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Regulation of Enzyme Levels in the Blood

Influence of Environmental and Genetic Factors on Enzyme Clearance

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Since its discovery, lactic dehydrogenase virus (LDV) has remained unique as a model of long-term enzyme elevation due to impairment of enzyme clearance. The present study shows that mice inoculated with silica develop an increase in plasma lactate dehydrogenase (LDH) lasting for at least 6 months and that the enzyme elevation is due, at least in part, to impairment of clearance. The extent of the enzyme elevation is dependent on both the dose and route of silica administration and mice that had received both silica and LDV showed a more profound impairment of LDH clearance than mice that had received silica or LDV alone. Examination of the factors that regulate circulating enzyme levels in normal mice revealed that whereas there was no difference in resting enzyme levels among several inbred strains of mice (BALB/cAnN, NZBWF1/J, B10.D2/nSnN, and A/J mice), when mice were stressed by the administration of an enzyme load, certain inbred strains (BALB/cAnN) cleared the enzyme rapidly and others (B10.D2/nSnN) cleared the enzyme slowly. Moreover, in B10.D2/nSnN mice, enzyme clearance was age-related. When different strains of mice were infected with LDV, LDH levels were substantially higher in the circulation of slow enzyme clearers as compared to rapid enzyme clearers. It is concluded that both environmental and genetic factors influence the clearance of LDH and that impairment of enzyme clearance may be a more important factor than previously suspected in regulating enzyme levels in disease states. (Am J Pathol 1988, 132:503–511)

IN HUMANS, THE level of enzymes in the blood, particularly lactate dehydrogenase (LDH), has been widely used to diagnose and follow the course of certain diseases.[1] In mice, LDH has been used to follow the growth of a number of transplantable tumors.[2,3] Initially, the increase in circulating LDH was thought to reflect an increase in the synthesis and release of enzyme from growing tumors. Subsequent studies showed that many transplantable mouse tumors were infected with lactic dehydrogenase virus (LDV).[2–4] When this virus was isolated and separated from mouse tumors, it became apparent that LDV was responsible for much of the enzyme elevation.

LDV is a member of the togavirus family. In mice, LDV infects cells of the reticuloendothelial system (RES), produces a life-long viremia, and circulates in the blood in the form of an infectious virus-antibody complex.[4–6] Perhaps its most unusual property is its capacity to produce a lifelong five-to-tenfold increase in the blood of a number of enzymes, including LDH. Analysis of the factors responsible for LDV-induced enzyme elevation revealed that the virus impairs the clearance of certain endogenous enzymes and that this is largely responsible for the enzyme elevation in both tumor-bearing and tumor-free animals.[3,4,7]

The findings with LDV raised the possibility that other diseases or environmental insults might similarly raise LDH levels by impairing enzyme clearance. The present investigation was initiated to study, in greater depth, environmental and genetic factors controlling the clearance of LDH.

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Materials and Methods

Animals

BALB/cAnN, A/J, B10.D2/nSnN, and NZBWF1/J male mice (5 to 8 weeks old) were obtained from either the Animal Production section of the National Institutes of Health (Bethesda, MD) or the Jackson Laboratory (Bar Harbor, ME). Groups of four or five BALB/cAnN mice of the same age and approximately the same weight were used for each set of experimental conditions unless otherwise stated.

Plasma

All blood specimens were obtained by retro-orbital bleeding with heparinized micropipettes.

Virus

A stock preparation of the LDV was used throughout the experiments. Mice were infected with virus by intraperitoneal injection of $10^{6.5}$ infectious doses median (ID_{50}). The assay for infectious virus was described elsewhere.[5] In brief, samples tested for LDV were serially diluted tenfold in phosphate-buffered saline (PBS), and 0.1 ml of each dilution was inoculated intraperitoneally into three mice. Mice were bled retroorbitally 72 to 96 hours later, and the LDH activity was measured.[8] The number of animals infected at each dilution was determined, and the ID_{50}/ml was calculated by the method of Reed and Muench.[9]

Heterologous Enzymes

Porcine muscle LDH-5 and porcine heart LDH-1 were obtained from the Sigma Chemical Company (St. Louis, MO). The enzymes were dialyzed extensively with 0.02 M sodium phosphate buffer, pH 7.4, before determination of activity. An appropriate dose of enzyme was administered intravenously to each mouse in a volume of 0.2 ml.

Endogenous Enzymes

The hepatotoxin carbon tetrachloride (CCl_4; Fisher Scientific, Fair Lawn, NJ) was used to produce an increase in endogenous LDH activity in the plasma. Each mouse received 0.002 ml/15 g body weight of CCl_4 in a volume of 0.05 ml intraperitoneally.

Clearance of Enzymes

The term clearance, as used throughout this paper, refers to the disappearance of enzyme activity from the peripheral circulation.[7] Clearance was studied by injecting heterologous enzyme intravenously into mice and measuring at intervals the plasma LDH level. The number of units of heterologous enzyme in the plasma at a particular time after injection was calculated by subtracting the number of units of enzyme found in the plasma of the uninjected control animals (usually <400 units/ml) from the number of units of enzyme in the plasma of the injected animals. To determine the percent of heterologous enzyme remaining in the blood, the resulting figure was divided by the number of units of heterologous enzyme present at 1 minute postinjection and multiplied by 100.

Silica

Silica, which has a particle size distribution of 0.5 to 10 μ (with approximately 80% between 1 and 5 μ), was obtained from Sigma Chemical Company. Silica was suspended in PBS (pH 7.4), sterilized by autoclaving, and dispersed by ultrasonic vibration (3 to 5 seconds at power setting 4 on a Branson ultrasonifier, Branson Sonic Power Co., Farmingdale, NY). Silica was injected intravenously at 1 mg/g body weight, unless otherwise stated.

MDP

Muramyl dipeptide Mur-NAc-L-Ala-D-isoGln (MDP), obtained from Sigma Chemical Company, was suspended in PBS and injected intravenously and intraperitoneally.

Statistics

Unless otherwise stated, in all experiments data points are expressed as the mean of four or five mice ± standard error. Differences between means with P values less than 0.05 ($P < 0.05$) were considered significant. Multivariate analysis of variance models were used to describe the data for each experiment in which animals were observed at multiple time points. The analyses were performed on the $\log_{10}$ scale for data representing units of enzyme activity and on the arcsin (square root) scale for data given in percentages. Generally, a one-way multivariate analysis was used, but in certain experiments (Figures 3, 5A, 5B, and 10) a two-way model was employed. Within experiments, when comparing data from groups of animals, Bonferroni t statistics were used when four or fewer comparisons were made and Scheffe's method was used when five or more comparisons were made.[10,11]

Results

Silica-Induced Enzyme Elevation

The effects of silica, LDV, and the combination of the two on plasma LDH levels were determined by injecting mice with these agents and measuring LDH levels at different times thereafter. Figure 1 shows that the plasma LDH level of control mice was less than 400 units/ml. Silica caused an almost tenfold increase of LDH within 24 hours after injection compared with controls ($P < 0.001$). Forty-eight hours after silica injection, LDH levels had declined but still remained nearly fourfold higher than control values (1300 to 1500 units/ml, $P < 0.001$) for the duration of the experiment. Mice injected with LDV showed approximately a tenfold increase in LDH. In contrast to silica, however, the increase in LDH caused by LDV did not decline during the 7 days of the experiment. When mice were inoculated with both silica and LDV, plasma LDH levels rose 20-fold.

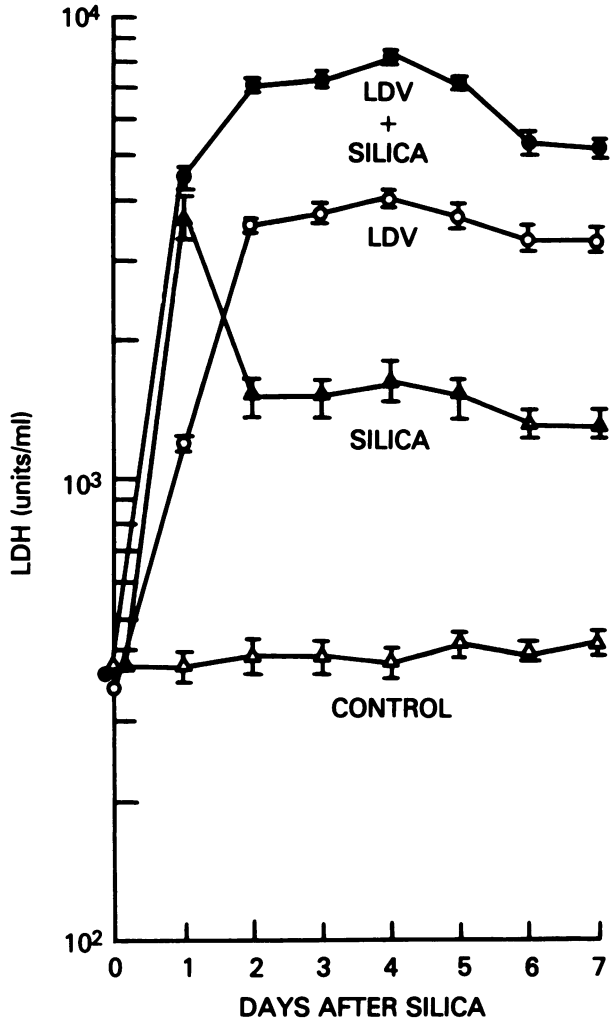

Figure 1—Effect of silica and LDV on plasma enzyme levels. Mice were injected with PBS (control), silica, LDV, or LDV plus silica. Plasma LDH levels were determined at 24-hour intervals. In all experiments, vertical bars represent the standard error of the mean.

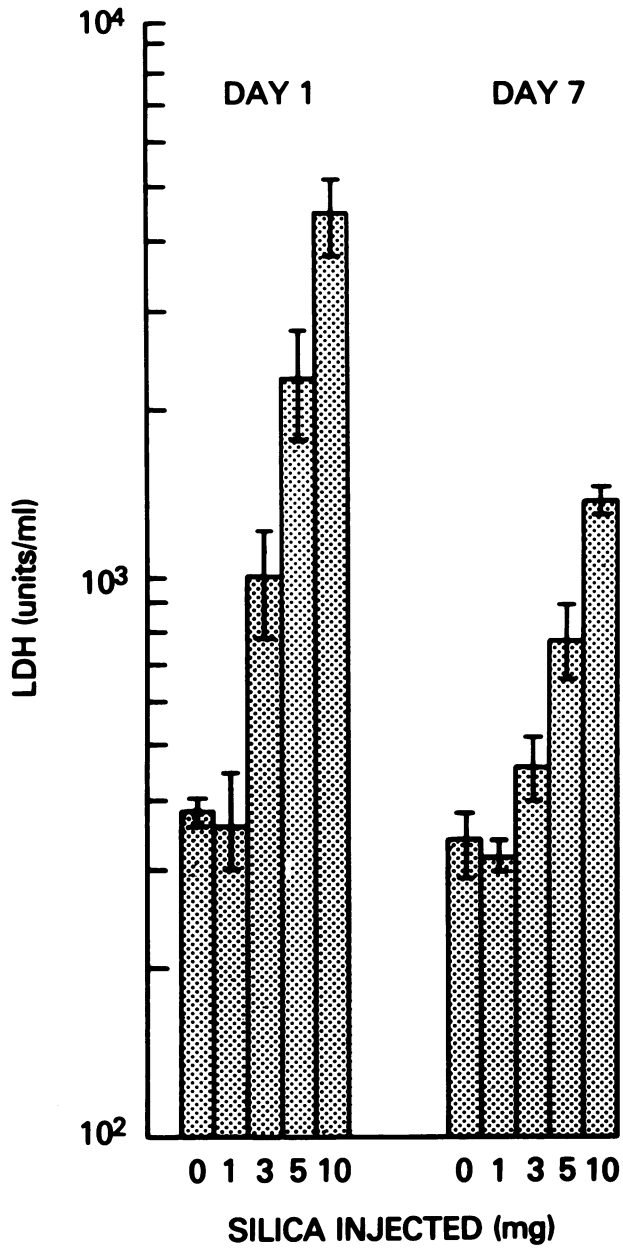

Figure 2—Dose response of silica on plasma LDH levels. Mice were injected intravenously with 0, 1, 3, 5, or 10 mg silica/10 g body weight. Plasma LDH levels were determined 1 and 7 days postinjection.

To study in more detail the effect of silica on LDH levels, mice were inoculated intravenously with different concentrations of silica and LDH levels in the circulation were measured at 1 and 7 days postinoculation. Figure 2 shows that there is a dose-dependent relationship between silica and LDH levels. Enzyme levels were higher on day 1 than on day 7 as also observed in Figure 1. Further experiments showed

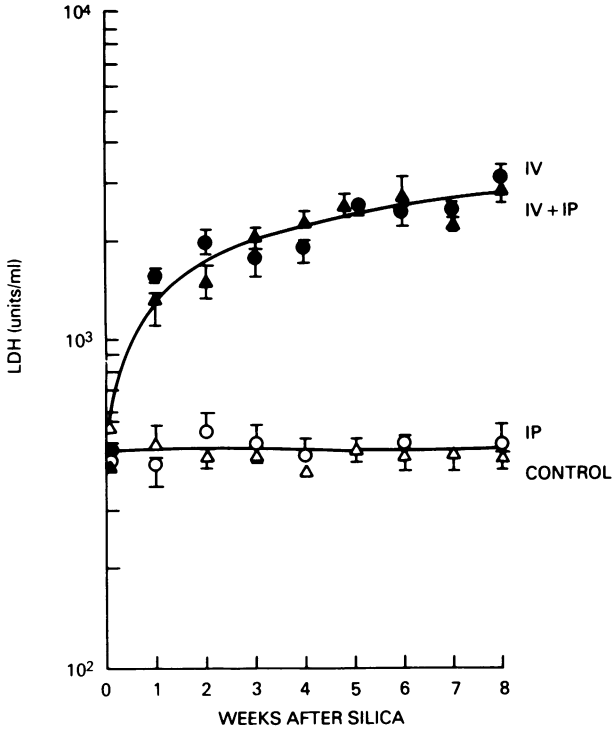

Figure 3—Effect of route of silica administration on plasma LDH levels. Mice were injected with silica intravenously (●), intraperitoneally (○), or by both routes (▲). Control mice (△) received only PBS by the intravenous route. Plasma LDH levels were determined at weekly intervals.

that the route by which silica was administered profoundly affected LDH levels (Figure 3). Mice given silica by the intraperitoneal route showed no increase in LDH levels for at least 8 weeks ($P < 0.001$). In all subsequent experiments mice were injected intravenously with 1.0 mg silica/g body weight.

The duration of silica-induced LDH elevation is shown in Figure 4. LDH levels rose from about 1200 units/ml at 1 week postinoculation to close to 4000 units/ml at 8 weeks and remained at approximately that level for at least 6 months. These values were all statistically different than controls ($P < 0.001$). To be sure that the rise in LDH was not due to inadvertent LDV infection, blood samples were obtained from mice on five occasions during the 6 months of the experiment and tested for infectious LDV. None of the samples contained virus.

Impairment of Enzyme Clearance

Previous studies showed that much of the increase in LDH levels in LDV-infected animals is due to virus-induced impairment of enzyme clearance.[7] Moreover, LDV has a differential effect on the clearance of LDH isoenzymes.[12] Figure 5 shows that LDV impairs the clearance of LDH-5, but has little effect on the clearance of LDH-1. To see if any of the silica-induced increase in LDH was due to impairment of enzyme clearance, purified LDH was injected intravenously into silica-treated mice. Uninfected and LDV-infected animals served as controls. Compared with uninfected controls, LDH-5 clearance in silica-treated animals was significantly impaired ($P < 0.05$) at all times except at the 10-hour time point. LDV-infected mice showed a more dramatic impairment of enzyme clearance as compared with uninfected controls ($P < 0.001$ at all time points). When animals received both LDV and silica, LDH clearance was even more profoundly impaired ($P < 0.001$ at all time points). In contrast to the differential effect of LDV on the clearance of LDH isoenzymes, silica-treated animals showed impaired clearance of both LDH-5 and LDH-1 as compared with uninfected controls ($P < 0.05$ for LDH-5 and $P < 0.02$ for LDH-1). It should be noted that LDH-1 is cleared considerably more slowly in normal animals than LDH-5.

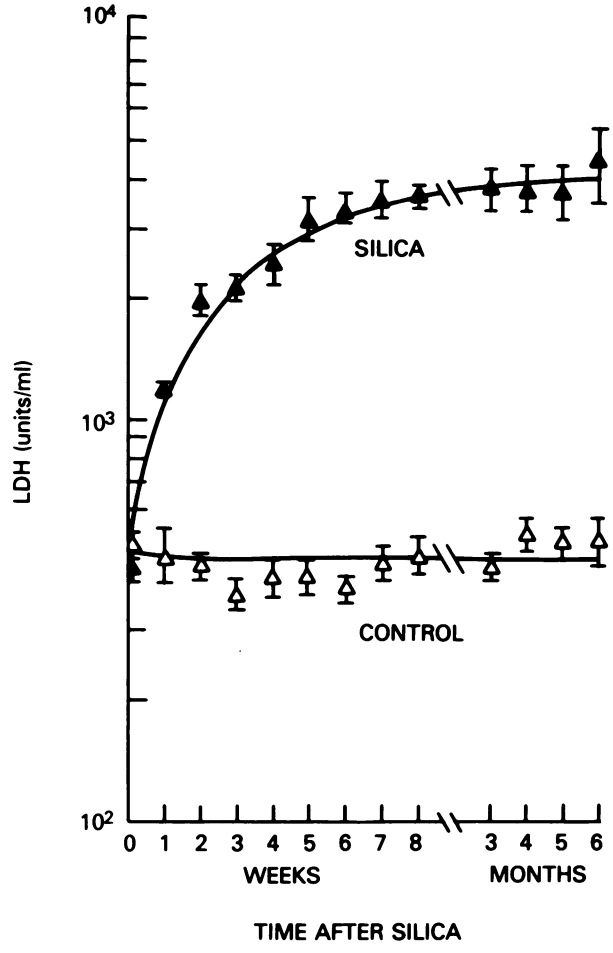

Figure 4—Duration of silica-induced plasma LDH elevation. Groups of mice were injected intravenously with silica or PBS (controls). Plasma LDH levels were determined on each animal at the times indicated.

In the experiment described, exogenous LDH was used to study enzyme clearance. To evaluate the clearance of endogenous LDH, mice were treated with the hepatotoxin CCl_4 that releases LDH into the circulation.[7] As seen in Figure 6, all groups (controls, silica-treated, LDV-infected, and LDV-infected and silica-treated) exhibited a large increase in plasma LDH within 24 hours. The control animals cleared LDH rapidly with the enzyme returning to almost normal levels within 48 hours. In contrast, the clearance of LDH in the silica-treated animals was impaired ($P < 0.05$). Even more dramatic impairment of enzyme clearance occurred in the LDV-infected animals ($P < 0.001$) and the most profound effect was observed in animals inoculated with both LDV and silica ($P < 0.001$).

Because LDV and silica, known suppressors of the RES,[3,13] impair enzyme clearance, the possibility that agents that activate RES functions, such as MDP,[14] might enhance enzyme clearance was investigated. Figure 7 shows that MDP-treated mice cleared LDH-5 faster than control mice. Statistically significant differences were found at 3, 4, 5, and 6 hours post-LDH injection ($P < 0.05$).

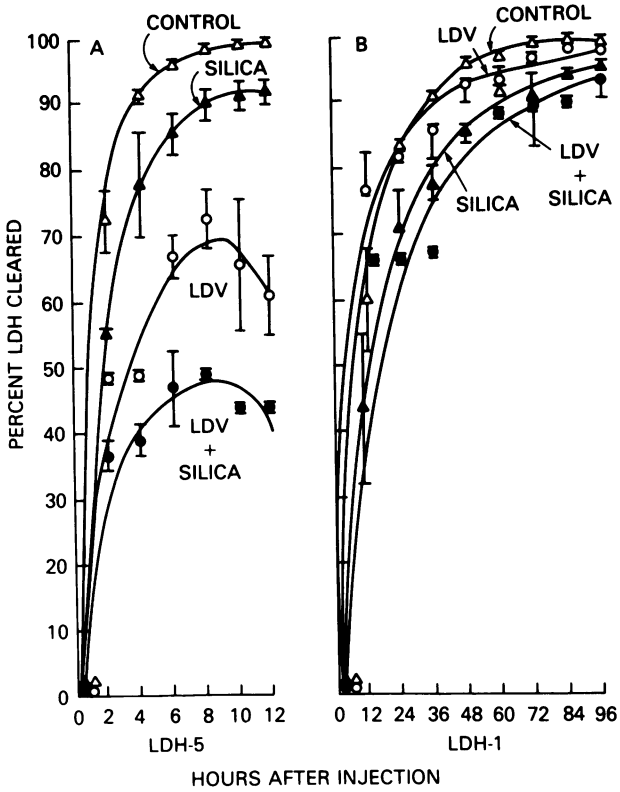

Figure 5—Clearance of exogenous LDH-1 and LDH-5. Mice inoculated with PBS (control), silica, LDV, or silica plus LDV, were injected intravenously with porcine LDH-5 (54,000 units/mouse) or porcine LDH-1 (69,000 units/mouse). At intervals the percent LDH cleared was determined. Mice were inoculated with LDV 3 weeks before or silica 4 weeks before the enzyme clearance studies.

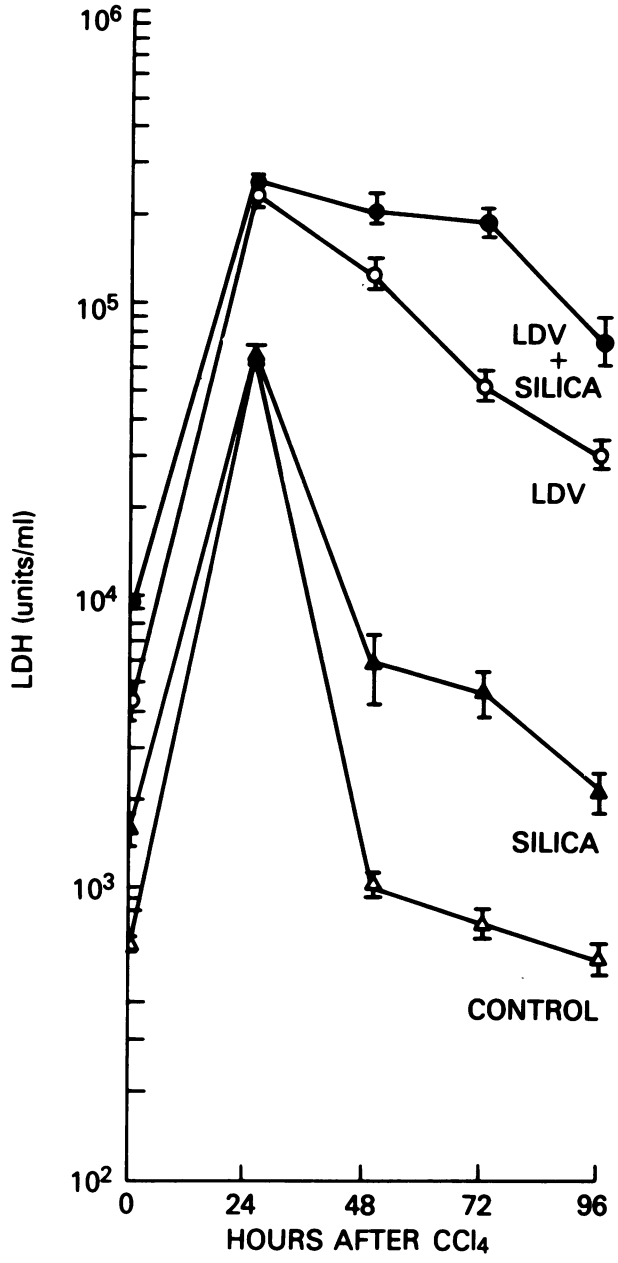

Figure 6—Clearance of endogenous LDH. Plasma LDH levels were determined at intervals after intraperitoneal injection of CCl_4 in mice injected with PBS (control), silica, LDV, or silica plus LDV. Animals were inoculated with LDV 21 days before or silica 5 days before CCl_4.

Genetic Differences in Enzyme Clearance

To see whether there were any genetic differences in the clearance of LDH, the plasma LDH level of several inbred strains of mice was first determined. Figure 8 shows little or no difference among BALB/cAnN, NZBWF1/J, B10.D2/nSnN, and A/J mice at 5 or 8 weeks of age. The possibility that differences in clearance might become apparent if the animals were stressed with an enzyme load was evaluated in BALB/

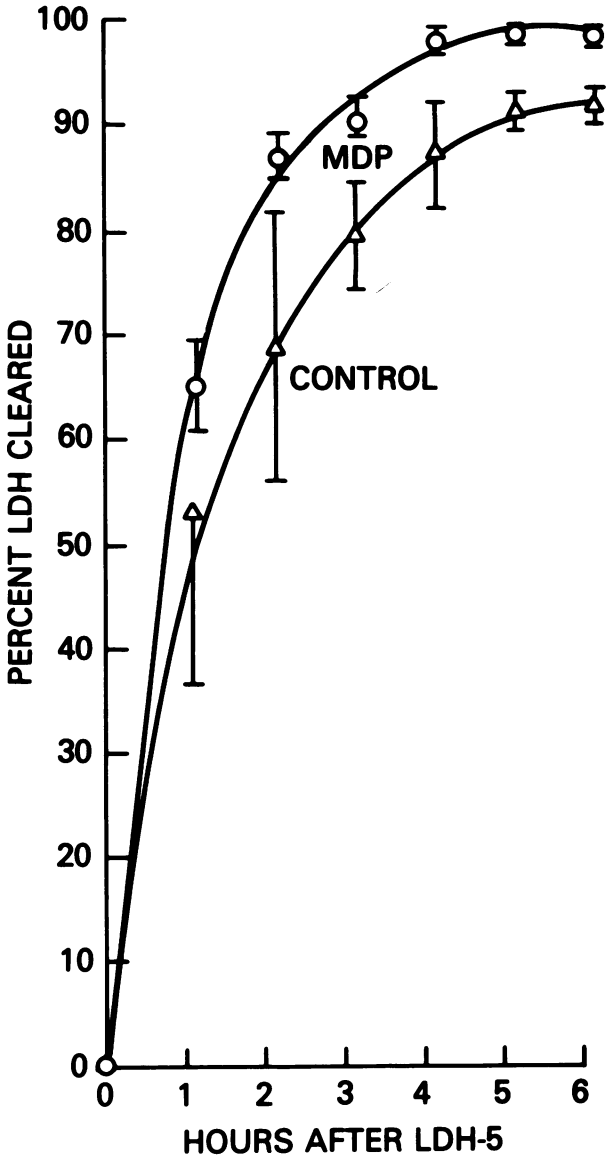

Figure 7—Effect of MDP on clearance of LDH-5. Mice were inoculated with MDP (200 μg intravenously and 200 μg intraperitoneally per mouse for 3 days) or PBS. Animals were then injected intravenously with 9000 units of LDH-5 and, at intervals, the percent LDH cleared was determined.

cAnN and B10.D2/nSnN mice. As seen in Figure 9, at 5 weeks of age, B10.D2/nSnN mice cleared LDH at a somewhat slower rate than BALB/cAnN mice ($P < 0.05$ at all times except 2 hours post-LDH injection). At 8 weeks of age, however, a marked impairment of the clearance of LDH by B10.D2/nSnN mice was observed ($P < 0.01$ at 2 to 6 hours post-LDH injection).

The demonstration that there was a difference among inbred strains of mice in the clearance of an enzyme load suggested that insults that released enzymes into the circulation or impaired LDH clearance might have a more pronounced effect on B10.D2/nSnN mice than on BALB/cAnN mice. Figure 10 shows that silica treatment or LDV infection impaired LDH clearance in both BALB/cAnN and B10.D2/nSnN mice compared with untreated controls ($P < 0.03$). Moreover, clearance of LDH is significantly slower in B10.D2/nSnN mice compared with BALB/cAnN mice under all experimental conditions (controls, silica treated or LDV infected) ($P < 0.03$).

Based on the observation that impairment of enzyme clearance was more pronounced in B10.D2/nSnN mice than in BALB/cAnN mice, and that it was more severe in 8-week-old than 5-week-old B10.D2/nSnN mice, the effect of LDV infection on plasma LDH was determined in animals infected at 5 weeks of age and followed for 3 weeks. As seen in Figure 11, LDV-infected BALB/cAnN mice showed the typical ten-fold increase in LDH within 1 week and the enzyme elevation persisted for the 3 weeks of the experiment. In contrast, B10.D2/nSnN mice showed a higher LDH level than BALB/cAnN mice at 1 week after infection. Moreover, the LDH level continued to

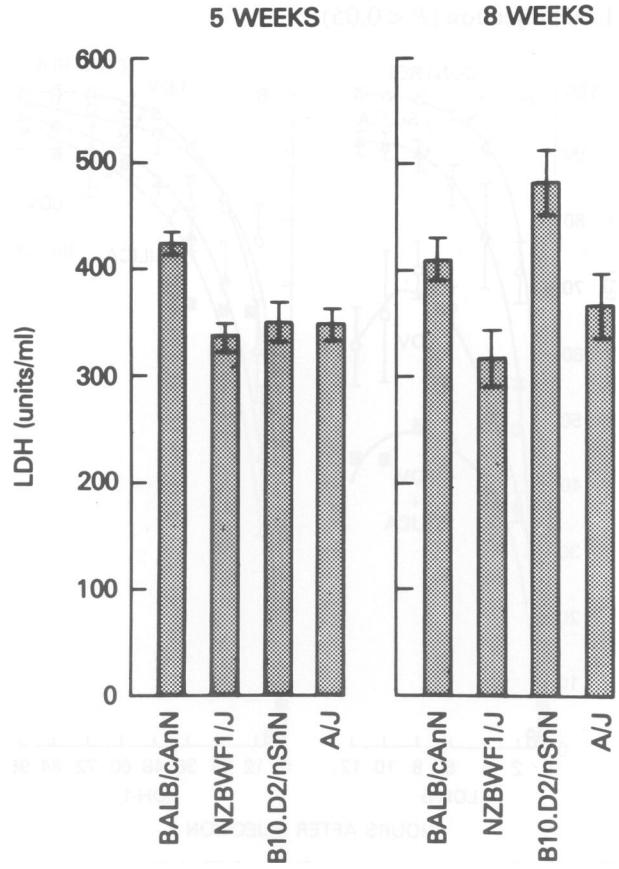

Figure 8—Plasma LDH levels of four inbred strains of mice. Plasma LDH levels were determined at 5 and 8 weeks of age (N = number mice tested). BALB/cAnN: 5 weeks N = 100, 8 weeks N = 20. NZBWF1/J: 5 weeks N = 35, 8 weeks N = 19. B10.D2/nSnN: 5 weeks N = 25, 8 weeks N = 16. A/J: 5 weeks N = 28, 8 weeks N = 14.

rise in B10.D2/nSnN mice and was significantly higher than in BALB/cAnN mice at 1, 2, and 3 weeks ($P < 0.05$). Uninfected controls of the same age from both strains showed no increase in enzyme level with time (data not shown).

Discussion

The level of a particular enzyme in the circulation is determined by the balance between influx and clearance. Nonetheless, fluctuations in enzyme levels in human disease are almost always attributed to altered influx (ie, the release of enzymes from proliferating or dying tissues). Earlier experiments with LDV provided the first unequivocal evidence that elevation of an enzyme could be the result of impaired clearance.[3,7] The current experiments provide another example. They show that silica raises enzyme levels and that, at least in part, this is due to impaired enzyme clearance.

There is relatively little information about the precise mechanism by which enzymes are cleared from the blood. The earlier work with LDV showed that this virus infects cells of the RES and suggested that

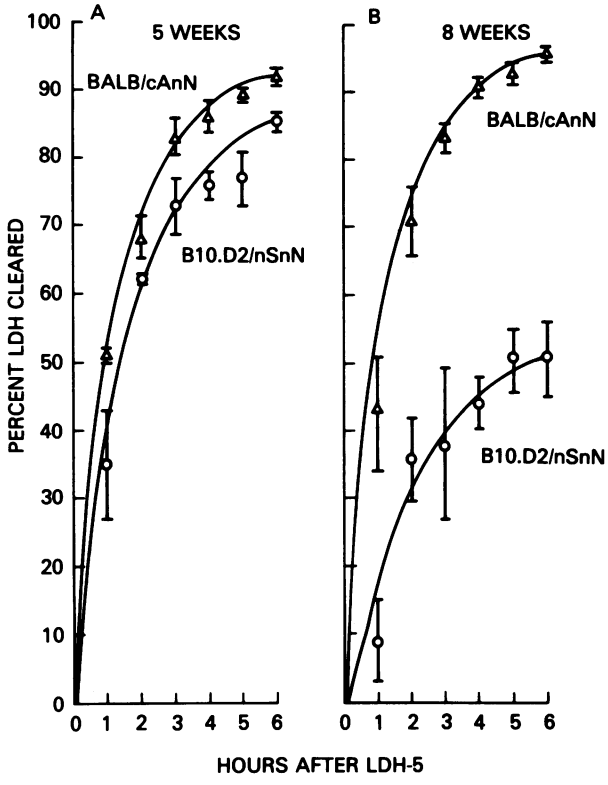

Figure 9—Clearance of LDH-5 in BALB/cAnN and B10.D2/nSnN mice. Mice 5 and 8 weeks of age were injected intravenously with porcine LDH-5 (50,000 units/mouse) and at intervals the percent LDH cleared was determined.

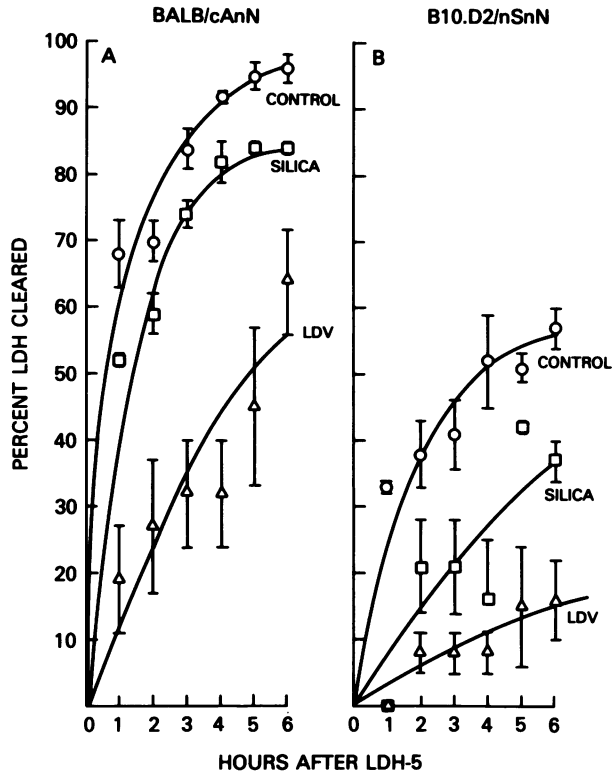

Figure 10—Effect of silica and LDV on clearance of LDH-5 in BALB/cAnN and B10.D2/nSnN mice. Eight-week-old mice inoculated with PBS (control), silica, or LDV were injected intravenously with porcine LDH-5 (9000 units/mouse) and the LDH clearance was determined. Animals were inoculated with LDV or silica 5 days before enzyme clearance studies.

these cells were involved in enzyme clearance.[3,7,12,15,16] Since that time, more direct evidence has accumulated that RES cells are involved in the clearance of LDH[17-20] and that several other enzymes are elevated in LDV-infected animals.[2,3] The rate of enzyme clearance depends on a number of factors including the pI or charge of the molecule.[12,21-23] Macrophages endocytose materials actively and some materials are taken up as part of the fluid engulfed by the cell during fluid phase pinocytosis, whereas other materials are taken up via receptor-mediated pinocytosis.[24,25] In fact, a variety of receptors having different biologic properties have been identified on RES cells.[26-29]

If, as the evidence now suggests, cells of the RES are involved in enzyme clearance, then damage to these cells by any one of a variety of factors might alter the function of these cells, resulting in impaired enzyme clearance and raised plasma enzyme levels. It is well known that silica is taken up by and can be cytotoxic for macrophages.[13,30] Moreover, silica is not readily eliminated from the body and is harbored by the host for months with the silica particles being repeatedly taken up by new crops of RES cells.[13] This could ac-

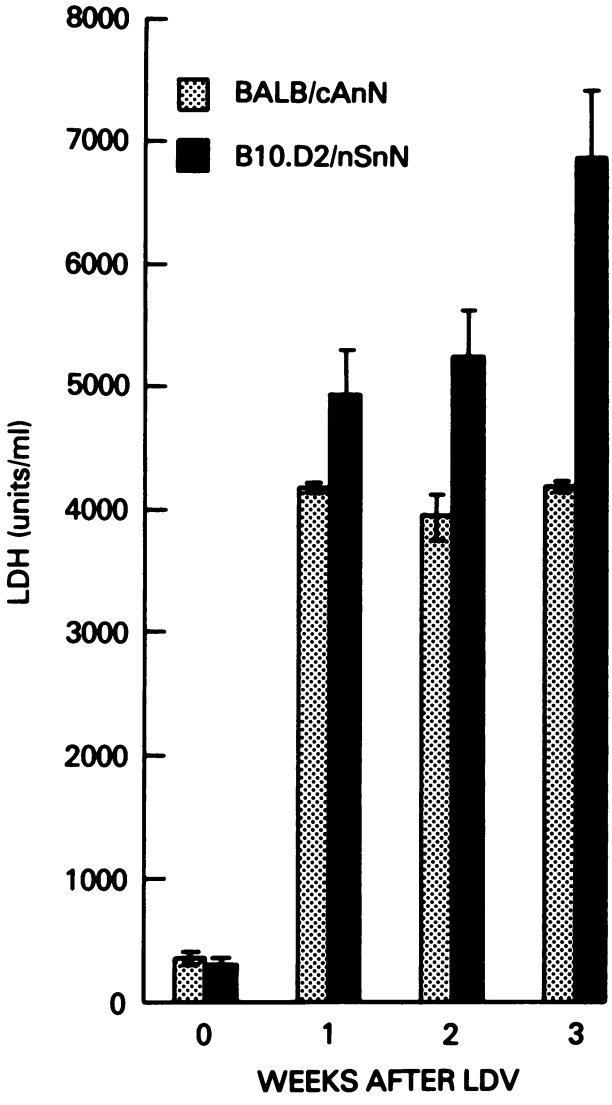

Figure 11—Effect of LDV infection on LDH levels in BALB/cAnN and B10.D2/nSnN mice. Mice, 5 weeks of age, were infected with LDV and at 0, 1, 2, and 3 weeks, thereafter, plasma LDH levels were determined.

count for the prolonged enzyme elevation (ie, greater than 6 months) observed in the silica-inoculated animals. In fact, it is known that silica can suppress the clearance of substances such as colloidal carbon, colloidal tin, and albumin microspheres.[13,30,31] Because silica can also damage cells, the possibility that LDH released from damaged cells might make some contribution to the influx and perhaps elevation of LDH in the plasma of silica-inoculated animals cannot be excluded.

These findings also raise the question as to the relation between enzyme clearance and other assays for measuring the functional capacity of the RES. LDV appears to infect a small subset of macrophages comprising about 15 to 20% of the macrophage population.[15,16] The authors do not know whether this subset has a greater or lesser capacity to clear enzymes than other cells of the RES. Because, however, enzyme clearance is more impaired in animals receiving both LDV and silica than in animals receiving either one alone, this argues that the subset of cells capable of clearing enzymes is far broader than just the LDV-infected subset. The sensitivity of the LDH clearance assay and the degree to which it does or does not overlap with already known assays of RES function also remains to be determined.

In addition to the environmentally-induced alteration in enzyme clearance, the current studies show that there are differences in enzyme clearance among inbred strains of mice. Thus, in diseases where the enzyme influx is increased due to tissue injury, animals that are slow enzyme clearers would be expected to have higher plasma enzyme levels than animals that are fast enzyme clearers. These experiments in animals, therefore, may have relevance to human diseases and the use of enzymes as diagnostic markers. Just as a glucose tolerance test is a more sensitive index of beta cell function than simply measuring fasting glucose levels, these studies show that the administration of an LDH load is a more sensitive and specific index of enzyme clearance then simply measuring resting LDH levels. Thus, without substantiation, a rise of plasma enzymes in animals or humans cannot be attributed exclusively to influx. In some situations, release of enzymes from acutely damaged tissue (eg, myocardial infarction) and their influx into the circulation is almost certainly the prime factor responsible for enzyme elevation,[32] but in other situations, where the disease process involves cells of the RES, impairment of enzyme clearance may be an important factor. This might apply to the enzyme elevation associated with diseases of the bone marrow, liver, and immune system such as anemia, hepatitis, acquired immune deficiency syndrome, and tumors.[1,33-35] The magnitude of enzyme elevation is thought to reflect the extent of acute tissue damage. But if, as demonstrated in these animal experiments, there are genetic and age-dependent differences in the rate of enzyme clearance among humans, then the magnitude of plasma enzyme elevation, even in acute tissue injury, may also reflect differences in enzyme clearance.

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