Leukocyte Lactate Dehydrogenase Changes as an Indicator of Infection Prior to Overt Symptoms

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Received for publication 1 May 1968

Lactic acid dehydrogenase (LDH) concentration of rabbit serum and leukocytes was followed during the course of an acute infection with *Diplococcus pneumoniae*. Control values were obtained prior to infection, and again 4, 24, and 48 hr later. LDH isozymes were characterized by acrylamide gel electrophoresis and quantitated by densitometry. An increase in serum LDH was observed as early as 4 hr after infection. These levels returned to normal in 24 hr and rose again 48 hr after infection. The LDH level of leukocytes, from 10 of 12 infected rabbits, rose rapidly during the 24-hr period after exposure. The levels were two to three times the original preinoculation level for that animal. In six of these rabbits, this LDH elevation occurred 4 hr after inoculation and preceded the onset of fever. Change in the type of leukocyte did not account for the increase in cellular LDH. All infected rabbits showed approximately the same increase in polymorphonuclear leukocytes, but not all developed comparable increases in LDH. The isozyme patterns obtained, when defined amounts of enzyme were applied to the gel for electrophoresis, were characterized for the most part by a three-enzyme pattern. Increasing amounts of enzyme occasionally revealed a fourth, more cathodal, enzyme. The more cathodic enzymes appear to be the most responsive when sudden shifts in enzyme concentration occur within the cell.

The clinical usefulness of serum lactate dehydrogenase (LDH) analyses has been established (8). Studies on 5,000 patients resulting in 15,000 analyses demonstrated the limitations resulting from the variability of the concentration of this enzyme in serum, as well as the variability in the distribution of its isozymes (11), and led to the consideration of leukocytes as a tissue source for the study of intracellular enzyme changes during the course of an infection. Small quantities of cells suffice for multiple analytical procedures, and the change in the enzyme content and isozyme distribution can be followed at frequent intervals during control and experimental periods. Small quantities of cells would allow for expansion to studies on human subjects. This report is concerned with the change in leukocyte LDH and its isozymes during the course of an acute pneumococcal infection in the rabbit and its role in the early demonstration of this condition.

**Materials and Methods**

Twenty-four male rabbits, weighing approximately 3 kg each, were divided randomly into four groups. Three rabbits (group I) received no treatment other than being bled according to the schedule set up for the experimental animals. Three rabbits (group II) were injected with 0.5 ml of brain heart infusion medium, the culture medium in which the organisms were grown. Six rabbits (group III) received 0.5 ml of culture filtrate containing pneumococcal polysaccharides. Twelve rabbits (group IV) received 0.5 ml of a 4-hr suspension of *Diplococcus pneumoniae* type I (strain A-5) containing 600 to 800 organisms (originally obtained from W. D. Sawyer, Johns Hopkins Medical School). All injections were administered subcutaneously.

Rabbits were bled before they were inoculated, and again after 4, 24, and 48 hr. A 7-ml amount of blood was removed from a marginal ear vein. A 2-ml amount was allowed to clot and the serum was separated for enzyme and protein assay. For separation of leukocytes, the remaining 5 ml was immediately added to a screw-capped plastic test tube (16 × 150 mm) containing 5 ml of 3% Dextran 150 (obtained from Pharmacia Fine Chemicals, Inc., New Market, N.J.) made up in EDTA-saline (0.06% disodium ethylenediaminetetraacetate in physiological saline).

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1 Presented in part at the 64th Annual Meeting of The American Association for the Advancement of Science, New York, N.Y., December 1967.
The pH was adjusted to 7.0 with 0.01 N HCl before the final solution was made. The tubes were mixed immediately by inversion and secured with a clamp at a 45° angle. They were maintained in this position, at room temperature, for 30 to 45 min. When it appeared that the erythrocytes had settled, the leukocyte-rich supernatant fluid was removed with a Pasteur pipette. To improve the yield of leukocytes, 5 ml of dextran solution was added to the sedimented cells and the procedure was repeated. After the erythrocytes had settled, the supernatant fluids of the first and second separations were pooled and the suspensions were centrifuged at 4°C at 2,000 × g for 10 min. Contaminating erythrocytes in the leukocyte pellet were removed by the method of Fallon (4) modified according to Kampaign (5), in that 3.6% NaCl was added to restore isotonicity. Cells were maintained at the lowered level of salt concentration for 20 sec. The cell suspensions were centrifuged again at 4°C at 2,000 × g for 5 min. The supernatant fluids were removed and each leukocyte pellet was suspended in 2 ml of EDTA-saline. The cells were counted in a hemocytometer (9). The suspensions were centrifuged again at 2,000 × g for 5 min. The supernatant fluids were discarded and a volume of glycerol and saline (50% by volume) was added to the cells, so that the final extract contained the equivalent of 10^9 cells per ml. Despite the lytic effect of the glycerol and the hypotonic nature of the solution, each suspension was homogenized in a small tissue grinder to ensure extraction of soluble enzymes. The homogenates were centrifuged at 4°C for 15 min at 20,000 × g and the pellets were discarded. A 5-ml amount of blood usually provided 10^9 to 1.5 × 10^10 cells.

Quantitative determinations of LDH were carried out by the method of Wroblewski and LaDue (10). Isozymes were separated in acrylamide gel, after the application of a specified number of units of enzyme to the columns, by the original method of electrophoresis outlined by Davis (2). Electrophoresis was carried out in a Canagco model 12 disc electrophoresis apparatus at 4°C, at a constant current of 5 ma per column, with a starting voltage of 20 v per column. The current was discontinued after 40 to 45 min when the tracking dye, bromophenol blue, reached the lower end of the tube. For staining isozymes, the gels were incubated for 30 min at 37°C in a 0.05 M Na lactate substrate in pH 7.5 tris(hydroxymethyl)aminomethane (Tris) buffer (0.05 M) containing KCN, nitro-blue tetrazolium, phenazine methosulfate, and diprophosphopyridine nucleotide in concentrations recommended by Allen (1).

Estimation of isozymes was carried out by scanning each gel in a Beckman Analyst modified for this purpose.

Proteins were determined by the Lowry method (6).

**RESULTS**

**Course of the infection.** Rabbits in the control groups remained afebrile throughout the experiment. Their leukocyte suspensions consisted of an average of 75% lymphocytes and 25% polymorphonuclear (PMN) leukocytes. At 4 hr after inoculation, leukocytes from group IV rabbits exhibited a rise in percentage of PMN leukocytes to 77%. They maintained this increase during the course of the disease. Temperature elevations of infected rabbits were noted 16 hr after exposure. Elevations in temperature persisted until their death 52 to 72 hr after inoculation.

**Concentration of LDH in serum.** The average serum LDH concentration in the control or pre-inoculation bleedings ranged from 42 to 130 units per ml (mean 87 ± 28). On repeat bleedings, the average value of the three control groups did not increase. In the pneumococcal-infected group IV, serum LDH concentration increased 100% in the 4-hr samples, returned to the original control value in 24-hr samples, then increased again 2.5 times in sera obtained 48 hr after infection (Fig. 1).

**Concentration of LDH in leukocytes.** The average LDH concentration of the leukocytes of 24 normal rabbits before any treatment and of leukocytes obtained from rabbits after injection with medium, culture filtrate, and a suspension of a culture of _D. pneumoniae_ is shown in Fig. 2. The average LDH content of normal rabbit leukocytes was 145 ± 62 units per 10^9 cells. Leukocytes obtained from rabbits bled at the four scheduled times during the 72-hr test period showed changes in LDH concentration of about the same order of magnitude in the control and in the medium- and filtrate-injected groups. In contrast, leukocytes of rabbits injected with _D. pneumoniae_ showed an increase in enzyme concentration as early as 4 hr after inoculation, a further

![Fig. 1. LDH concentration of rabbit serum (Wroblewski and LaDue units per ml of serum) prior to and during the course of an acute pneumococcal infection. Control group includes sera from uninoculated rabbits, rabbits inoculated with medium, and those inoculated with culture filtrate.](http://jb.asm.org/Downloaded from http://jb.asm.org/)

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increase 24 hr after inoculation, and a return, at 48 hr after inoculation, to the 4-hr level.

The range of LDH values as well as the mean in each group are summarized in Table 1. Variations in group IV, the pneumococcus-infected group, are worthy of note. Eight of the twelve infected rabbits exhibited significant increases in leukocyte LDH content within 4 hr after infection. The increase continued for the next 24 hr before the cellular enzyme level began to decline. Two rabbits exhibited a decline in leukocyte LDH level 4 hr after exposure, followed by a rise in LDH level 24 hr after exposure. On the other hand, leukocytes from two rabbits exhibited no elevation in LDH content after infection, but only a steady persistent decline, until 52 hr after exposure, just before their death, the enzyme level of their leukocytes was 10 units per 10^6 cells.

**Concentration of soluble cellular protein in leukocytes.** Soluble cellular protein did not remain constant during the 72-hr experimental period. All control groups showed similar changes. There was a decrease of about 14% in the average protein value of the 4-hr samples, with return to normal values at 24 and 48 hr. Proteins in leukocytes from infected rabbits increased, but not significantly, in all postinoculation leukocyte preparations, with one exception. This occurred in one of the rabbits that showed the persistent enzyme decline after infection. Figure 3 depicts these changes, as well as the changes in specific enzyme activity, that is, the ratio of cellular LDH content to protein content, during the course of infection. Despite the slight increase in soluble cellular protein, the ratio of LDH to protein in the leukocytes of infected rabbits exceeds the ratio of the controls throughout the experimental period.

**Isozymes of leukocyte LDH.** When separated by means of acrylamide gel electrophoresis, and when applied in defined enzyme concentration, the LDH isozymes of leukocyte extracts present very consistent patterns. When 100 units of LDH was applied to the column, 20 zymograms of the 24 leukocyte preparations obtained from preinoculation bleedings consisted of 3 closely spaced isozymes that appeared midway down the gel. The two most anodic of these isozymes were about equal in intensity and provided 75 to 85% of the total enzyme. The relative concentration of isozymes from leukocytes appeared to vary even in control rabbits after repeated bleedings. The most cathodic of the three major isozymes doubled in concentration. This isozyme decreased in relative concentration almost 50% from control concentration in leukocytes obtained from infected rabbits 24 and 48 hr after exposure. Typical examples of these changes are shown in Fig. 4.

The general consistency of this three-line pattern at 100 units makes exceptions noteworthy. In one group III rabbit injected with culture filtrate, a very anodic isozyme appeared in post-

![Fig. 2. LDH concentration of rabbit leukocyte extracts during the course of a pneumococcal infection. Symbols: (O) group I, control; (■) group II, medium injected; (△) group III, filtrate injected; (●) group IV, pneumococcus injected.](image)

### Table 1. LDH in rabbit leukocyte extracts in pneumococcal infection

<table>
<thead>
<tr>
<th>Time</th>
<th>Normal (1)</th>
<th>Medium-injected (3)</th>
<th>Filtrate-injected (6)</th>
<th>Pneumococcus-infected (12)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Range</td>
<td>Mean</td>
<td>Range</td>
</tr>
<tr>
<td>Preinoculation</td>
<td>155</td>
<td>107–180</td>
<td>120</td>
<td>100–132</td>
</tr>
<tr>
<td>4 hr postinoculation</td>
<td>166</td>
<td>108–234</td>
<td>147</td>
<td>121–159</td>
</tr>
<tr>
<td>24 hr postinoculation</td>
<td>199</td>
<td>156–234</td>
<td>151</td>
<td>143–159</td>
</tr>
<tr>
<td>48 hr postinoculation</td>
<td>127</td>
<td>85–184</td>
<td>207</td>
<td>196–218</td>
</tr>
</tbody>
</table>

* Number of rabbits in parentheses. Mean of 24 preinoculated rabbits = 145 ± 62 units per 10^6 of cells.
* Statistical significance compared with mean of P < 0.05.
* Statistical significance compared with mean of P < 0.01.
* Statistical significance compared with mean of P < 0.05.
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When more than 100 units of LDH was applied (up to 400 units), the majority of the leukocyte preparations maintained a three-isozyme pattern, with the added enzyme causing an increase in diffuse staining between each isozyme. This made quantitation by densitometry less accurate. On the other hand, 2 of 12 leukocyte preparations from infected rabbits, 24 hr after infection, had a 4th isozyme of more than 7%. One preparation obtained after 24 hr from a filtrate control, exhibited a similar zymogram (Fig. 5).

DISCUSSION

Most of our knowledge of organ LDH isozymes comes from tissues obtained after death. Leukocytes offer an active tissue, available for repeated analyses, so that changes under normal conditions, as well as conditions of stress and disease, can be studied. In contrast to the serum enzyme, leukocyte LDH does not shift in concentration as rapidly, and does not reflect the composite change of the whole individual. The changes that appear in the leukocyte reflect increases and decreases in response to the changing physiological requirement of the cells themselves.

Five LDH isozymes have been said to exist in tissues. By actual determination, the variability in concentration of individual isozymes in tissues under normal conditions and conditions of stress and infection has not been as extensively studied as have descriptive patterns of different organs injection leukocyte preparations. It appeared at the level of the albumin fraction in the first third of the gel, and represented about 10% of the total enzyme. In two leukocyte preparations obtained from a primary bleeding, an isozyme representing 1 to 2% of the total enzyme appeared at the cathode end of the gel, above the three major isozymes normally seen after electrophoresis of 100 units.

FIG. 3. (top) Protein concentration of leukocyte extracts during the course of a pneumococcal infection. Dotted line: average protein concentration of leukocyte extracts in the combined control groups. Solid line: average protein concentration of leukocyte extracts in the pneumococcus-infected group. (bottom) Specific activity of lactate dehydrogenase in leukocyte extracts during the course of a pneumococcal infection. Dotted line: average of the three control groups (normal, medium, and filtrate-injected). Solid line: average value of the pneumococcus-infected group.

FIG. 4. Isozymes of leukocyte LDH after electrophoresis of 100 units of enzyme. Zymogram 1: pattern of LDH isozymes from a leukocyte extract obtained from a preinoculation bleeding of a normal rabbit in group I. Zymogram 2: pattern of LDH isozymes from a leukocyte extract obtained from the third bleeding of the same normal rabbit. Zymogram 3: pattern of LDH isozymes from a leukocyte extract of the preinoculation bleeding of a rabbit in group IV. Zymogram 4: pattern of LDH isozymes from a leukocyte extract of the third bleeding, taken 24 hr after infection from the same rabbit as in zymogram 3.
and different species. Most studies have been carried out with starch-gel electrophoresis. Polyacrylamide gels offer the advantage of controlling the concentration of the gel and thus the pore size. It offers a gel that permits definitive separation of proteins that migrate through it, as well as one that selectively sieves out and prevents some molecules from entering it.

It has been pointed out by Dietz and Lubrano (3) that the method of Davis (2), which includes the use of a 7.0% separating gel as well as a stacking and sample gel, does not permit the separation of all five LDH isozymes. By omitting the stacking and sample gels and by substituting 40% sucrose as a sample diluent, they point out that all five isozymes become apparent. The number of isozymes obtained by using both methods was compared in several leukocyte preparations. We did not always obtain an increase in the number of isozymes by using the less-concentrated gel. There was considerable loss in the sharpness of the resolution of individual bands. For this reason, it was decided to use the method of Davis throughout this study. Under rigidly standardized conditions, differences in migration and concentration of isozymes could be compared and full advantage could be taken of the sharpness of separation obtained with the original method.

The number of isozymes that appear in the leukocyte LDH zymogram is, to some extent, characteristic of a particular individual. Most rabbit leukocyte zymograms at 100 units are characterized by a three-isozyme pattern. Some possess a small amount of a fourth enzyme. Differences in zymograms can be considered significant only if they are derived from like number of enzyme units. Their quantitative, as well as qualitative changes, can then be assessed. Whereas the mild stress of repeated bleedings appeared to bring about an increase in the most cathodal of the three major isozymes, an acute fulminating pneumococcal infection, accompanied by elevated temperatures, gave rise to a decrease of this same enzyme. Development of zymograms at different levels of enzyme concentration makes it possible to observe the appearance of isozymes present in too low a concentration for color development when only 100 units of enzyme are electrophoresed.

Changes in the leukocyte isozyme pattern are not wholly dependent on the type of cell in a mixed suspension. Differentials done on the final leukocyte suspensions indicated an average of 30% PMN leukocytes and 70% lymphocytes on all suspensions before infection. This ratio was not altered in the control groups, whereas a change in distribution of isozymes was noted with repeated bleedings. A reversal of the ratio of PMN to lymphocytes was noted immediately after and during the infection, yet the increase in total LDH was even greater at 24 hr than at 4 hr or 48 hr postinoculation. The higher concentration of LDH in PMN (7) could, in part, account for the elevation. However, it should be noted that (i) the two injected rabbits that showed the continuous decline in LDH activity of the leukocytes had the higher level of PMN throughout, and (ii) when the LDH activity declined in the other animals, the PMN level remained elevated. Still, the source of the increase in LDH activity is important. Work is continuing to determine the relative contribution of separated PMN and lymphocytes.

Thus, it appears that changes in enzyme con-

![Figure 5. Effect of concentration of enzyme on zymograms of leukocyte extracts. Zymograms 1, 2, and 3: patterns obtained after electrophoresis of increasing amounts of enzyme from a control leukocyte preparation. Zymograms 4, 5, and 6: patterns obtained after electrophoresis of increasing amounts of enzyme from a leukocyte preparation obtained 24 hr after pneumococcal infection.](http://jb.asm.org/Downloaded from http://jb.asm.org/)

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centration of circulating leukocytes, alone or together with changes in isozyme distribution, may demonstrate an exposure to an infectious agent prior to the appearance of clinical symptoms.

Acknowledgments
This study was supported by research contract DA 49193 MD 2598, U.S. Army Medical Research and Development Command.
We thank Nancy Green and Frances LeSane for excellent technical assistance.

Literature Cited