Differential Response of Friend Leukemia Virus and Lactate Dehydrogenase Virus to Chemotherapy and in Vitro Neutralization

P. S. Ebert, M. A. Chirigos, and P. A. Ellsworth
Viral Leukemia & Lymphoma Branch, National Cancer Institute, National Institutes of Health, and Microbiological Associates, Inc., Bethesda, Maryland

SUMMARY
Preparations of pooled Friend virus used in this laboratory were found to contain lactate dehydrogenase (LDH) virus. Interrelationships between these viruses and the effects of drug treatment in vivo and virus neutralization in vitro were studied. Both 6-mercaptopurine and streptonigrin administered to Friend virus-infected mice delayed the onset of the 10- to 100-fold normal elevation of plasma LDH seen in untreated animals. The suppressed LDH levels in the treated mice were accompanied by a three to four log reduction in Friend virus titer. Drug treatment did not reduce the titer of Friend virus-associated LDH virus in mice nor was it effective against a preparation of Riley's LDH virus. A preparation of Friend virus neutralizing antibody was tested for its ability to modify the typical Friend virus LDH response in vivo. A neutralized Friend virus preparation produced LDH levels identical to those of mice inoculated with LDH virus alone, whereas mice receiving non-neutralized Friend virus showed a 90-fold normal elevation of plasma LDH after 14 days. The Friend virus neutralizing antibody preparation, though effective against Friend virus, did not significantly reduce the titer of the LDH virus. The results suggest that chemotherapy and virus neutralization procedures delay or prevent Friend virus replication, but are ineffective against LDH virus. Friend virus was unable to modify the infectivity of the LDH virus.

INTRODUCTION
A virus which causes a sustained 10-fold elevation of plasma LDH2 above normal is associated with as many as 50 transplantable murine tumors and leukemogenic viruses (20). The LDH virus was shown to exert its enzyme-elevating activity by impairing the clearance of certain enzymes (2, 12, 17, 23). A synergistic 10-200 fold elevation of LDH in peripheral circulation has been reported to occur in mice carrying different neoplasias which are contaminated with LDH virus (1, 19, 20). The Friend virus pool in this laboratory was found to contain LDH virus. Experiments were designed to determine possible interactions between Friend virus and LDH virus and to observe the responses of the two viruses to drug therapy and to neutralization with specific antibody.

MATERIALS AND METHODS
Mice. Adult BALB/c male and female mice 4–6 weeks old were used for all experiments.
VIRUSES. Friend virus which induces polycythemia in mice was supplied by Dr. J. B. Moloney, National Cancer Institute (see Reference 15). The virus was passaged and inoculated according to procedures described by Chirigos et al. (7). The designation “LDH virus” refers to that virus naturally occurring in the Friend virus pool. LDH virus (Riley) was kindly supplied by Dr. V. Riley, Sloan-Kettering Institute, N. Y. LDH virus (Riley) stock (109.7 ID50/ml) was diluted with Eagle's MEM containing 20% veal infusion broth (18), and 0.2 ml of diluted virus was inoculated i.p. into mice for observations of LDH levels and to provide plasma for LDH virus titer determinations.

Drugs. Streptonigrin (NSC-45383) 0.3 mg/kg; and 6-mercaptopurine (NSC-755) 60 mg/kg.

Drug Treatment. Drugs were administered at 0.01 ml/gm of body weight s.c. in the scapular region. Mice were treated once daily for four consecutive days beginning on the fourth day following virus infection.

Friend Virus and LDH Virus Titer Determinations. Reduction of Friend virus titer between drug-treated and infected control mice was determined by the spleen weight assay of Chirigos et al. (7). LDH virus titers were determined by the method of Notkins and Shochat (18). Plasma obtained from 3–5 donor mice was serially diluted and each dilution inoculated into a group of six mice.

LDH Assay Procedures. Plasma samples for LDH assays and for determination of LDH virus titers were obtained from mice by the orbital bleeding technique of Riley (22). Hemolyzed plasma samples were discarded. Plasma LDH was determined essentially as described by Wroblewski and LaDue (25). To initiate the reaction pyruvate was added to a cuvet containing NADH2, phosphate buffer, and 40 μl or dilutions of plasma
held at 29°C for five minutes. The rate of decrease in optical density was observed at 340 mμ and 29°C in a Gilford recording spectrophotometer for 30 to 90 seconds. Plasma samples showing decreases in optical density outside of the range of 0.05 to 0.1 O.D. units per minute were rediluted. Each daily series of readings included multiple determinations of an LDH standard employed to correct experimental values for differences in the amount of inhibitor in various lots of NADH. One unit of LDH activity was defined as a change in optical density of 0.001 per minute at 29°C.

Neutralization of Friend Virus by Antibody. Ascitic fluid containing neutralizing antibody against Friend virus (IAF) (13) was employed for neutralization experiments. The ability of this antibody preparation to inhibit replication of Friend virus at various stages of viremia has previously been reported (6). Ascitic fluids (IAF and NAF) were incubated one hour at 56°C, then for 24 hours at 37°C to inactivate LDH virus. Penicillin (100 units/ml) and streptomycin (100 units/ml) were included to prevent bacterial growth. Stock Friend virus was diluted 1:5 with 0.05 M sodium citrate and then combined with equal parts of ascitic fluid from immunized or nonimmunized mice, or with fetal calf serum to provide a Friend virus control. LDH virus (Riley) was diluted appropriately so that the titer would be similar to the LDH virus titer of the Friend virus pool. Ascitic fluids were combined with equal parts of citrate to be tested in vivo for possible residual LDH virus contamination. Antibody-virus mixtures, antibody alone, and LDH virus (Riley) were incubated for one hour at room temperature and 0.2 ml aliquots of each were immediately injected i.p. into mice for subsequent determination of LDH levels. Inocula were also bioassayed for Friend virus and LDH virus titers.

RESULTS

Chart 1 shows the effect of streptonigrin and 6-mercaptopurine treatment on the plasma LDH levels of Friend virus-infected mice. Friend virus-infected mice showed progressive increases of LDH activity for 21 days. Treatment of infected mice with streptonigrin resulted in plasma enzyme levels which remained constant at approximately 5000 units until the 21st day. 6-Mercaptopurine therapy caused enzyme activity to remain at the same level only until the 14th day at which time LDH activity began to increase rapidly. Differences in plasma enzyme activity between infected control mice and infected mice receiving either 6-mercaptopurine or streptonigrin were significant at eight days after infection (P < 0.02) and highly significant at 11 and 14 days after infection (P ≤ 0.001).

Changes in the Friend Virus and LDH Virus titers as a result of drug treatment are shown in Table 1. Drug therapy caused a reduction of 2.9 to 3.9 logs in Friend virus titers until the 11th day after virus inoculation. By Day 14 the virus titers had increased to within one log of infected control mice. Bioassay of the Friend virus pool employed for inoculation of donor mice confirmed the presence of LDH virus. The LDH virus titer in Friend virus-infected mice remained essentially constant (ID₅₀/ml of 10⁻⁷.₆⁻¹.₈) from Day 4 through Day 14. Bioassays for LDH virus in plasma on Days 8 and 14 indicated that

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Effect of chemotherapy on Friend and LDH virus titers of Friend virus (FV)-infected mice.

* Treatment started four days after virus inoculation and was given daily for four days.

+ On the indicated day plasma was collected from five donor mice in each group of infected animals and injected into groups of five female recipient mice for subsequent determination of spleen weights after 14 days.

+ Spleen weight response of mice inoculated with undiluted donor plasma.

+ The log reduction of titer was estimated by comparing the mean log spleen weight of recipient mice sub-inoculated with drug-treated donor plasma to the nontreated control standard curve (7).

+ 0.2 ml of a 10⁻¹.₃ dilution of Friend virus stock was inoculated i.p. into donor mice. The LDH virus titer was determined on this dilution of virus.

treatment with either streptonigrin or 6-mercaptopurine did not depress LDH virus titers. Since, in a duplicate experiment, equal virus titers were obtained for infected and streptonigrin-treated mice on Day 14, the difference of 0.5 log ID₅₀/ml of virus between these two groups is not considered to be significant.

Although the plasma LDH levels in Friend virus-infected mice were suppressed with therapy (Chart 1), the consistent LDH virus titers obtained during treatment did not indicate any suppression of LDH virus replication. It was then considered necessary to determine the susceptibility of LDH virus alone to chemotherapy. The effect of drug treatment upon LDH levels of LDH virus (Riley)-infected mice is shown in Chart 2. Four treatments with streptonigrin or 6-mercaptopurine at
Chart 1. Effect of chemotherapy on lactate dehydrogenase levels in Friend virus-infected mice. Three groups of mice were inoculated with Friend virus and one additional group received no virus. On the 4th through 7th day after virus inoculation one group of infected mice received single daily doses of 6-mercaptopurine s.c. and a second group was similarly treated with streptomycin. Normal mice received no drug. Each point in the chart represents the mean of 5 samples of plasma.

Chart 2. Effect of chemotherapy on lactate dehydrogenase (LDH) levels in LDH (Riley)-infected mice. Three groups of mice were inoculated with 0.2 ml of a 10^-6 dilution of LDH virus (Riley) stock. Drug treatments were the same as those described in the legend to Chart 1. Each point represents the mean LDH activity of 5 plasma samples.

doses which significantly reduced Friend virus titers (Table 1) failed to reduce the enzyme levels in LDH virus (Riley)-infected mice as compared to controls. After 14 days, enzyme activity of the 6-mercaptopurine-treated mice showed a transient elevation above untreated controls. The difference between the activities of the two groups of mice was not statistically significant and, in addition, a duplicate experiment did not show a similar elevation.

The effect of chemotherapy on LDH virus (Riley) titers in this same experiment is shown in Table 2. LDH virus (Riley) caused little or no splenomegaly. In agreement with the observations on the plasma LDH levels, the LDH virus titers in the drug-treated mice were not reduced below control levels on either Day 8 or 14.

To verify the observation that increased rates of Friend virus replication could be detected in the presence of LDH virus by monitoring LDH activity, Friend virus was incubated in vitro in the presence or absence of IAF and the mixtures were subsequently inoculated into mice. Chart 3 shows the progression of LDH activity in groups of donor mice which were injected with aliquots of the six different inocula. Groups of mice receiving Friend virus, Friend virus plus IAF, Friend virus plus NAF, and LDH virus (Riley), respectively, showed identical LDH activities until the sixth day after inoculation. Beginning at the sixth day, typical LDH synergism appeared in mice rejected with Friend virus inoculum and with Friend virus plus NAF inoculum. The neutralized Friend virus preparation did not produce a synergistic elevation of LDH levels, but rather showed only the 10-fold above normal level typical of LDH virus (Riley) infection. This observation showed that Friend virus antibody specifically neutralized Friend virus without affecting associated LDH virus. IAF and NAF preparations alone caused no increases in plasma LDH above normal levels, indicating that these preparations contained no residual LDH virus.

The results of Friend virus bioassays showed that the preparation containing Friend virus antibody exhibited a 3.8 log reduction in virus titer compared to the Friend virus control inoculum. The data therefore indicate that the Friend virus antibody significantly reduced the Friend virus titer during the in vitro incubation. In contrast, the Friend virus incubated with fluid from nonimmunized mice showed a titer similar to that of the Friend virus inoculum.
Chart 3. Effect of inoculation of neutralized Friend virus upon plasma lactate dehydrogenase (LDH) activity. Each point in the chart represents the mean LDH activity of 5 plasma samples from mice infected with the appropriate virus-antibody mixture. IAF, immune ascitic fluid containing antibody to Friend virus. NAF, normal ascitic fluid from nonimmunized mice.

Table 3

<table>
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<th>Test inoculum after incubation for 1 hr at 35°C</th>
<th>LDH virus titer (ID50/ml log 10)</th>
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<td>Friend virus</td>
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</tr>
<tr>
<td>Friend virus + IAF</td>
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<td>Friend virus + NAF</td>
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<tr>
<td>LDH virus (Riley)</td>
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<tr>
<td>IAF</td>
<td>0</td>
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<td>NAF</td>
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Lactate dehydrogenase (LDH) virus titer of Friend virus inoculum neutralized in vitro with immune ascitic fluid (IAF). Following the 1 hour incubation of inocula at room temperature each preparation was either inoculated directly or diluted as described in Materials and Methods and the LDH virus titer determined (18). NAF, normal ascitic fluid, from nonimmunized mice.

DISCUSSION

Though enzyme elevation seems to be a characteristic peculiar to the LDH virus and not the Friend virus per se (11), it appears that the combination results in a synergistic and progressive elevation of LDH activity. Drug-treated Friend virus-infected mice showed an absence of a synergistic enzyme response as well as a reduction in Friend virus titer. LDH levels in these mice were typical of levels observed during LDH virus infection (Charts 2, 3). Apparently the two drugs specifically suppressed the sites of Friend virus replication and, as a result, also prevented the synergistic elevation of LDH. The lack of effect of the drugs on the LDH virus titer of the Friend virus preparation, as well as that of the LDH virus (Riley), was reflected also by the 10-fold normal level of LDH exhibited by these groups of treated mice. The data suggest that the two drugs, known to decrease Friend virus replication (7), do not affect the accompanying LDH virus.

Results in Chart 1 and Table 1 show that Friend virus titers in drug-treated mice approached levels of nontreated virus control animals before any change was noted in the LDH synergistic response. Some lag in the expression of increased LDH activity is not surprising in view of the fact that a peak of enzyme activity in the plasma of LDH virus-infected mice does not appear until three days after the appearance of the titer peak and the onset of enzyme accumulation (18). However, the delayed synergistic response observed in the 6-mercaptopurine-treated group did not appear in the streptonigrin-treated group within the observation period. A possible explanation for this difference may be that the retarded splenomegaly observed in the streptonigrin-treated mice may have delayed the splenic rupture and hemorrhage which occur in more enlarged spleens.

Normal mice were treated with 6-mercaptopurine and streptonigrin to show that the elevated plasma LDH observed following treatment of Friend virus-infected mice with high doses of drugs (Chart 1) was not due to damage to host cells. No apparent increases in plasma LDH were observed. However, it must be noted that slight increases in the release of endogenous LDH due to drug toxicity may not be observed readily because of efficient enzyme clearance in normal animals. Chart 2 showed the effect of drug treatment upon mice which exhibited impaired enzyme clearance. Again, despite the use of mice which should be more sensitive to increases in the release of endogenous LDH, no significant increases of plasma LDH were noted following drug treatments.

The source of LDH accumulating in the peripheral circulation of Friend virus-infected mice is difficult to establish. Notkins (19) and Riley (24) have suggested that the synergistic elevation of plasma LDH in tumor-bearing or *Eperythrozoon coccoides* -infected mice also infected with LDH virus was due to an elevated influx of enzyme resulting from damaged tissue and red cells. The early stages of Friend virus infection are characterized by erythropoiesis, erythrocytosis, and splenic rupture (3, 14, 16). Increased erythrocyte destruction within the first 48 hours after infection also has been observed (4). Hemorrhage in the spleen and rupture of red cells with resultant release of intracellular LDH could be responsible for the synergistic accumulation of LDH appearing in the circulation of Friend virus-infected mice after the impairment of clearance is established by the LDH virus infection.

Though both Friend and LDH viruses have been shown to replicate within organs of the reticuloendothelial system, a differential effect was seen to occur as a result of drug treat-
ment. Chirigos and March (5) have shown that the decrease in Friend virus titer following treatment with drugs or total-body X-irradiation was the result of a reduction in the number of infected spleen cells replicating virus, and that the spleen is the primary organ involved in Friend virus replication. du Buy and Johnson (8) reported that the LDH virus titer in the plasma of X-irradiated mice was similar to that of control mice, suggesting that radiation-sensitive cells such as those contained in the spleen were not target cells of this virus. Macrophages, which are relatively radiation-resistant, are probable host cells for LDH virus (8, 10). Since drug treatment reduced the plasma virus titers of Friend virus but not LDH virus, the data are compatible with the finding that drug treatment reduced the number of host spleen cells in which Friend virus replicates.

No apparent dependence of the LDH virus upon the Friend virus appears to exist. LDH virus titers remain the same in the presence and absence of Friend virus; thus the Friend virus apparently cannot modify LDH virus titers. In addition, a differential effect on the two viruses was produced both by drug treatment and virus neutralization with no effect upon the LDH virus titer. Plagemann and Swim (21) observed no differences in the growth rate and transplantability of four tumors in normal mice and in mice additionally infected with LDH virus. The LDH virus apparently exists as a benign contaminant. Whether the Friend virus requires the LDH virus to manifest its pathogenicity is at present undetermined. Although Friend virus (containing LDH virus) readily elicits early increases in spleen weight in mice, the LDH virus alone has negligible ability to induce splenomegaly (20). Preliminary experiments with rat-passaged Rauscher virus free of LDH virus indicate that recombination with LDH virus did not restore the property of the Rauscher virus to promote early splenomegaly in mice or induce greater than 10-fold normal elevations of plasma LDH (unpublished data).

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REFERENCES

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