Effect of Intramuscular or Intrahepatic Injections of Clostridium perfringens on Rabbit Lactate Dehydrogenase

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Serum lactate dehydrogenase (LDH) activity, LDH isoenzyme pattern, phospholipase C activity, phosphorous level, hemoglobin, and erythrocyte osmotic fragility were followed in rabbits after intramuscular (IM) or intrahepatic (IH) injections of Clostridium perfringens. On the first day after IM injection, there was a drop in LDH activity; this was followed by an increase of LDH activity on the third and sixth day. On the seventh day, LDH activity began to decline, and by the ninth day it had almost returned to normal. On the sixth day after IM injection, there was an increase in serum LDH isoenzyme 5, hemoglobin, and erythrocyte osmotic fragility, but the increase of erythrocyte osmotic fragility and serum hemoglobin could not be attributed to phospholipase C activity since that enzyme was not detected nor was there an increase in serum phosphorus. C. perfringens was recovered by culturing the wound of IM-injected rabbits but not recovered from IH-injected rabbits. Rabbits injected IH showed no change from normal values in any of the tests performed.

Clostridium perfringens possesses a hemolytic and lethal alpha-toxin which has been found to be identical to phospholipase C (6), which catalyzes the hydrolytic cleavage of phosphatidylcholine into a diglyceride and phosphorylcholine. Phosphatidylcholine plays an important role in the membrane structure of mammalian erythrocytes, and it has been found that the sensitivity of various mammalian erythrocytes to C. perfringens increases with phosphatidylcholine content (7). Hemolysis of rabbit erythrocytes from the action of C. perfringens results in the release of various intracellular enzymes, one of which is lactate dehydrogenase (LDH; reference 9). The diagnostic value of LDH has been studied extensively. In patients exhibiting myocardial infarction there is an increase of LDH isoenzyme-one (LDH1; reference 8); in cases of liver disease, there is an increase of LDH2 and LDH3 (10); and, when rabbits were injected subcutaneously with Diplococcus pneumoniae, there was an increase of LDH activity and a shift toward the cathodic isoenzymes (5). The purpose of this work was to see whether, after intramuscular (IM) or intrahepatic (IH) injections of C. perfringens, there would be an increase in serum LDH and which isoenzyme was predominant. If there was an increase in LDH3, then muscle or liver injury would be suspected; if LDH1 had an increase, then the action of phospholipase C upon erythrocytes may be suspected.

MATERIALS AND METHODS

Organism, culture conditions, and injection procedure. C. perfringens (ATCC 3629) was incubated at 37 C for 24 hr in Cooked Meat Medium (Difco). The bacterial suspension was adjusted to 1.8 × 10^3 cells/ml by centrifugation and dilution with Cooked Meat Medium broth. An equal volume of this suspension of C. perfringens and 8 ml lactic acid was made to establish a necrotic environment for the growth of C. perfringens in rabbits; this suspension was injected immediately into the rabbits. Resting muscle was used, and the left gluteal muscle was the site of IM injections. Eighteen, 9-month-old, female, New Zealand white rabbits (Small Stock Industries, Lamoni, Iowa) were bled from the posterior marginal vein at 0, 3, 6, and 9 days prior to injection of C. perfringens. Three rabbits were injected IM and three were injected IH with 0.25 ml of the suspension, and then four rabbits were injected IM and four IH with 0.50 ml of the suspension. Two rabbits were injected IM and two IH with 0.50 ml of 8 ml lactic acid as an injection control.

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Determination of total serum LDH activity. Total activity of LDH was determined by the method of Wacker, Ulmer, and Vallée (9), and isoenzymes were separated and quantitated by the method used by Wright, Cavely, and Eberhardt (12).

Osmotic fragilities of erythrocytes. Osmotic fragilities were measured by the method of Dacie and Vaughan (1). Blood which showed hemolysis was washed once in isotonic saline and added to the various concentrations of sodium chloride with a 0.025 ml microtiter dropper (Linbro Chemical Co., Inc., New Haven, Conn.). Serum hemoglobin was determined by the method recommended by Fielding and Langley (3).

Phospholipase C activity determination. Portions of 1.0 ml and 2.0 ml of serum were added to test tubes containing: 2.0 ml of tris(hydroxymethyl)aminomethane-maleate buffer (0.1 M, pH 7.3), 0.5 ml of calcium chloride (0.50 M), and 1.5 ml of lecithin (2.0%). The serum substrate mixture was incubated in a 37°C water bath for 30 min, precipitated with 5.0% trichloroacetic acid, and centrifuged at 1,800 \times g for 5 min at room temperature. A 1-ml volume of the supernatant was evaporated with 1 ml of 5 N sulfuric acid at 80°C for 5 min, followed by the addition of 1 ml of 2 N nitric acid and heated to fuming. When the solution had cooled, 1 ml of deionized water was added, and the solution was boiled for 5 min. When this had cooled, 1 ml of 2.5% ammonium molybdate and 6.5 ml of deionized water were added to the tubes. At timed intervals, 0.4 ml of the reducing agent of Fiske and Subbarow was added. Absorbance was measured at 600 nm with a Beckman DU spectrophotometer after 10 min of incubation at room temperature.

RESULTS

Course of infection. An elevation of rectal temperature was recorded on the first through the seventh day after IM injection of 0.50 ml of the suspension of C. perfringens. Rabbits that were injected IM with 0.25 ml or IH with either 0.25 or 0.50 ml of the bacterial suspension showed no elevation of rectal temperature on any day after injection. Rabbits injected IM with 0.25 or 0.50 ml had large amounts of pus and sanguinous fluid in the area of injection on the sixth and ninth days, and bacilli were observed in all hematoxylin-stained tissue sections. Although there was a correlation between the size of the abscess and the amount injected IH, there was no correlation between the size of the abscess and the amount injected IM. It was also observed that on the sixth and ninth days after IM injection, C. perfringens was recovered by culture from the site of injection but was not recovered on any day from IH-injected rabbits (Table 1).

Total serum LDH activity. Rabbits injected IM with 0.50 ml of the suspension demonstrated a decrease in LDH activity at 24 hr after injection. Forty-eight hours after injection, there was an increase in LDH activity of approximately two times that of the pre-injected controls and of the lactic acid-injected controls. By the sixth day after injection, LDH activity reached its maximum, and by the seventh day a decrease in LDH activity was evident (Fig. 1).

LDH isoenzyme pattern. On the third day after IM injection of 0.50 ml of C. perfringens, LDH5 showed a slight increase in percentage of activity, by the sixth day LDH5 reached maximum activity, on the seventh day LDH5 began to decline, and on the ninth day LDH5 had almost returned to normal (Fig. 1). This elevation and decline of LDH5 was concomitant with the elevation and decline of total serum LDH activity. Rabbits that were injected IM with 0.25 ml or IH with either 0.25 or 0.50 ml of the suspension of C. perfringens showed no change from normal values.

<table>
<thead>
<tr>
<th>Method of injection</th>
<th>Vol ml</th>
<th>Size of abscess cm</th>
<th>Necrosis of tissue</th>
<th>C. perfringens recovered by culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>IM</td>
<td>0.25</td>
<td>TD</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>IM</td>
<td>0.50</td>
<td>TD</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>IH</td>
<td>0.25</td>
<td>0.8 × 1.0</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>IH</td>
<td>0.50</td>
<td>2.0 × 2.1</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

*TD, Too diffuse to determine.

![Fig. 1. Rabbit serum LDH activity after IM injections of lactic acid (■), C. perfringens (●); and percent activity of LDH5 after IM injection of C. perfringens (▲).](http://iai.asm.org/)
Osmotic fragility of erythrocytes and serum hemoglobin levels. Only on the sixth and seventh days was there any change in osmotic fragility of erythrocytes or serum hemoglobin. There was increased osmotic fragility of erythrocytes and serum hemoglobin in rabbits that were injected IM with 0.50 ml of the bacterial suspension on these days (Fig. 2). The increased serum hemoglobin did not affect the reading of osmotic fragility, because there is increased osmotic fragility with increased hypotonicity (Fig. 3) and the method used to determine osmotic fragility is not adequately sensitive to detect such small amounts of serum hemoglobin. Rabbits that were injected IM with 0.25 ml or IH with 0.25 or 0.50 ml of the bacterial suspension showed no change from normal values.

Phospholipase C activity and serum phosphorus determinations. There was no detectable phospholipase C activity in the sera nor was there an increase in serum phosphorus of any of the animals. The suspension of C. perfringens used for injection did have phospholipase C activity, and the cultures obtained when rabbits were sacrificed also had phospholipase C activity.

**DISCUSSION**

*C. perfringens* was more pathogenic in rabbits that were injected IM than in rabbits that were injected IH. This is evident since only in IM-injected rabbits was *C. perfringens* recovered by culture, an increase in LDH activity, and a change in LDH isoenzyme pattern. The pathogenicity of *C. perfringens* is apparently due to the ability of the microorganism to survive in the wounds of rabbits, and the ability of *C. perfringens* to survive in the wounds may be due to a number of factors. One of these factors may be the lower oxygen tension in muscle than in liver tissues (2), thus preferentially providing better growth conditions for *C. perfringens*. Another factor contributing to the pathogenicity of *C. perfringens* in muscle tissue is the lack of antibody-producing cells in muscle tissue and the relative lack of available phagocytes. Whereas the liver does possess immunocytes and phagocytes, the muscle is not known for its ability to produce antibody or phagocytes.

The increased osmotic fragility of erythrocytes and serum hemoglobin indicates that something attacked the erythrocyte cell membrane, altered its structure, and released hemoglobin into the serum. Since phospholipase C activity was not detected and there was no increase in serum phosphorus, the increased osmotic fragility of erythrocytes and the elevated serum hemoglobin could not be attributed to phospholipase C activity in serum. Zamecnik, Nathanson, and Aub (13) did not report phospholipase C activity in the blood of dogs that were injected IM with purified phospholipase C. However, dogs that were administered phospholipase C by slow intravenous (IV) drip did show a rise in the level of free phospholipase C activity. The rise in phospholipase C activity in IV-injected dogs was paralleled by a rise in plasma hemoglobin and a fall in the hematocrit; this rise in activity was reported in lethal doses and not in specific units of activity. The increased osmotic fragility of erythrocytes and

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**Fig. 2.** Osmotic fragility (○) and serum hemoglobin (□) of rabbits after IM injections of lactic acid, and osmotic fragility (△) and serum hemoglobin (●) after IM injections of *C. perfringens*. Rabbits were bled just prior to injection on day zero.

**Fig. 3.** Osmotic fragility of rabbit erythrocytes before (△) and 6 days after IM injection with 0.05 ml of *C. perfringens* (○).
elevated serum hemoglobin may be the result of nondetectable quantities of phospholipase C, the action of other toxins of *C. perfringens*, and of toxic by-products, or a combination of these.

Other infections also influence LDH activity. A rise in liver LDH activity subsequent to liver lesions produced by *Pasteurella multocida*, *Mycoplasma tuberculosis*, or peritonitis from sterile quartz powder was discovered by Hauss, Leppelman, and Planitz (4). When *P. multocida* was injected IM into rats, there was a decrease in liver LDH activity at 24 hr after injection, followed by a steady increase in liver LDH for 5 days after injection. Serum of rabbits injected subcutaneously with *D. pneumoniae* by Liese, Gray, and Ward (5) showed an increase in total activity of serum LDH as early as 4 hr after injection. This was followed by an onset of fever by 16 hr after injection, a decrease of total serum LDH activity by 24 hr after injection, and an increase of the total activity of serum LDH at 48 hr after injection. The next 48 hr revealed a slight decline in LDH activity, but the decline in LDH activity never fell to that level of preinjected controls. Electrophoresis of leukocyte extracts revealed the presence, at 24 hr after injection, of a more cathodic isoenzyme that was not present before infection. In my results there was a decrease of total LDH activity and a slight increase in LDH$_5$ at 24 hr after injection of *C. perfringens*. This was followed by an increase of total LDH and LDH$_5$, until the sixth day, when these values began to decline. Liese, Gray, and Ward (5) reported a decrease in total LDH activity and the appearance of a new, more cathodic, isoenzyme 24 hr after injection of *D. pneumoniae*. This was followed by a decline in total LDH activity and the disappearance of the isoenzyme within the next 24 hr. Although there is no explanation for the decline in the total LDH activity, this phenomenon has been observed by Hauss, Leppelman, and Planitz (4), Liese, Gray, and Ward (5), and myself. The slight discrepancy in results may be due to different organisms used, the different sites of injection, and the different methods of LDH determination and electrophoresis.

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**LITERATURE CITED**