MULTIPLE ENZYME CHANGES IN THE PLASMA OF NORMAL AND TUMOR-BEARING MICE FOLLOWING INFECTION WITH THE LACTIC DEHYDROGENASE AGENT

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In search of a diagnostic tool for the early detection of cancer, many investigators have looked for differences in the plasma enzyme activity of the normal and tumor-bearing animal. Although alterations in plasma enzyme activity have been reported with a number of tumors these findings have not always been consistent. However, with certain transplanted tumors, there appeared to be a close correlation between tumor growth and the increase in the plasma activity of the enzyme lactic dehydrogenase (LDH) (1–4). Pertinent to this observation was the report by Riley (5) of a transmissible lactic dehydrogenase-elevating agent (LDH agent) which was found in the plasma and tissues of many tumor-bearing mice. Injection of this agent into normal mice produced a 5- to 10-fold increase in the plasma LDH activity of these animals within 72 hours. Although there was no evidence of tumor growth or gross pathology (except slight splenomegaly) in the recipients (6), the increased plasma LDH activity persisted for months and the LDH agent could be recovered and transferred serially to normal mice.

Initially, Riley studied 26 different types of mouse tumors and recovered a transmissible LDH agent from all of these tumors (5). Because of this high recovery, the relationship of the LDH agent to the tumor process was of considerable interest. Recently, however, studies from a number of laboratories have shown that not all tumors contain this agent (7–11). In addition, Notkins *et al.* (7) showed that the Moloney leukemia agent could be separated from the LDH agent by passage through rats without losing its ability to produce leukemia. This and other evidence suggested that the LDH agent was not integrally related to the tumor but was a contaminant which was being carried by many serially transplanted mouse tumors (7).

Because of the wide distribution and enzyme elevating capacity of the LDH agent, it was decided to reinvestigate the relationship between tumor growth and the increase in plasma LDH activity, employing a mouse tumor (SS-70429) which was

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free of the LDH agent. Similar experiments have also been reported by Riley (8). The studies from our laboratory (12, 13) showed that: (*a*) the plasma LDH activity of the infected tumor-bearing animal was not only many times higher than that of the uninfected tumor-bearing animal but was also several times greater than could be accounted for by simply adding the plasma LDH activity of the normal animal infected with the LDH agent to that of the uninfected tumor-bearing animal. (*b*) The early rise in plasma LDH prior to the actual appearance of the tumor was shown not to be due to the tumor but was the result of infection with the LDH agent. (*c*) There was a gradual increase in the plasma LDH activity of the uninfected tumor-bearing animal which paralleled tumor growth, but a transmissible agent was not associated with this elevation. From these studies it became clear that much of the increased plasma LDH activity previously thought to have been the result of tumor growth was in fact the result of contamination of the tumor with the LDH agent.

Since the LDH agent produced a major and long lasting increase in plasma LDH activity, it was pertinent to determine whether other enzymes were equally elevated or whether LDH was the only enzyme influenced by this agent. In addition, we wished to study the plasma activity of these enzymes in the infected tumor-bearing animal as compared to the uninfected tumor-bearing animal. The results of our experiments, reported below, show that other enzymes in addition to LDH are elevated and that quantitative differences in plasma enzyme activity do exist between the infected and uninfected tumor-bearing animal.

Materials and Methods

Animals.—CAF-1 and C_3H/HeN male mice 4 to 6 weeks old, bred at the National Institutes of Health, Bethesda, were used throughout the experiments.

Tumors.—Mammary carcinoma C_3HBA and plasma cell tumor SS-70429 both of C_3H origin were kindly supplied by Dr. Morris Barrett of the National Cancer Institute.

LDH Agent.—The LDH agent was originally isolated from the plasma of a mouse which had received the Moloney leukemia agent. At approximately weekly intervals thereafter, the LDH agent was passed by intraperitoneal injection of 0.1 ml of a 10^{-1} saline dilution of plasma from infected animals into 4 to 6 week old normal CAF-1 male mice. A plasma pool from the 7th serial passage of the LDH agent, P-7, was used in the earlier experiments. The plasma from the 8th serial passage, P-8, was used to prepare a pellet of the LDH agent, which was resuspended to original volume in Eagle's basal medium with 20 per cent veal infusion broth. The procedure described by Moloney to concentrate virus from plasma was employed to prepare the pellet (14). P-7 and P-8 were stored in individual vacuum sealed vials at −55°C.

Plasma for Enzyme Determinations.—Blood was obtained by orbital bleeding as described by Riley (15). Heparinized micropipettes were used to obtain 0.1 to 0.2 ml of blood from each mouse. The blood was centrifuged at approximately 1500 *g* for 15 minutes and the plasma was separated from the cells and kept at 4°C until assayed. Hemolyzed specimens were not used.

Enzyme Determinations.—Lactic dehydrogenase (LDH) was assayed as described by Wroblewski and LaDue (16). One unit of activity is defined as the amount of enzyme which produces a decrease in the optical density of reduced nicotinamide-adenine dinucleotide ($NADH_2$) at 340 mμ of 0.001 per 1 minute. Malic dehydrogenase (MDH) was assayed as described by Mehler (17). One unit of activity is defined as the amount of enzyme which produces a decrease in the optical density of $NADH_2$ at 340 mμ of 0.001 per 1 minute. Isocitric dehydrogenase

(ICDH) was assayed according to the method of Ochoa (18). The change in optical density resulting from the reduction of nicotinamide-adenine dinucleotide phosphate (NADP) was measured at 340 mμ. One unit of activity is defined as the amount of enzyme which produces an increase in optical density of 0.001 per 1 minute. Aldolase was assayed by the method of Sibley and Lehninger (19) as modified by Beck (20), except that the chromogen development reaction was shortened to 30 minutes. One unit of aldolase activity is defined as the amount of enzyme which will form 1 μM of triose phosphate from fructose-1,6-diphosphate in 15 minutes at 37°C. Assays were done on a Beckman DU spectrophotometer. Glutamic-oxalacetic transaminase (GOT) was assayed by the method of Steinberg, Baldwin, and Ostrow (21). One unit of activity is defined as the amount of enzyme which produces a decrease in the optical density of $NADH_2$ at 340 mμ of 0.001 per 1 minute at 25°C. Phosphohexose isomerase (PHI) was assayed according to the procedure described by Bodansky (22). The data is expressed in Bodansky units. Alkaline phosphatase was assayed as described by Garen and Levinthal (23). The increase in optical density resulting from the dephosphorylation of *p*-nitrophenyl phosphate was followed spectrophotometrically. One unit of activity is defined as the amount of enzyme which produces an increase in optical density at 410 mμ of 0.001 per minute.

All data were expressed as units of enzyme per ml of plasma. All assays were done on the Bausch & Lomb spectronic 505 except where noted.

PROCEDURES

CAF-1 male mice, 6 weeks old, were placed 4 per cage in 8 cages. On day zero, the 4 animals in each cage were bled and the plasma was pooled. Eight specimens were thus obtained. This pooling procedure was followed throughout the experiments. Several hours after obtaining these control specimens, one-half of the animals (cages 1 to 4) received 0.1 ml intraperitoneally (ip) of a 10^{-3} dilution of the LDH agent in 0.85 per cent NaCl and were labeled experimental animals. The remaining animals (cages 5 to 8) received 0.1 ml of 0.85 per cent NaCl ip and were labeled control animals. On days 3, 7, 14, and 21 the mice were bled and a plasma pool was obtained from each cage. Each pool was assayed for malic dehydrogenase, phosphohexose isomerase, and isocitric dehydrogenase. A second group of animals was set up in exactly the same way but the plasma pools from these animals were used to assay lactic dehydrogenase, glutamic-oxalacetic transaminase, alkaline phosphatase, and aldolase. The enzyme activity of the control and experimental pools were determined, averaged, and the per cent increase was calculated.

In the second experiment C_3H/HeN male mice 5 weeks old were divided into 6 groups of 24 animals each. On day 0, groups 1 and 2 received 0.1 ml of 0.85 per cent NaCl ip. Groups 3 and 4 received 0.025 ml of a 50 per cent SS-70429 tumor suspension in 0.85 per cent NaCl. This material was injected subcutaneously into the right flank. Groups 5 and 6 received 0.025 ml of a 50 per cent C_3HBA mammary tumor suspension in 0.85 per cent NaCl also subcutaneously in the right flank. Several hours after implantation of the tumors, groups 2, 4, and 6 were inoculated intraperitoneally with 0.1 ml of a 10^{-2} dilution of the LDH agent (P-8). On days 3, 4, 10, 11, 17, and 18 each animal was bled and four plasma pools were obtained from each group of 24 mice. Pools collected on days 3, 10, and 17 were used to assay lactic dehydrogenase, malic dehydrogenase, and isocitric dehydrogenase. The pools collected on days 4, 11, and 18 were used to assay aldolase. The arithmatic average of each group in the appropriate enzyme unit is reported.

RESULTS

Infection of Normal Mice with the LDH Agent.—Within 72 hours after injection of the LDH agent, there was an increase in the plasma enzyme activity of five out of the seven enzymes studied (Fig. 1). GOT, PHI, MDH, ICDH,

and LDH were elevated from 50 to 800 per cent over the normal controls which were assayed at the same time. The increase in the plasma activity of these enzymes persisted for the 21 days of the experiment. Infection of normal mice with the LDH agent produced a 500 to 800 per cent increase in LDH activity, a 400 to 700 per cent increase in ICDH activity, a 100 to 150 per cent increase in MDH and PHI activity, and about a 50 per cent increase in GOT activity. Two enzymes, alkaline phosphatase and aldolase, remained within the normal range. MDH, LDH, and ICDH assays performed on a group of animals which

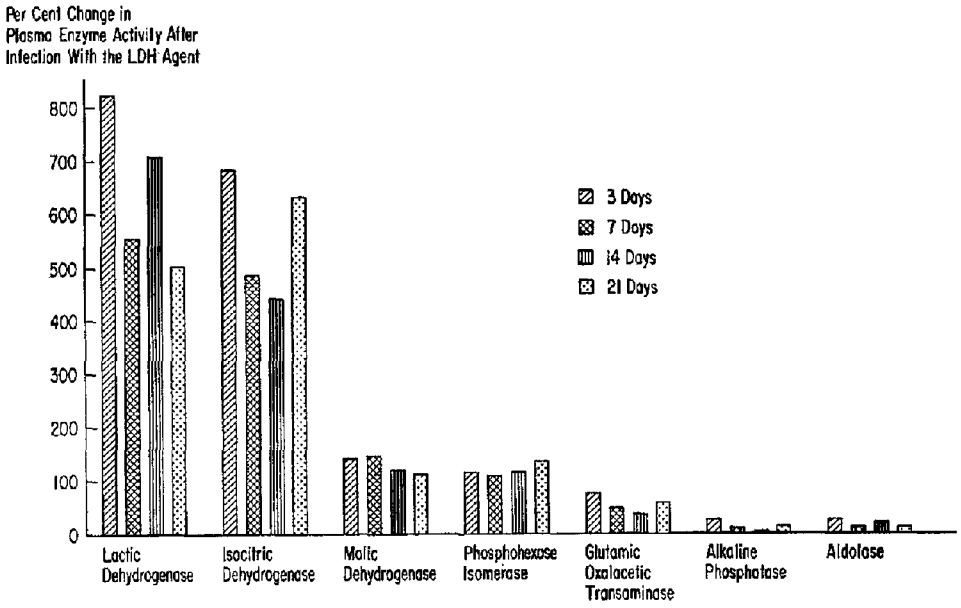

FIG. 1. Per cent increase in the activity of plasma enzymes of normal mice infected with the LDH agent.

had been infected with the LDH agent 7 months earlier showed that these enzymes were still elevated although to a slightly lesser extent than that seen in newly infected animals.

Infection of Tumor-Bearing Mice with the LDH Agent.—In the second experiment tumor-bearing animals with and without the LDH agent were followed for changes in plasma enzyme activity. On day 17 as seen in Fig. 2, the plasma LDH of infected mice bearing the SS-70429 tumor was increased 7-fold over that of animals with the same tumor but uninfected, while the plasma LDH of infected animals bearing mammary carcinoma C_3HBA was 2.5 times higher than its uninfected counterpart. As seen in Fig. 3, the plasma ICDH activity of animals with the SS-70429 tumor reached 8 times the level of its uninfected

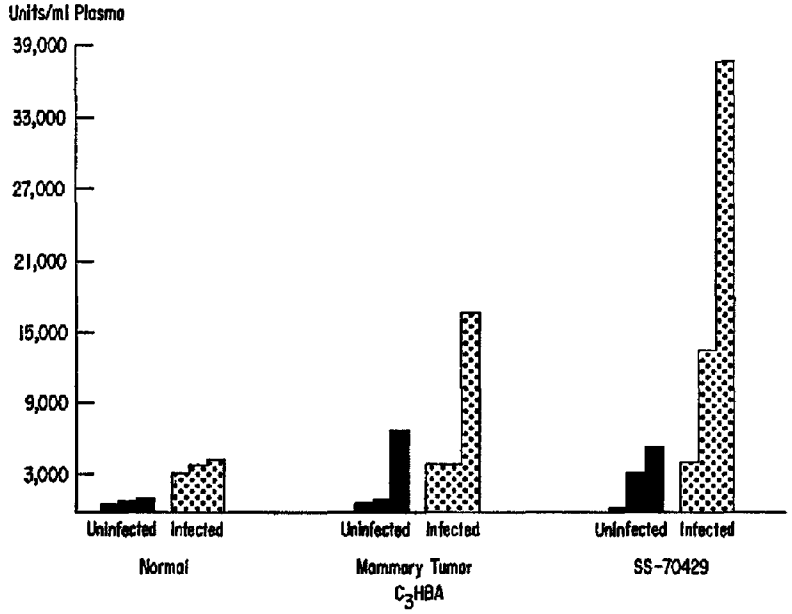

FIG. 2. A comparison of plasma lactic dehydrogenase activity in normal and tumor-bearing mice experimentally infected with the LDH agent. The bars within each group (left to right) correspond to days 3, 10, and 17.

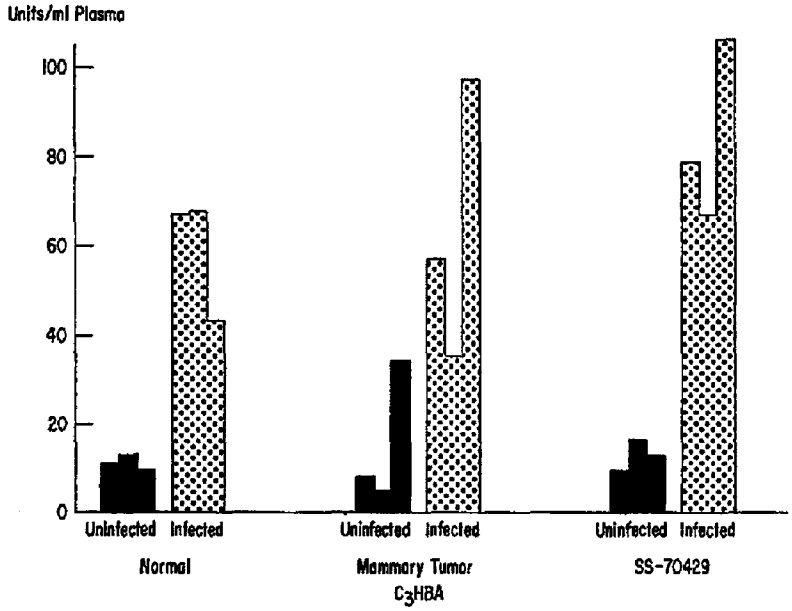

FIG. 3. A comparison of plasma isocitric dehydrogenase activity in normal and tumor-bearing mice experimentally infected with the LDH agent. The bars within each group (left to right) correspond to days 3, 10, and 17.

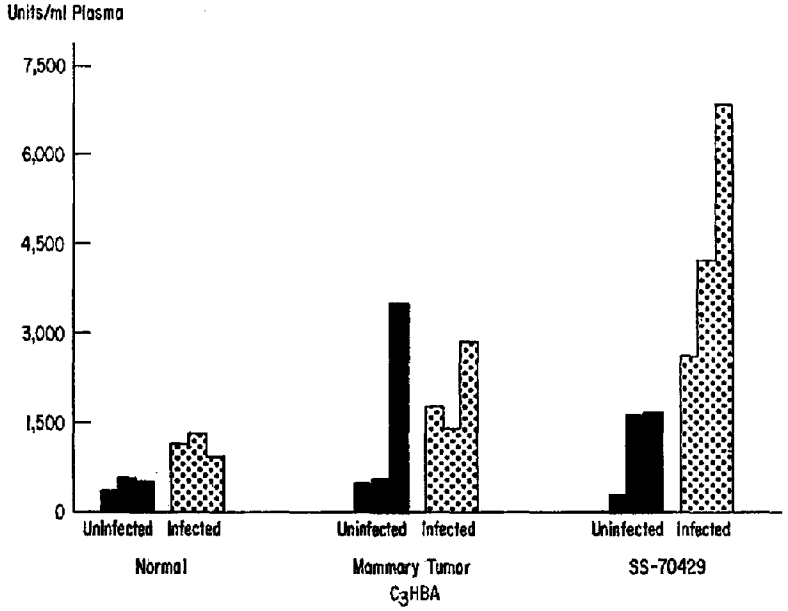

FIG. 4. A comparison of plasma malic dehydrogenase activity in normal and tumor-bearing mice experimentally infected with the LDH agent. The bars within each group (left to right) correspond to days 3, 10, and 17.

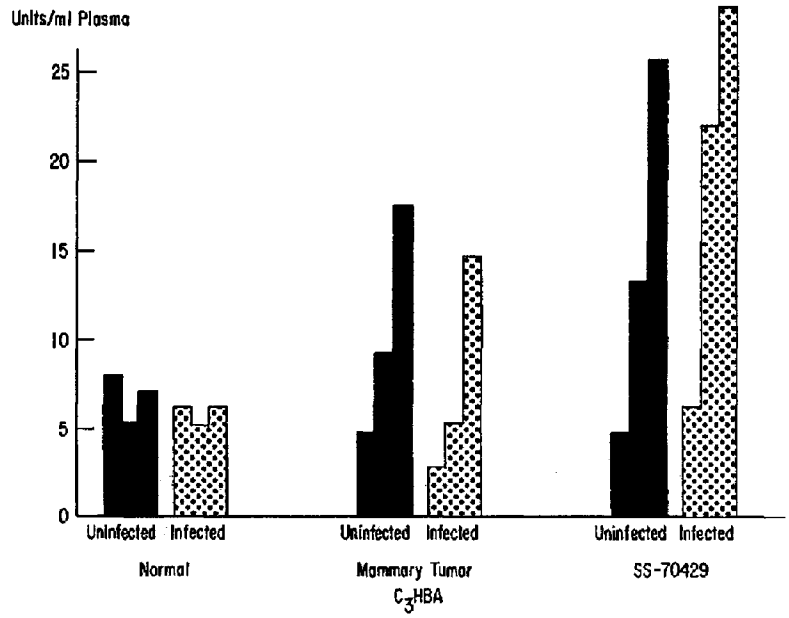

FIG. 5. A comparison of plasma aldolase activity in normal and tumor-bearing mice experimentally infected with the LDH agent. The bars within each group (left to right) correspond to days 4, 11, and 18.

counterpart. Plasma ICDH of infected animals bearing mammary carcinoma C_3HBA was 7 times above that of the uninfected tumor-bearing animal on day 10, but only 3 times higher on day 17. Plasma MDH, Fig. 4, from infected animals bearing SS-70429 reached 4 times the level of its uninfected counterpart. On days 3 and 10 the plasma MDH activity of the infected mice with mammary carcinoma C_3HBA was close to 3 times above its uninfected counterpart. However, on day 17, the plasma MDH activity of the infected and uninfected mammary tumor-bearing animal was about the same. The plasma aldolase activity, Fig. 5, of infected animals bearing either mammary carcinoma C_3HBA or plasma cell tumor SS-70429 did not differ greatly from that of its uninfected counterpart. However, this was not completely unexpected since the LDH agent did not produce an increase in the plasma aldolase activity of the normal animal.

Enzyme determinations on plasma from the uninfected tumor-bearing mice showed that none of the four enzymes studied (LDH, MDH, ICDH, and aldolase) were elevated on day 3. However, with the exception of ICDH from animals bearing the SS-70429 tumor, all of these enzymes were elevated by day 17. Transfer of plasma (0.1 ml ip of a 10^{-1} dilution) from these uninfected tumor-bearing animals to normal mice failed to produce an increase in the plasma enzyme activity of the recipients.

DISCUSSION

Riley has shown that infection of mice with the LDH agent produced a 5- to 10-fold increase in plasma LDH activity within 72 hours (5). The experiments reported above showed that, in addition to LDH, four other enzymes were elevated. The per cent increase in enzyme activity differed with each enzyme but was relatively constant for that enzyme over the 21 days of the experiment. Further studies showed that LDH, ICDH, and MDH were still elevated at 7 months but at a slightly lower level.

The mechanism by which the LDH agent produces the increase in plasma enzyme activity remains obscure. The two most attractive hypotheses are that the LDH agent (*a*) stimulates cellular enzyme production or (*b*) produces cell damage and subsequent release of enzymes into the circulation. Since the enzymes that were studied are found in most cells of the body, an agent or process which destroys or damages cells could produce an increase in plasma enzyme activity. Such an increase could also result from destruction of cells concerned with the clearance or catabolism of enzymes. However, the animals infected with the LDH agent showed no gross pathology, except slight splenomegaly (6, 24), and apart from the elevation of plasma enzymes could not be distinguished from the uninfected animals. Although gross pathology has not been reported, subtle changes in cell wall permeability could exist and permit enzyme leakage. The alternative to the cell damage hypothesis is that the LDH

agent acts on a genetic or metabolic level so as to stimulate enzyme production and subsequent release into the circulation. Increase in enzyme activity following virus infection has been reported in a number of situations including mouse fibroblasts in tissue culture infected with vaccinia (25), embryonate eggs infected with influenza virus (26), rabbit epithelium infected with the Shope papilloma virus (27), and bacteria infected with phage (28–30).

At the present over 40 types of transplanted mouse tumors have been reported to carry a transmissible LDH-elevating agent (5, 7–11). The widespread distribution of this agent makes it particularly important in the study and evaluation of enzyme changes in the tumor-bearing animal. In the above experiment, the plasma from uninfected and infected tumor-bearing animals were compared, and it was shown that the early increase in plasma enzyme activity (MDH, ICDH, LDH) was not due to the tumor but the LDH agent. However, by days 10 and 17 the uninfected tumor-bearing animals also began to show an increase in the plasma activity of these enzymes which paralleled the gross increase in tumor size. Transfer of plasma from these animals to normal recipients failed to reveal a transmissible enzyme elevating agent, thereby suggesting that the increase in plasma MDH, LDH, ICDH, and aldolase of the uninfected tumor-bearing animals was related to the growing tumor and was independent of the LDH agent.

As in previous experiments (12) the increase in plasma LDH activity of the infected tumor-bearing (SS-70429) animal was several times greater than that which could be explained by simply adding the plasma LDH activity of the uninfected tumor-bearing animal to that of the infected non–tumor-bearing animal. The experiments reported above demonstrate a similar, although less pronounced, increase in the plasma MDH activity of the infected tumor-bearing (SS-70429) animal. On the other hand, a more than additive increase in plasma enzyme activity was not seen with aldolase or substantiated with ICDH. In addition, animals with mammary carcinoma C_3HBA and infected with the LDH agent failed to show a more than additive increase with any of the four enzymes studied (LDH, MDH, ICDH, and aldolase).

In those infected tumor-bearing animals showing a more than additive increase in plasma enzyme activity, the tumor would appear to be the most likely source for these enzymes by providing additional cells (targets) upon which the LDH agent could act. However, many secondary changes are known to take place in an animal during tumor growth. The tumor may stimulate the production of certain non-tumor cells such as those of the reticuloendothelial system or immature blood cells (due to anemia), some of which may serve as target cells for the LDH agent. An increase in the number of these cells following tumor implantation, and not the tumor, may therefore be responsible for the more than additive increase in the plasma enzyme activity of the infected tumor-bearing animal. Thus, if a tumor such as SS-70429 directly or

indirectly provided additional target cells, infection of that animal with the LDH agent would result in a greater increase in plasma enzyme activity (LDH and MDH) than would be expected by adding the plasma activity of the uninfected tumor-bearing animal to that of the infected non-tumor-bearing animal. The lack of such an increase in plasma ICDH and aldolase may be due to a relatively low concentration of these enzymes in the target cells as compared to MDH and LDH. Furthermore, a tumor which did not provide additional target cells (the localized C_3HBA may be such a tumor) would not be expected to produce a more than additive increase in plasma enzyme activity when infected with the LDH agent. In addition to enzyme production and release, differences in the solubility and the rate of clearance of an enzyme from the circulation may be responsible for some of the observed plasma enzyme differences.

From the above experiments it can be seen that an unsuspected contaminant, carried by as many as 40 different types of mouse tumors, may have been responsible for much of the increased plasma enzyme activity seen in tumor-bearing mice and previously attributed to the tumor. In addition, Riley believes that this agent may also have some influence on the metabolism of the tumor (31, 32). Furthermore, Rausher and Notkins (33) and Rowe (34) have evidence that the LDH agent interferes with infection by other viruses and Yaffe (10) has succeeded in propagating the LDH agent in tissue culture. At present, this agent has been isolated only from mice but further studies on its host range are warranted.

SUMMARY

Within 72 hours after injection of the LDH agent into normal mice, five (LDH, ICDH, MDH, PHI, and GOT) out of the seven plasma enzymes studied were elevated. This elevation persisted for the duration of the experiment. Alkaline phosphatase and aldolase were not elevated.

Plasma from mice bearing tumor SS-70429 and infected with the LDH agent showed 7 times more LDH, 8 times more ICDH, and 4 times more MDH activity than the plasma from mice with the same tumor but uninfected. The plasma aldolase activity from the infected tumor-bearing animal was approximately the same as that from the uninfected tumor-bearing animal. Somewhat similar results, but lower in magnitude, were found with mice bearing mammary carcinoma C_3HBA.

The early rise in plasma enzyme activity (LDH, MDH, ICDH) prior to the actual appearance of the tumor was shown to be due not to the tumor, but to the LDH agent. Uninfected tumor-bearing mice showed a late increase in plasma enzyme activity which appeared to be related to tumor growth.

The findings reported above suggest that contamination with the LDH

agent may have been responsible for much of the increased plasma enzyme activity previously attributed to the tumor.

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