than $10^{-8}$. Although this investigation is preliminary and more direct morphologic and quantitative evidence is desired, it seems very unlikely that a nonself-reproducing agent could be responsible for these results. The disappearance of the agent from the cultures under certain experimental conditions is by no means any argument against this hypothesis. On the contrary—highly specific requirements are characteristic of many viruses.

However, the association of malignant tissue with a virus or virus-like particle is by no means a proof of any causative relationship. Tumors have been shown to be a good vehicle for viruses (2, 17 and others). Even when carcinogenic viruses were extracted from tumors, such as Moloney leukemogenic virus from Sarcoma 37 and Graffi and Friend viruses from Ehrlich ascites tumor (5, 8), it is probable that these tumors served only as “innocent” carriers for these viruses, which were picked up from a host some time in their long transplantation history.

No definite proof of the nature of LA has as yet been obtained. The association of such an agent with tumors was found to be of wide occurrence, but the finding that this agent occurs in all experimental tumors was not confirmed. All the data by now support the hypothesis that LA is a virus, not associated with the etiology of the malignant process and most probably a passenger of exogenous origin introduced into the tumor-host system by accidental infection. Had the agent been associated with the etiology of the malignant process, one should expect it to be present more frequently in primary tumors and transplanted tumors of recent origin than in old tumors. In fact, it was found mainly in tumors which had a very long transplantation history or which had some kind of contact with such tumors. Primary tumors and transplanted tumors which were kept under relatively isolated conditions were apparently free of the agent. No LDH-augmenting activity was found to be associated with any of the carcinogenic viruses tested. Although LA was found in animals which had been in contact with Moloney leukemogenic virus, it has been shown that it is an entity separable from the leukemogenic virus: After the material has been passed through tissue culture, LA-free leukemias were
obtained. A similar “curing” effect has been obtained also with Hep. 134 and SBL1/p, chemically induced tumors.

Since this manuscript was submitted, two additional papers on LDH-augmenting agent have been published (16, 19). The data in both papers are consistent with the data and interpretations presented here.

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REFERENCES

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