Prolonged Remissions of Lymphatic Leukemia in DBA/2 Mice Induced with Endogenously Produced Lactate Dehydrogenase Antibody

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SUMMARY

Induction of lactic dehydrogenase (LDH) antibody with porcine LDH in L1210 leukemia-bearing DBA/2 mice can induce remissions of the disease. The concurrent, limited use of a cytotoxic agent (azathioprine) in combination with LDH has been investigated and found to be advantageous in terms of obtaining a greater number of prolonged remissions (over 150 days) than does the use of LDH alone.

The protocols developed used 12 to 25 DBA/2 mice, inoculated with either 200 or 2000 L1210 tumor cells, per group. Animals were given injections of from 92 to 275 pmoles of LDH in adjuvant. Some groups received injections postinoculation only, others both pre- and postinoculation. Azathioprine (two injections) was added to some of the treatment regimens. Control groups comprised untreated L1210-inoculated animals, L1210-inoculated animals treated with azathioprine only, and animals treated pre- and postinoculation with ovalbumin (788 to 1480 pmoles) and porcine albumin (217 to 650 pmoles), respectively.

Untreated L1210-bearing mice die of leukemia 10 to 12 days postinoculation. Prolonged survival times (over 150 days) in the other groups ranged from 18% for azathioprine control groups, 0 to 10% for various albumin and albumin plus azathioprine combinations, 0% for LDH treatment given postinoculation; 0 to 43% for LDH given pre- and postinoculation, and 92% in one experiment in which azathioprine (two injections) was added to a pre- and post-LDH injection schedule. Survival rates in LDH experiments appeared to correlate with the pattern of anti-LDH engendered in a given experiment.

INTRODUCTION

While little is understood of the significance of lactic acid in malignant transformation, its formation from carbohydrate sources in the presence of an ample supply of oxygen is one of the most general and distinctive features of many malignant tissues (13, 15, 16). One of the authors (5) became interested in the possibility of inhibiting neoplastic tissue proliferation in vivo by parenteral administration of LDH some years ago. In vitro work by Ng and Gregory suggests that a unique immunological mechanism involving entry of anti-LDH into the tumor cell (9, 10) might make LDH a peculiarly suitable foreign protein with which to suppress the growth of malignant tissue. Porcine LDH was used to test these concepts in DBA/2 mice bearing L1210 ascitic lymphatic leukemias.

MATERIALS AND METHODS

LDH. The porcine muscle LDH isoenzyme was obtained from Boehringer-Mannheim (New York, N. Y.) suspended in glycerol:water, 50:50, 10 mg or 5000 IU/ml. This material was diluted for injection with 7.5% NaHCO₃ or 0.85% NaCl solution to yield concentrations of 133 IU of LDH activity per ml. This in turn was thoroughly mixed 1:1 with complete or incomplete Freund's adjuvant, Difco Laboratories, Inc. (Detroit, Mich.) with the aid of a VirTis homogenizer. One-tenth ml of the appropriate freshly mixed emulsion containing 91.5 pmoles LDH (or a multiple thereof) was given i.p. per injection.

Ovalbumin. This protein Baker (Detroit, Mich.) was dissolved in 0.85% NaCl solution and diluted appropriately so that 0.1 ml of final emulsion containing adjuvant was equivalent to 296 pmoles of ovalbumin. Injections, as with LDH, were given i.p.

Porcine Albumin. This protein Sigma Chemical Co. (St. Louis, Mo.) was handled similarly to the ovalbumin. Quantities administered varied from 650 to 1480 pmoles/0.1 ml injection.

Azathioprine. Two 50-mg tablets of azathioprine (Imuran; Burroughs Wellcome Co., Research Triangle Park, N. C.) were finely ground and suspended in 50 ml of 7.5% NaHCO₃ solution. Azathioprine was given i.p. in doses of 2 mg/injection. The volume of these, as of all injections, was maintained at 0.1 ml.

The L1210 lymphatic leukemia cell line was obtained courtesy A. D. Little Laboratories (Cambridge, Mass.). It
was maintained by transplantation through a series of DBA/2 mice. For inoculation of the experimental animals, ascites fluid containing approximately $1 \times 10^8$ cells/ml was counted on a Coulter counter and diluted with 0.85% NaCl solution to yield concentrations of 2,000 or 20,000 cells/ml. Viability of the tumor cells, as determined with a trypan blue supravital dye exclusion procedure, was maintained at the 98% level in all experiments. Experimental animals were inoculated i.p. with 0.1 ml suspension containing 200 or 2,000 cells.

Eight-week-old DBA/2 mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) and were fed ad libitum with Purina mouse chow throughout the experiment.

In 1 experiment, 99 8-week-old DBA/2 mice were distributed in groups of 5 to 6 animals into 19 plastic cages and allowed to acclimatize for 2 weeks. Five experimental groups were established, each of which received 200 L1210 cells as the tumor inoculation. These included: Group 1, a control group which received the L1210 inoculation but no further treatment; Group 2, which received 2 injections of azathioprine after inoculation with the L1210 line; Group 3, in which LDH treatment was started after inoculation with the tumor line; Group 4, in which LDH treatment was started 2 to 3 weeks prior to inoculation with the L1210 line; and Group 5, in which LDH treatment, started before inoculation with the tumor line as in Group 4, was supplemented by 2 injections of azathioprine shortly after introduction of the L1210 cells. A detailed protocol is shown in Table 1.

A 2nd series of DBA/2 mice received an inoculation of 2,000 L1210 leukemia cells. LDH for this experiment was diluted with 0.85% NaCl solution, rather than with bicarbonate. All 4 groups of animals in this series of studies were treated with LDH (or ovalbumin, in the case of the controls) both pre- and post-tumor cell inoculation. Pre-inoculation antigen injections were given on Days -24 and -16 in complete Freund's adjuvant and on Day -8 in incomplete Freund's adjuvant. Post-inoculations were all in incomplete Freund's adjuvant and were given on Days 1, 8, 16, and 24 where there were survivors. (As before, the day of the L1210 inoculation was labeled as Day 0.) No azathioprine was given in this experiment. Group 6 received 91.5 pmoles, Group 7 received 183 pmoles, and Group 8 received 274.5 pmoles of LDH per 0.1 ml injection; Group 9 received 296 pmoles of ovalbumin per 0.1 ml injection.

Finally, a similar series of control studies was undertaken, with porcine albumin in adjuvant as the foreign protein. As described above, most of the animals received injections both pre- and post-L1210 inoculation; the amounts of porcine albumin used varied from 217 to 650 pmoles/injection.

All animals were offered 2% NaHCO₃ in lieu of drinking water for 10 days after inoculation with the L1210 line. Blood was obtained from the animals periodically by orbital sinus puncture with heparinized capillary tubes, and the following parameters were monitored: count and morphology of the leukocytes, serum LDH levels, and serum LDH antibody development and maintenance. One hundred μl of blood for plasma antibody determinations were obtained from all animals on the day prior to the L1210 inoculation. Blood was also drawn for this purpose after inoculation, in the 1st experiment, on Days 7, 10, 16, and 44 after the L1210 injection. For the other parameters, an attempt was made to rotate the animals that were bled on any given occasion to avoid the development of anemia due to excessive bleeding.

Antibody was identified and an approximation of relative titers was obtained by utilizing nitro blue tetrazolium staining (8) of immunodiffusion plates. Each mouse serum was allowed to diffuse against 11 concentrations of antigen varying from 5 to 0.0015 μg LDH per μl.

Table 1
Treatment protocol used for the 1st series of DBA/2 mice
A 2% aqueous NaHCO₃ solution was substituted for drinking water for all animals from Day 0 through Day 10. In all cases the amount of LDH injected was 0.013 mg or 6.7 IU/injection. For further details, see text.

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* Controls.
* Numbers in parentheses, number of animals.
* Administered in complete Freund's adjuvant.
* Administered in incomplete Freund's adjuvant.
* With 200 L1210 ascites cells.
RESULTS

Survival data for both sets of animals have been collected in Table 2. All of the control animals of the initial experiment (Group 1) died between Days 10 and 11 after inoculation with the tumor line. Anti-LDH was not discovered in the serum of any of these animals. While prolonged remissions were not obtained in any of the mice in which LDH treatment was started after inoculation with the L1210 line, 17 of 20 of these mice lived at least 1 day longer than the controls, and 9 of 20 lived at least 2 days longer. Four survived through Day 13 and 1 survived to Day 21 of the experiment. Even by Day 9, none of these animals had sufficient antibody in their serum to be detectable under the conditions of the immunodiffusion test.

Prolonged remissions were obtained in 43% of the mice pretreated with LDH (Group 4). These animals were alive and apparently healthy some 150 days after the inoculation with the L1210 line. The introduction of 2 injections of azathioprine shortly after inoculation with the leukemia cells (Group 5) greatly potentiated the effect of the LDH treatment, increasing the 150-day survival rate of these animals to 92%. This represents a considerable improvement over the 18% of animals in which prolonged remissions were obtained with the use of azathioprine alone (Group 2).

The first check for presence of antibody in all of the animals was made 1 day before inoculation with the L1210 line. At this time, anti-LDH was demonstrable in all of the LDH-pretreated mice (i.e., Groups 4 and 5), and only in these animals. LDH antibody continued to be in evidence in animals in Groups 4 and 5 on Days 10, 32, and 66, with a suggestion that titers were maximal on Day 32 (16 days after the last LDH injection). There was no discernible difference between the antibody production of the 2 groups. In general, stained precipitin lines were found opposite antigen wells containing from $7.8 \times 10^{-4}$ to 1.25 $\mu$g LDH per $\mu$l although, occasionally, both higher and lower antigen wells also yielded the characteristic line.

For the first few days after L1210 inoculation, white blood counts remained relatively normal, i.e., 5,000 to 15,000/cu mm. As the leukemia develops, the WBC count rises gradually to between 10,000 and 40,000/cu mm. Shortly, i.e., within hours before death, WBC counts often rise dramatically to values near and over 100,000/cu mm. As expected, azathioprine depressed leukocyte counts, initially, in at least 1 instance, to as low as 2,600/cu mm. Toward the end of the 2nd week, this suppression is overcome and the count is in the normal range or, in animals in which the leukemia is developing, is elevated.

Differential blood smears were prepared utilizing a May-Grünwald-Giemsa stain. The acute lymphatic leukemia induced with the L1210 line is readily recognizable by the presence of large tumor cells. These are up to 3 times the size of the normal mouse lymphocytes. Large nuclei may or may not be segmented, and they frequently appear to fill almost the whole cell so that very little cytoplasm is seen.

Two or 3 weeks after inoculation, there was no evidence of tumor cells in the peripheral blood of surviving LDH-

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Table 2
Survival of DBA/2 mice\(a\) after inoculation with L1210 ascites cells

\(a\) Treatment: Group 1 (control), none; Group 2, azathioprine 2 times; Group 3, LDH 4 times postinoculation; Group 4, as in Group 5, plus azathioprine 2 times; Group 5, LDH 3 times pre- and 4 times postinoculation; Group 6, LDH (91.5 pmol/injection); Group 7, LDH (183 pmol/injection); Group 8, LDH (274.5 pmol/injection); Group 9 (control), ovalbumin (296 pmol/injection).

\(t\) Numbers in parentheses, number of L1210 cells inoculated.

\(t\) These animals were lost due to trauma associated with the injections or the bleeding procedure.

\(t\) This animal was lost to a volvulus apparently associated with neither the leukemia nor the treatment.
treated mice. At this time isolated intact tumor cells did remain in evidence among the mice treated with azathioprine only, and deaths, apparently due to the leukemia, continued to occur.

Antibody development in the 2nd series of mice was marginal; even with the use of highly sensitive staining procedure, only 60% of the animals of Group 6 showed LDH antibody on the day before inoculation, and titers were lower than those encountered in the previous experiment. All of the animals in Group 8 showed staining antibody at this time, in titers comparable to those found in the previous experiment.

Data for the rate at which prolonged survival times were obtained using porcine albumin as the foreign protein have been gathered in Table 3. Administration methods, i.e., the use of adjuvant, azathioprine, and injection schedules, all were analogous to those used with LDH. The best long-term survival rate obtained was 14%.

Base line (i.e., before any treatment) plasma LDH levels for our DBA/2 mice ranged from 90 to 185 modified Dade units per liter. The L1210 tumor line carries the Riley virus (14), which presumably accounted for increases in serum LDH levels to from 500 to 650 units/liter within 48 hr after inoculation with the tumor line. Subsequently, a comparison of LDH levels in Groups 4 and 5 (375 and 452 units, respectively) showed lower postinoculation plasma LDH levels precisely in those groups in which LDH antibody was demonstrable. Analogous results were obtained for Groups 7 and 8, when compared with Groups 6 and 9. Thus, while definitive proof is lacking and other interpretations remain possible, the data suggest that some cross-reactivity between antiporcine antibody and the host LDH existed.

There were no marked variations in weight which can be ascribed to either foreign protein treatment or the L1210 inoculation until shortly before death. In 1 series of experiments, weight averages at the beginning of the experiment (Day -30) were 22.1, 21.5, and 22.1 g for animals destined to be treated with LDH, albumin, and nothing, respectively. On the L1210 inoculation day (Day 0), the same group showed average weights of 24.4, 24.5, and 24.4 g, respectively. About the time the controls start to die (Day 10), the same groups showed weights of 25.0, 24.6, and 25.5 g, respectively. In the last 2 days or so before mice die of the leukemia, they eat less, but ascites frequently develops so that the weight loss due to lack of appetite tends to be balanced by the weight gain due to the ascites.

A summary of results, including a number of less successful LDH experiments, has been included in Table 4.

**DISCUSSION**

The protocol used in this study evolved from a series of preliminary unpublished experiments conducted with rabbit muscle LDH. The preferential use of porcine muscle LDH in the current work rested with the vehicle (glycerol: water for the porcine versus ammonium sulfate suspension for the rabbit preparation) rather than on any recognized intrinsic antigenic difference between the 2 preparations. During the earlier experiments, the principal points that were established included the following. (a) A slight acidosis develops in the L1210-inoculated animals, hence the introduction of the 2.0% NaHCO3 solution in lieu of drinking water. (b) No prolonged remissions were obtained, although there were modest increases of survival times. However, all of the initial experiments paralleled the Group 3 treatment recorded here, i.e., LDH treatment was begun only after inoculation with the L1210 line. It was judged that the virulence of the leukemia inoculation was such that there was insufficient time for development of adequate antibody production to deal with the tumor cells. This assumption appears to have been borne out by the Group 3 animals, which showed no significant antibody development by Day 9 and no prolonged remissions. (c) The original data prompted us to include a cytotoxic drug in some of the therapy regimens. Better survival times were obtained with the 91.5 pmole/LDH per injection dose adopted for Groups 3 through 6 than with one-third of this dose. In fact, the latter dosage of LDH did not yield any increases in survival time. From Groups 6 and 7, it is also apparent that increasing LDH dosages are increasingly effective within the framework of a given experiment.

The use of the LDH-M (muscle) isozyme is somewhat arbitrary. In the preliminary work, it was established that the overwhelming serum LDH of the DBA/2 mouse is LDH-3, whereas field mice display all 5 of the normally

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**Table 3**

Prolonged survival rate of DBA/2 mice inoculated with L1210 ascitic lymphatic leukemia cells and treated with porcine albumin in adjuvant

The effects of challenging the L1210 tumor line with a nonspecific protein used under conditions similar to those used with LDH are summarized below. As in the LDH experiments, the 1st 2 injections were given in complete Freund’s adjuvant and the balance, in incomplete adjuvant.

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encountered LDH isoenzymes. However, it has been suggested that anti-LDH-(M) does cross-react with the other isoenzymes of LDH, although progressively more weakly as the number of M-monomers in a given tetramer decline (3, 9). The action of other isoenzymes of LDH is currently under study.

One of the striking features of the control animals is that, for a given group of mice and a given L1210 preparation, death of all the animals comprising the group occurs within a 48-hr period. It is as a consequence of this that even modest increases in survival time, such as demonstrated by the Group 3 mice, become significant, and prolonged remissions, as demonstrated by Group 4, are striking.

The combination of azathioprine with LDH, as exemplified by Group 5, is somewhat less paradoxical if its cytotoxic as well as immunosuppressive capabilities are taken into account. Mathé (6) and others (1) who have been interested in the potential of an immunological approach to cancer have found inclusion of 6-mercaptopurines in their essentially immunological regimens for leukemia not only useful but mandatory. Mathé et al. (7) have postulated that the immunological but not the chemotherapeutic approach to cancer therapy can reach the very last tumor cells, provided the total number of tumor cells in the organism lies below a certain experimentally determined maximum. The crucial problem in dealing with a combination cytotoxic and immunotherapeutic regimen probably lies in the timing of administration of the 2 agents.

The immunological approaches to cancer have depended largely on attack by way of antigenic determinants in the tumor cell membrane (2). The speculation underlying the current work is that an intracellular enzyme, vital to the tumor cell, may be inhibited by the extracellularly formed LDH antibody. Support for the concept has been provided by the in vitro work of Gregory et al. (4) who have shown significant inhibition of anaerobic glycolysis in homogenates of rabbit liver, rabbit VX-2 carcinoma, and mouse Ehrlich ascites carcinoma, as well as inhibition of aerobic glycolysis in rabbit tumor homogenates by chicken antibody to LDH. With VX-2 tumor cells growing in vitro, both anti-LDH and sodium oxamate (a chemical inhibitor of LDH) significantly inhibited lactic acid formation and almost totally inhibited tumor cell reproduction (9). These authors, using indirect fluorescent antibody studies, were able to show that homologous anti-LDH was taken up and accumulated in active form within rabbit VX-2 carcinoma cells, mouse Ehrlich carcinoma cells, and BWS147 leukemic cells growing in vitro. Neither normal rabbit leukocytes, kidney and muscle cells, nor mouse kidney and muscle cells accumulated these antibodies (10). Also of importance to the work reported here is the discovery by Rajewsky (12) that a considerable part of rabbit antibodies to porcine LDH cross-react with the rabbit's own enzyme, both in vivo and in vitro. Our own serum LDH data are compatible with this finding.

Leukemia in some mouse strains, including DBA/2 mice, may be unusually sensitive to foreign protein reactions (7, 11). Nevertheless, in our hands, LDH seems to be a superior antigen against leukemia as compared to other foreign proteins such as ovalbumin and porcine albumin. It is evident from our data that all of the LDH experiments were not equally successful. There is a suggestion that survival rates in the LDH experiments correlated with the anti-LDH pattern engendered in any given experiment. Thus, in all cases in which a reasonable number (> 25%) of long-term survivors was observed, an antibody population which stained readily with tetrazolium blue obtained in 100% of the animals. In experiments in which a lesser percentage of long-term survivors was observed, the antibody production was poor and/or a nonstaining antibody appeared that has not been characterized otherwise to date.

In DBA/2 mice, then, LDH antibody induction can be used to obtain prolonged remissions of acute lymphatic leukemia. Whether this is an idiosyncratic phenomenon peculiar to DBA/2 mice or whether the findings reported here can be extrapolated to other species is the subject of further investigations in this laboratory.

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Prolonged Remissions of Lymphatic Leukemia in DBA/2 Mice Induced with Endogenously Produced Lactate Dehydrogenase Antibody

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