# Enzymatic and Immunologic Alterations in Mice Infected with Lactic Dehydrogenase Virus

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In 1954, Sigurdsson introduced the term *slow infections* to describe several chronic diseases in sheep.[1] These infections were characterized by a long latent period, lasting from a few months to several years, followed by a protracted illness resulting in severe disease or death. Subsequently, a number of viruses have been classified under the heading *slow viral infections*. These included lymphocytic choriomeningitis virus, Aleutian mink disease virus, the murine leukemia viruses, scrapie, lactic dehydrogenase virus and a number of others.[2] In general, these viruses have been poorly characterized and many have been difficult to study in tissue culture because they failed to induce a cytopathologic effect. Recently, however, several of these viruses have been studied in greater detail, and the accumulated evidence suggests that the term slow infection may be a misnomer. In fact, certain of these viruses replicate very rapidly. The latent period may be hours rather than months or years, and subtle pathologic changes can be detected early during the course of infection. The late manifestations of the disease also may be subtle and in certain cases may be immunologically induced. Thus, the concept of slow infections, as originally defined, was extremely narrow in scope and needs extensive modification to encompass new findings and concepts. In fact, the terms *persistent* or *recurrent viral infections* might be more applicable to this area of virology than is the term slow infections.

In our laboratory, we used lactic dehydrogenase virus (LDV) as a model for studying persistent viral infections. The purpose of this talk is to review briefly what is known about LDV, to discuss some of the factors involved in the persistence and pathogenesis of this infection, to show how the unsuspected presence of LDV might have interfered with experimental results and to report some of our very recent findings on the replication of this virus *in vitro*.

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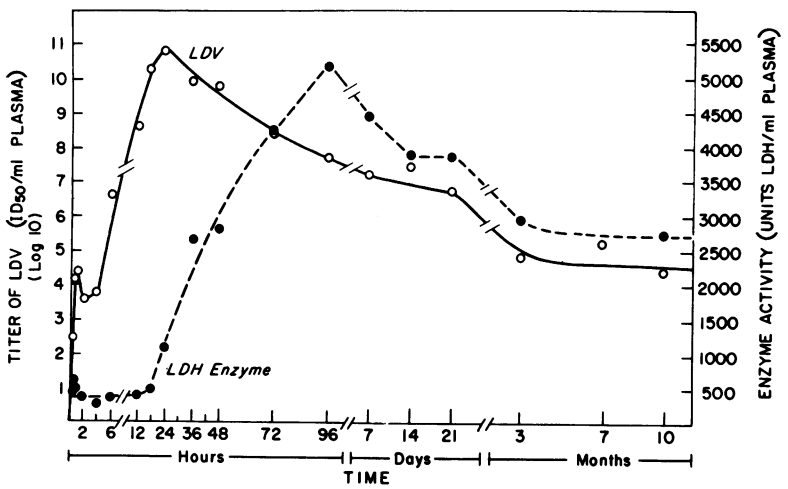
LDV is an ether-sensitive RNA virus.[3] It is about 40 mμ in diameter and has a density in sucrose of 1.17 g/ml. The natural host is the mouse, and the virus replicates in macrophages. Infection of mice with LDV results in a five- to tenfold increase in the activity of plasma lactate dehydrogenase (LDH). The increase in enzyme activity serves as the basis for detecting the virus, and the virus titer is determined by injecting serial tenfold dilutions of the virus into recipient mice and calculating the dose that infects 50% of the animals ($ID_{50}$). Text-fig 1 represents a typical growth curve.[4] Virus replication began within 6 hours after infection and reached $10^{10.8}$ $ID_{50}$/ml at 24 hours. The titer dropped over the next 72 hours but remained at about $10^{5.0}$ $ID_{50}$/ml for the rest of the animal's life. Despite the extremely high virus titer during the acute phase of the infection, the animals appeared perfectly healthy. The enzyme elevation was highest at 96 hours postinfection and remained at five- to eightfold above normal for the rest of the animal's life.

The fact that the LDH activity was elevated suggested that other plasma enzymes also might be elevated. Table 1 shows that the activity of isocitric dehydrogenase was elevated to about the same extent as that of LDH, whereas the activities of malic dehydrogenase, phosphohexose isomerase, glutamic oxalacetic transaminase and glutathione reductase were elevated to a lesser extent. Activities of aldolase, alkaline and acid phosphatase, leucine amino peptidase, glucose-6-phosphate dehydrogenase and α-glycerophosphate dehydrogenase were not elevated.

Next, we attempted to study the mechanism of enzyme elevation.[5]

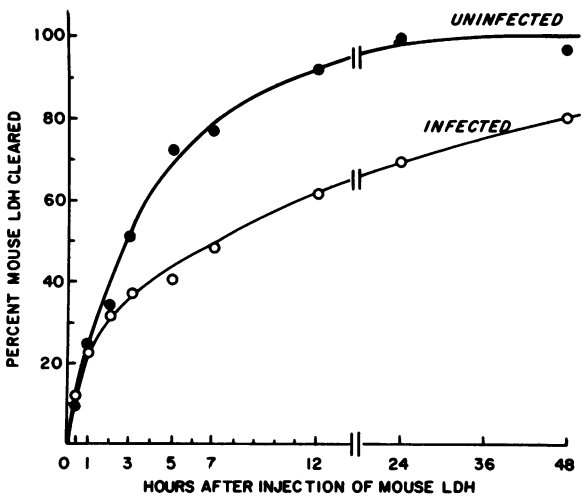



TEXT-FIG 1—Virus titer and enzyme activity in plasma of LDV-infected mice.[4]

Table 1—Effect of LDV on Plasma Enzyme Activity

| Enzymes | Percent increase |
|---|---|
| Lactate dehydrogenase | 500–1000 |
| Isocitric dehydrogenase | 500–1000 |
| Malic dehydrogenase | 100–200 |
| Phosphohexose isomerase | 100–200 |
| Glutamic oxalacetic transaminase | 50–100 |
| Glutathione reductase | 50–100 |
| Aldolase | 0 |
| Alkaline phosphatase | 0 |
| Acid phosphatase | 0 |
| Leucine amino peptidase | 0 |
| Glucose-6-phosphate dehydrogenase | 0 |
| α-Glycerophosphate dehydrogenase | 0 |

Preliminary experiments failed to demonstrate leakage of enzymes from infected cells, enzyme synthesis *de novo* or increased activation of existing enzymes. These negative findings suggested that the increase in plasma enzyme activity might be due to impaired clearance of endogenous enzymes. To test this possibility, LDH was injected intravenously into infected and uninfected mice and the rate at which enzyme activity disappeared from the peripheral circulation was studied. As seen in Text-fig 2, infected mice cleared intravenously administered LDH at a substantially slower rate than did uninfected mice. Within 7 hours, the uninfected mice had cleared over 75% of the injected enzyme, whereas it took the infected mice almost 48 hours




TEXT-FIG 2—Clearance of LDH from plasma of infected and uninfected mice.[5]

to clear the same amount of enzyme. Additional experiments showed that isocitric dehydrogenase also was cleared from the circulation more slowly by infected animals, whereas alkaline phosphatase, an enzyme whose activity was not elevated after infection with LDV, was cleared at the same rate in infected and uninfected animals. These findings supported the hypothesis of impaired clearance and suggested that any disease state, infection, chemical or toxin that damaged cells and released endogenous enzymes into the peripheral circulation would result in a higher and more prolonged elevation of enzyme activity in LDV-infected animals than in normal animals. This is illustrated in Table 2. Injection of carbon tetrachloride results in severe liver damage and the release of endogenous LDH into the peripheral circulation. Within 24 hours after injection of carbon tetrachloride, the plasma LDH level was 95,000 units/ml in uninfected animals and slightly higher (110,000 units) in infected animals. At the end of 3 days, most of the enzyme had been cleared from the circulation of uninfected animals. In contrast, the LDH level in infected animals was approximately 40,000 units/ml and was still elevated above background at the end of 5 days. A similar situation occurs in tumor-bearing animals. Since the amount of enzyme entering the circulation of tumor-bearing animals is greater than that of normal animals, infection of tumor-bearing mice with LDV would impede the clearance of this additional enzyme and result in a more than additive or synergistic increase in plasma enzyme activity. The data in Table 3 show that infection with LDV raised the plasma LDH level from 500 to 4500 units in nontumor-bearing mice, whereas infection with LDV in tumor-bearing mice raised the plasma enzyme level from 5500 to 38,000 units. Since LDV replicates in cells of the reticuloendothelial system (RES), and since the RES has been implicated in the clearance of certain enzymes, it has been postulated that infection of the RES by LDV is responsible for the impaired

Table 2—Effect of LDV on Plasma LDH Activity after Administration of Carbon Tetrachloride

| Days after carbon tetrachloride | LDH (units/ml) | |
|---|---|---|
| | Uninfected | Infected |
| 0 | 400 | 4,000 |
| 1 | 95,000 | 110,000 |
| 2 | 2,000 | 70,000 |
| 3 | 700 | 40,000 |
| 4 | 400 | 12,000 |
| 5 | 400 | 8,000 |

Table 3—LDH Activity in the Plasma of Uninfected and Infected Tumor-Bearing Mice

| Group | LDH activity in plasma (units/ml) |
|---|---|
| Uninfected control | 500 |
| Infected control | 4,500 |
| Uninfected tumor-bearing | 5,500 |
| Infected tumor-bearing | 38,000 |

enzyme clearance.[3,5] Although other viruses can cause cell damage and release intracellular enzymes, LDV is the only viral infection that, thus far, has been shown to increase enzyme levels in peripheral circulation by decreasing the rate of enzyme clearance. Thus, LDV may prove to be a valuable tool for studying cells and factors involved in enzyme clearance.

Since LDV has been found as a contaminant in over 100 different mouse tumors and virus preparations in laboratories throughout the world, LDV may have influenced the results and interpretations of many experiments. For example, several laboratories reported a correlation between tumor growth and plasma enzyme activity.[3] These results had to be reinterpreted after it was recognized that many of the tumors were contaminated with LDV. Subsequent experiments showed that much of the increase in plasma enzyme activity, which had been attributed to tumor growth, was in fact due to LDV. More recently, attempts to determine the half-life of asparaginase in tumor-bearing mice resulted in conflicting reports, until it was recognized that certain of these tumors were contaminated with LDV and that LDV impaired the clearance of asparaginase.[6] In fact, LDV may have a theraputic value. Riley found that if leukemic mice were infected with LDV and then given asparaginase, 95% of the animals survived 40 days or longer, whereas if uninfected leukemic mice were given asparaginase, none of the animals survived 40 days. LDV alone had no effect on survival of leukemic mice. Riley suggested that LDV extended the half-life of asparaginase and thereby increased the effective theraputic dose. Whether the elevation of other plasma enzymes also have biologic consequences remains to be determined.

In addition to its effect on enzyme clearance, LDV can affect the functional capacity of the immune system (Table 4). Immune function tests revealed that under certain circumstances LDV acted as an adjuvant. Infected animals made more antibody to a foreign protein (human gamma globulin) than did uninfected animals, and infection

Table 4—Effect of LDV on Immune Function

| Function | Effect |
|---|---|
| Humoral immunity | |
| Antibody production | Enhanced |
| Induction of tolerance | Depressed |
| Immunoglobulins | Elevated |
| Cellular Immunity | |
| Host-versus-graft | Depressed |
| Graft-versus-host | Depressed |
| Phagocytosis | Depressed |

with LDV prevented adult mice from developing immunologic tolerance to aggregate-free human gamma globulin.[7,8] In addition, LDV produced marked proliferation of the germinal centers in the spleen and lymph nodes of germfree mice and increased the level of circulating immunoglobulins. Under other circumstances, LDV depressed cellular immunity, as measured by allograft rejection and the graft-versus-host reaction.[9] The depression in cellular immunity might, in part, be explained by recent observations of Snodgrass and Hanna.[10] These investigators found that within 48 hours after infection, LDV produced a marked depletion of lymphocytes in thymic-dependent areas of the spleen and lymph nodes. Factors responsible for the depletion of lymphocytes, however, are unknown. Other experiments in our laboratory showed that LDV depressed the functional capacity of the RES as measured by carbon clearance.[5] The depression in RES function might be related to the fact that LDV replicates in macrophages.

Recent experiments from a number of laboratories indicate that many other viruses can affect immune function.[11] Marked depression of humoral immunity has been reported in animals infected with the leukemia viruses, lymphocytic choriomeningitis virus and Aleutian mink disease virus. Depression of cellular immunity has been reported with the leukemia viruses and alterations of RES function have been observed with a variety of viruses. The effect of viruses on immune function may have several potentially important biologic consequences. Viruses that stimulate the immune response might be a factor in the initiation and potentiation of autoimmune disorders. Viruses that depress humoral and cellular immunity might make the host more susceptible to other infectious agents and might initiate or potentiate the development of certain tumors. Similarly, depression of humoral or cellular immunity might make the host less able to reject virus-infected

cells, and this might be one of the factors responsible for the chronic nature of certain viral infections.

Through its effect on the immune system, LDV might have affected the results and interpretations of a number of experiments. Several laboratories have reported accelerated growth of tumors in the presence of LDV.[10,12,13] Others have noted that infection with agents such as *Eperythrozoon coccoides* may be more severe in LDV-infected animals.[14] These effects could be explained on the basis of virus-induced immunosuppression.

Another way in which a viral infection might produce disease is through the deposition of circulating immune complexes. In many respects, a chronic viral infection offers the ideal situation for the development of immune complex disease. First, the virus is a foreign antigen. Second, it is a self-replicating agent that can produce a continuous supply of antigen over a long period of time. Third, the immune response of the host to viral antigens results in the formation of immune complexes; and fourth, some of these virus-antibody complexes retain their ability to infect and thereby perpetuate the infection. For a long time, however, it was not recognized that viruses could circulate in the blood of chronically infected animals as infectious virus-antibody complexes. In fact, it was thought that a persistent viremia represented a lack of antibody, presumably on the basis of immunologic tolerance. Our studies with LDV showed that persistently infected mice were not immunologically tolerant.[15,16] In addition to neutralizing antibody, we found that the virus was circulating in the form of an infectious virus–antibody complex. These complexes were detected within 8 weeks after infection and persisted for the animal's life. The anti-immunoglobulin neutralization technic was used to demonstrate these complexes.[15,16] In brief, anti-immunoglobulin reacts with immunoglobulins on the surface of the virion and results in neutralization by producing more extensive coverage of the surface of the virion than occurs with antiviral antibody alone. Virus that does not have antiviral antibody on its surface cannot be neutralized by anti-immunoglobulin. With specific anti-immunoglobulins it is possible to determine the class of immunoglobulin that has attached to the virion.

By use of the anti-immunoglobulin neutralization technic (Table 5), other investigators have found infectious virus-antibody complexes in the circulation of animals infected with lymphocytic choriomeningitis virus, Aleutian mink disease virus and the murine sarcoma and leu-

Table 5—Circulating Infectious Virus-Antibody Complexes and Glomerulonephritis

| Virus | Complexes of infectious virus–antibody | Glomerulonephritis |
|---|---|---|
| Lactic dehydrogenase | Yes | Yes |
| Lymphocytic choriomeningitis | Yes | Yes |
| Aleutian mink disease | Yes | Yes |
| Murine leukemia and sarcoma | Yes | Yes |
| Equine infectious anemia | Probable | Yes |

kemia viruses.[17–19] There is also evidence that infectious complexes exist in the circulation of horses infected with equine infectious anemia virus [20] and that hepatitis virus circulates as an immune complex.[21]

One of the most perplexing observations arising from our studies with LDV was the paradoxic demonstration that infectious virus persisted in the circulation in the presence of excess neutralizing antibody. Neutralization studies revealed that infectious virus-antibody complexes obtained from chronically infected animals were more resistant to subsequent neutralization by fresh antiviral antibody than virus that had not been previously exposed to antiviral antibody.[15,16] These and other experiments [22–24] suggest that the attachment of antibody to the virion sterically hinders or blocks additional antiviral antibody from reaching unoccupied receptor sites on the virion and/or from reaching infectious particles buried within virus–antibody aggregates. This might account, in part, for the chronic nature of certain of these virus infections.

Within the last few months, Porter and Porter [25] and Oldstone and Dixon [26] showed that the deposition of circulating virus-antibody complexes in the kidneys of LDV-infected animals led to the development of immune-complex type of glomerulonephritis. Immunofluorescent studies revealed viral antigens, specific antiviral antibody and complement in the kidneys of these persistently infected animals. Similary, the glomerulonephritis observed in animals infected with lymphocytic choriomeningitis virus, Aleutian mink disease virus, and the murine leukemia viruses has been attributed to the deposition of virus-antibody complexes.[17–19,27] Although complexes circulate in all of these infections, there is considerable difference in the severity of the glomerulonephritis. In contrast to the severe glomerular lesions

in animals infected with lymphocytic choriomeningitis virus and Aleutian mink disease virus, very mild glomerular lesions were found in LDV-infected mice. At present, there is very little information as to how the host responds to circulating virus-antibody complexes. It is not known whether the size of the complex, the nature of the viral antigen, the amount or type of antibody or the attachment of accessory factors such as complement [28] or rheumatoid factor [29] are responsible for the production of virus-induced immune-complex disease. Immune-complex disease is another example of how an unsuspected viral infection might produce pathologic changes and influence the interpretations of experimental results.

Next, I would like to summarize some very recent studies on the events involved in the replication of LDV *in vitro*. These experiments will be reported in detail elsewhere.[30] Primary mouse-embryo cells were infected with different multiplicities (20, 2, 0.2) of LDV and, at various times thereafter, the tissue culture fluid was harvested and assayed in mice for infectious virus. Replication could be detected within 6–9 hours after inoculating the cultures and the virus titer reached a maximum of $10^{8.0}$ $ID_{50}$/ml within 24 hours. At the lowest multiplicity of infection (0.2), the maximum titer was reached at 48 hours after inoculation. Despite the high virus titer in the tissue culture fluid, a cytopathologic effect was not observed.

The next experiment was designed to see whether the replication of LDV resulted in the production of interferon. Tissue culture fluid was harvested at different times after infection and assayed for interferon activity against vesicular stomatitis virus. No interferon activity was detected. Other experiments, however, showed that interferon could interfere with the replication of LDV. Primary mouse-embryo cells were incubated overnight with different concentrations of mouse interferon. The monolayers were infected with LDV and 24 hours later the virus titer was determined. At high concentrations of interferon (120 units), the virus titer was depressed by over 99%. Thus LDV is a poor produced of interferon, but is sensitive to the action of interferon. The low production of interferon may be another of the factors responsible for the persistence of this infection *in vivo*.

The next set of experiments was designed to study the effect of metabolic inhibitors on the replication of LDV. Different concentrations of actinomycin D were incubated with monolayers of primary mouse-embryo cells for 1 hour. The actinomycin D then was removed and

the cells were infected with LDV. The virus titer was determined 18 hours later. As little as 0.125 μg/ml of actinomycin D inhibited the virus titer by as much as 98%. Other experiments showed that the replication of LDV was inhibited most if the actinomycin D was added within the first few hours after initiating the infection. These experiments suggested that a DNA-dependent RNA step was involved in the replication of LDV. To see whether new DNA synthesis was required in the replication of LDV, monolayers of primary mouse-embryo cells were treated, prior to infection, with different concentrations of cytosine arabinoside. At $10^{-3}$ M, cytosine arabinoside inhibited cellular DNA synthesis by 99%, but had no appreciable effect on the replication of LDV. Similarly, 5-fluorodeoxyuridine did not inhibit virus replication. On the basis of these metabolic-inhibitor studies, one would not expect LDV to contain an RNA-dependent DNA polymerase. Dr. Masakazu Hatanaka kindly assayed our preparations of LDV for RNA-dependent DNA polymerase activity and found them to be negative.

Finally, LDV was labeled with an RNA precursor. Infected monolayers were pulsed with $^{3}$H-uridine, the supernatant fluid was concentrated 300-fold and layered on a 10–60% linear sucrose gradient. The tubes were centrifuged in an SW-39L rotor at 100,000 *g* for 20 hours, and fractions were collected and assayed for both infectivity and radioactivity. Our experiments showed that the peak of radioactivity corresponded to the peak of infectivity, both being at 1.17 g/ml. Material prepared and concentrated in the same way from uninfected cultures that also had been pulsed with $^{3}$H-uridine or from infected cultures that had been pulsed with $^{3}$H-thymidine failed to show a peak of radioactivity at 1.17 g/ml. Attempts now are being made to determine the molecular weight of the viral nucleic acid.

In conclusion, LDV produces a persistent infection in mice and subtle alterations in function rather than gross cytopathologic changes. The virus replicates rapidly and the infection results in both early and late disease. Early disease is characterized by impairment of enzyme clearance and derangement of immunologic function. Both of these alterations may be due to the replication of the virus in the cells of the RES. Late disease is characterized by circulating virus-antibody complexes and the development of immune-complex type of glomerulonephritis. Both early and late disease are mild and the infection does not result in death. However, because of the effect of the virus on enzyme clearance and immune function, the

ability of the host to handle a second infectious agent or a tumor may be significantly altered. It is under conditions of environmental stress that LDV might be most injurious to the host.

The factors responsible for the persistence of viral infections are still poorly understood. The studies with LDV suggest that virus-induced immunosuppression, the persistence of infectious virus in the presence of neutralizing antibody and poor interferon production might be contributing factors.

Evidence also is accumulating that virus-induced immunopathology may be an important factor in the pathogenesis of both acute and persistent viral infections. In addition to producing immunologic derangement and immune-complex disease, viruses can induce new cell-surface antigens and the interaction of these antigens with specific antiviral antibody and complement can result in cell damage.[31] The fact that the immune response to viral antigens may be injurious to the host and that infectious virus can persist in the presence of neutralizing antibody poses a challenging and potentially difficult problem for the development of vaccines against persistent viral infections.

Finally, it should be emphasized that persistent viral infections are not limited to experimental animals. It is becoming increasingly evident that under certain circumstances many common viruses such as herpes simplex virus, cytomegalovirus, Epstein-Barr virus, herpes zoster, rubella, rabies, hepatitis, wart virus and others can produce persistent or recurrent infections in man.[32,33] Moreover, certain chronic degenerative neurologic diseases such as subacute sclerosing panencephalitis, Kuru, Jacob-Krutzfeld and progressive multifocal leukoencephalopathy appear to be of viral origin.[2,32] In addition, it has been speculated that viral infections might be involved in "autoimmune" disorders [34] and diseases of the endocrine system.[35] It is hoped that the information emerging from animal models will provide new insight into the etiology, pathogenesis and prevention of these human diseases.

## Summary

Lactic dehydrogenase virus (LDV) is an ether-sensitive RNA virus of mice. It is about 40 mμ in diameter and has a density, in sucrose, of 1.17 g/ml. The virus replicates rapidly *in vitro,* but fails to produce any gross cytopathology. Studies using metabolic inhibitors indicate that a DNA-dependent RNA step is involved in its replication. *In vivo,* LDV produces a life-long viremia and an increase in the activity of

a number of plasma enzymes. The early manifestations of infection are characterized by impaired enzyme clearance and altered immunologic function. The late manifestations are characterized by circulating infectious virus-antibody complexes and the development of immune complex–type glomerulonephritis. Both early and late manifestations are mild but may alter the host's ability to handle a superimposed infection or tumor. The studies with LDV suggest that the term *slow infection* is a misnomer and that virus-induced immunopathology may be an important factor in the pathogenesis of certain persistent viral infections.

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