INFECTION AND IMMUNITY, Apr. 1970, p. 363-372
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Vol. 1, No. 4
Printed in U.S.A.

Biochemical and Immunological Effects of *Listeria monocytogenes* Hemolysin

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Received for publication 26 November 1969

The biochemical and immunological effects of *Listeria monocytogenes* hemolysin in CD-1 mice were studied. Intraperitoneal injection of 256 complete hemolytic units (CHU) caused a twofold increase in plasma β-glucuronidase levels but was not lethal. In contrast, 256 or more CHU caused 100% lethality in 4 to 5 min when administered intravenously. Intravenous administration of 50 CHU caused a 10- to 11-fold increase in plasma β-glucuronidase levels and was lethal for a variable percentage of the animals. Carbon clearance experiments showed the phagocytic index to be depressed by relatively small amounts of intravenously administered hemolysin and suggested that hemolysin may function as a leucocidal agressin during listeric infection. Increased plasma levels of ornithine carbamyltransferase after intravenous injection of hemolysin indicated hepatocellular damage. Liver carbohydrate and blood glucose determinations on fasted mice showed a reduced gluconeogenic capability in hemolysin-treated animals. Mice immunized with purified hemolysin or live vaccine were more resistant to several of the toxic parameters studied. The data indicate that hemolysin is produced during listeric infection and is antigenic, but not necessarily a protective immunogen.

Some of the granulomatous lesions produced by *Listeria monocytogenes* suggest the action of a toxin. *Listeria* has not been shown to produce a classical exotoxin; however, toxic fractions have been prepared by disruption of the bacterial cell or extraction of the cell with organic solvents.

Although the derived fractions have been shown to possess varying degrees of toxicity for laboratory animals, their participation in the formation of granulomatous lesions has not been demonstrated. It seems improbable that these fractions would initiate the cellular damage observed in listeriosis, since extensive lysis of the bacterium in the primary lesion or later in the infection has not been shown. This suggests that cellular damage might be initiated by a toxin excreted during the active growth of *Listeria*.

Harvey and Faber (J. Bacteriol, p. 45-46, 1941) first reported the production of a soluble hemolysin by *L. monocytogenes*. A number of workers have partially characterized the physical and chemical properties of the lysin (11, 13, 22; M. Rogul and A. D. Alexander, Bacteriol. Proc., p. 82, 1964). The biological properties of the lysin have been less well studied.

Preliminary toxicity tests of hemolysin in guinea pigs, rabbits, and mice tended to exclude hemolysin as a classical exotoxin but not its functioning as an accessory factor in the pathogenesis of *Listeria* infections (11, 22).

In the preceding paper, we described the activity of *Listeria* hemolysin on release of hydrolytic enzymes from isolated lysosomes and degranulation of peritoneal monocytes in vitro (14). The present study deals with toxic effects of *Listeria* hemolysin in experimental animals.

MATERIALS AND METHODS

Bacterial cultures. *L. monocytogenes* strain 9-125, serotype 4b, was employed for production of hemolysin. *L. monocytogenes* strain A4413, serotype 4b, was used for active immunization of mice and for infection in LD_{50} determinations. Stock cultures were maintained on frozen tryptose agar (Difco) slants. Cultivation for production of hemolysin was in Brain Heart Infusion broth (Difco) at 37 C for 24 hr.

Experimental animals. White female mice (CD-1 strain, pathogen-free, from Charles River Mouse Farms, Wilmington, Mass.) weighing 14 to 18 g were maintained on Purina Laboratory Chow and water ad lib.

Purification of hemolysin. Hemolysin was prepared as described previously (16). Protein content of hemolysin preparations was determined by the method of Lowry et al. (16).

Titration of hemolysin. Hemolysin (0.5 ml) was

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added to 0.5 ml of phosphate-buffered saline (PBS) containing 6mM cysteine and incubated at 37 C for 30 min for reactivation. Doubling dilutions were made in PBS, and a 1% suspension of washed rabbit erythrocytes (0.5 ml) in PBS was added to each tube with immediate mixing. The suspensions were incubated at 37 C for 1 hr and observed for hemolysis. The titer was recorded as complete hemolytic units (CHU), i.e., the reciprocal of the dilution of the last tube showing complete lysis. The supernatant fluid after low-speed centrifugation (800 × *g*) was on occasion tested for residual hemolytic activity by addition of 0.1 ml of a 5% erythrocyte suspension. The residual hemolytic activity was consistently negative. Erythrocytes were prepared from rabbit blood obtained by aseptic cardiac puncture and added v/v to sterile Alsever's solution (5). Suspensions were stored at 6 C for 10 days prior to use and were discarded after 10 weeks.

Cholesterol inhibition. Cholesterol suspensions were prepared by the method of Cohen, Schwachman, and Perkins (6). Reactivated hemolysin was added to an equal volume of the cholesterol suspension and allowed to react for 30 min at 37 C. Cholesterol-inhibited hemolysin was used as a control in experiments with hemolysin.

Plasma enzymes. Plasma levels of β-glucuronidase were determined by a modification of the method of Fishman et al. (9), as described in the Worthington Manual (Worthington Biochemical Corporation, Freehold, N.J.). Plasma levels of ornithine carbamyltransferase were determined with commercially available kits (no. 1083, Sigma Chemical Co., St. Louis, Mo.).

Liver carbohydrate. For fasting experiments, food, but not water, was removed from mice in the evening prior to the period of maximal food intake. Hemolysin (50 CHU) was injected intravenously either at the beginning of the fast or 12 hr later. Fed animals were injected with hemolysin at 9:00 PM. Mice were bled from the orbital venous plexus with heparinized bleeding tubes and killed by decapitation. Livers were removed immediately and were frozen. The tissue was stored at −40 C until assayed for total liver carbohydrates. Carbohydrate was estimated by the anthrone method of Seifter et al. (24). Frozen livers were thawed, the excess moisture was removed by blotting on filter paper, and the tissue was weighed to the nearest 5 mg. Five livers were added to 30% KOH (15 ml) and digested by heating in a boiling water bath for 30 min. The digest was cooled and appropriate dilutions were made. A 10-ml amount of anthrone reagent (0.2% anthrone in 95% H_2SO_4) was rapidly added to 5.0 ml of dilute digest, and the color was allowed to develop for 10 min in a boiling-water bath. The tubes were cooled and read at 620 nm to determine the amount of carbohydrate present from a standard curve.

Blood components. Blood components were analyzed by using commercially available kits (Sigma Chemical Co., St. Louis, Mo.) according to the manufacturer's directions.

Blood glucose was determined colorimetrically at 620 nm from a protein-free filtrate after addition of copper and arsenomolybdate (Sigma Technical Bulletin no. 14).

Lactic acid was determined by using protein-free filtrates prepared immediately after blood was drawn. After the addition of lactic dehydrogenase and excess reduced β-nicotinamide adenine dinucleotide (β-NADH), the amount of β-NADH converted to β-nicotinamide adenine dinucleotide (β-NAD) was measured spectrophotometrically at 340 nm, and used to determine the amount of pyruvic acid originally present (Sigma Technical Bulletin no. 725-UV).

Carbon clearance. The method was a modification of that described by Biozzi et al. (4). Special biological ink C11/1431a (Guenther-Wagner Co., Hanover, Germany, obtained from John Henschell Co., Inc., Framingdale, N.Y.) was centrifuged at 3,000 × *g* for 30 min to remove large particles. Samples were dried to constant weight, and total solids were determined. The ink was diluted to a final concentration of 40 mg/ml in 1% gelatin. The carbon suspension was warmed to 40 C in a water bath prior to injection. Carbon (2.0 mg) was injected intravenously (0.05 ml), and the animals were bled from the retroorbital venous plexus after 5 and 10 min. Blood (0.05 ml) was transferred to 5.0 ml of 0.1% Na_2CO_3 and absorbancy was determined at 675 nm using a model 2000 recording spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). The amount of carbon per ml of blood was determined from a standard curve. The phagocytic index K was computed by using the following formula: $K = (\log C_1 - \log C_2)/T_1 - T_2$. C_1 and C_2 are the concentrations of carbon in micrograms per milliliter of blood at times T_1 and T_2.

Immunization. Female mice weighing 16 to 18 g at the start of the experiment were immunized according to the following schedule.

Group I. Nonreactivated hemolysin (200 CHU) was injected intraperitoneally at 3-day intervals for a total of three injections. Mice were bled and tested 7 days after the last injection.

Group II. Activated hemolysin (50 CHU) was injected intravenously at 3-day intervals for a total of three injections. Mice were bled and tested 7 days after the last injection.

Group III. Activated hemolysin (50 CHU) was injected intravenously as a single dose. Mice were bled and tested 7 days after the injection.

Group IV. Mice were injected intraperitoneally with 1.6×10^8 viable *L. monocytogenes* strain A4413. Animals were bled and tested 13 days later.

Group V. Normal animals received three intraperitoneal injections of PBS. Mice were bled and tested 7 days after the last injection.

Several animals from each immunized group and the control group were bled, and the sera were titered for neutralizing antibody. Protection was evaluated by using LD_{50} values, carbon clearance experiments, protection against lethal challenge with 100 CHU of hemolysin, and residual foot pad thickening.

Residual foot pad thickening. Mice were anesthetized with ether, the thickness of the left hind foot was measured, and 0.05 ml of culture filtrate was injected into the foot pad. The animals were sacrificed by ether overdose 24 hr later, and thickness of the feet was again measured.

Neutralizing antibody titration. Sera were diluted

1:10 in the first tube by using 0.9 ml of a 0.85% NaCl solution as diluent. Doubling dilutions were made thereafter. PBS (0.4 ml) containing 5 CHU of reactivated hemolysin per ml was added to each tube, with mixing, and incubated for 30 min at 37 C. A 0.1-ml amount of a 5.0% rabbit erythrocyte suspension was added to each tube, with mixing, and incubated an additional 1 hr at 37 C. Neutralizing antibody titer was recorded as the reciprocal of the highest dilution showing inhibition of lysis.

Statistical analysis. All data, where appropriate, were statistically analyzed. Statistical tests used were the *t* test and *z* test as described by Dixon and Massey (8) and the *u* test as described by Lewis (15).

RESULTS

Plasma β-glucuronidase and lethality. *Listeria* hemolysin has been shown to release hydrolytic enzymes from isolated lysosomes and initiate degranulation of peritoneal monocytes in vitro (14). To determine what effect, if any, hemolysin had on experimental animals, plasma β-glucuronidase levels were examined after intraperitoneal or intravenous administration of the lysin.

Figure 1 shows the changes in plasma β-glucuronidase levels at selected time intervals after an intraperitoneal injection of 256 complete hemolytic units. There was a twofold increase in the enzyme level 30 to 60 min after hemolysin injection. The level returned to near normal by 6 hr and remained there for at least an additional 18 hr. Nonreactivated and cholesterol-inhibited hemolysin controls exhibited normal values. Hemolysin concentrations up to 1,024 CHU were nonlethal when administered by the intraperitoneal route.

In contrast to intraperitoneal challenge, intravenous injection of 256 or more hemolytic units caused a convulsive, rapidly fatal reaction, with death occurring within 5 min of injection. Fifty CHU were lethal for a variable percentage of mice within 30 min of administration. Figure 2 shows plasma β-glucuronidase values of mice surviving this dose. Peak levels of 10- to 11-fold above normal were reached 1 hr after injection, with an apparent increase in clearance rate of the enzyme over that observed after intraperitoneal administration. The plasma levels returned to near normal by 4 hr and remained slightly elevated for 24 hr.

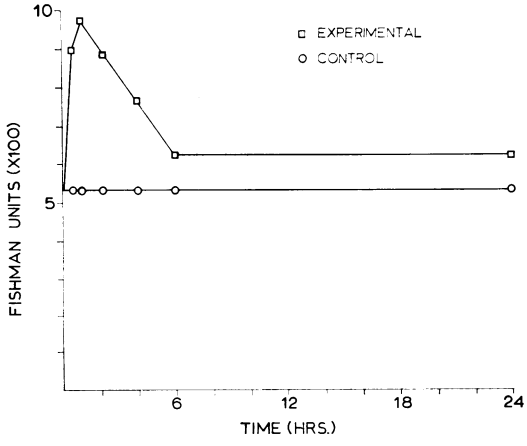

FIG. 1. *Plasma β-glucuronidase of mice receiving 256 hemolytic units intraperitoneally. Each point represents mean values from 15 to 20 mice.*

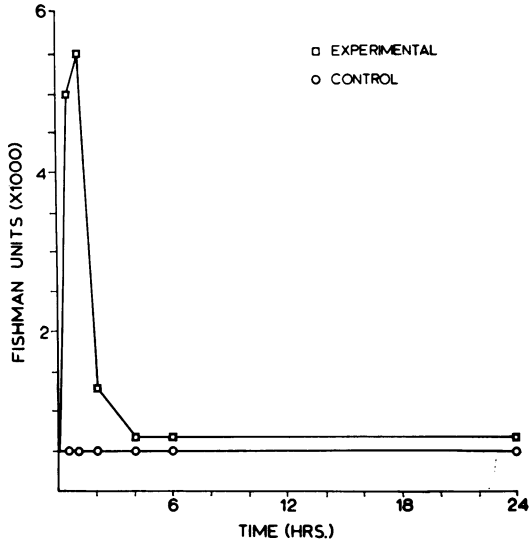

FIG. 2. *Plasma β-glucuronidase of mice receiving 50 hemolytic units intravenously. Each point represents mean values for 15 to 20 mice.*

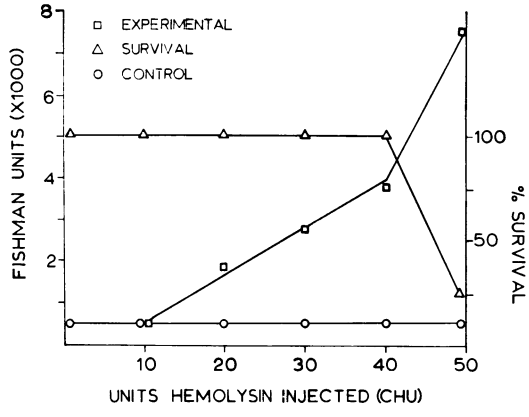

FIG. 3. *Plasma β-glucuronidase and per cent survival of mice receiving graded doses of hemolysin intravenously. Each point represents mean values from 20 to 30 mice.*

Intravenous injection of relatively small amounts of hemolysin caused an increase in plasma levels of β-glucuronidase. Figure 3 shows the response of enzyme levels and lethality of mice to graded doses of hemolysin. First evidence of damage was observed at 20 CHU with enzyme levels approximately fourfold greater than normal. A marked increase in plasma enzyme release and lethality (74%) occurred at 50 CHU. Nonreactivated, cholesterol-inhibited, and heat-inactivated hemolysin showed values identical to those of normal animals.

Carbon clearance. The preceding experiments suggested damage to the reticuloendothelial system (RES) as evidenced by release of lysosome-associated enzyme. To further substantiate this as a site of damage, carbon clearance experiments were performed on hemolysin-treated mice. The results (Fig. 4) showed a clearance rate reduced to near 20% of normal 2 hr after intravenous administration of 50 CHU. The phagocytic index showed a slow progressive increase but remained depressed significantly below normal for 48 hr after injection, returning to normal at 72 hr.

Small amounts of hemolysin were capable of diminishing reticuloendothelial activity. The depression of carbon clearance in response to graded amounts of intravenously administered hemolysin is shown in Fig. 5. Doses as low as 20 CHU was capable of reducing particle clearance. The phagocytic index fell to near 20% when 50 CHU was administered. Nonreactivated hemolysin and cysteine controls were not capable of depressing RES function.

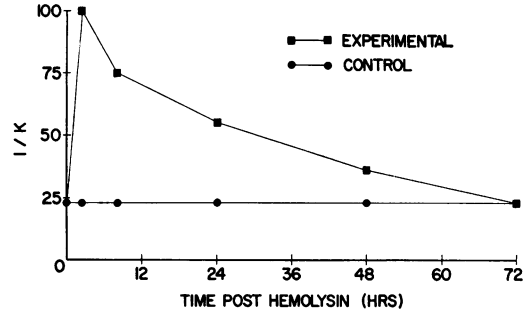

FIG. 4. *Carbon clearance in mice receiving 50 hemolytic units intravenously.*

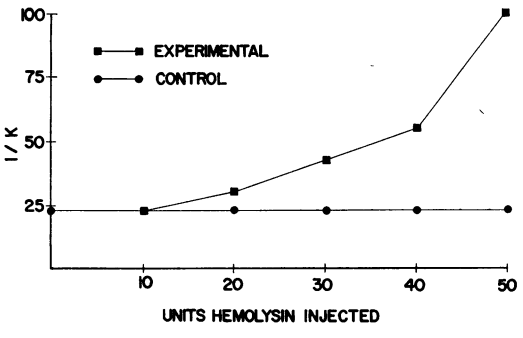

FIG. 5. *Carbon clearance in mice receiving graded doses of hemolysin intravenously.*

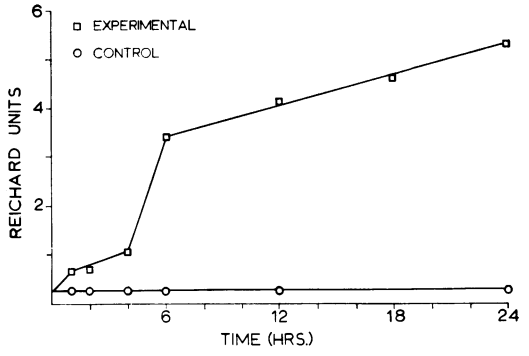

FIG. 6. *Plasma ornithine carbamyltransferase in mice receiving 50 hemolytic units intravenously. Each point represents mean values of 20 to 30 mice.*

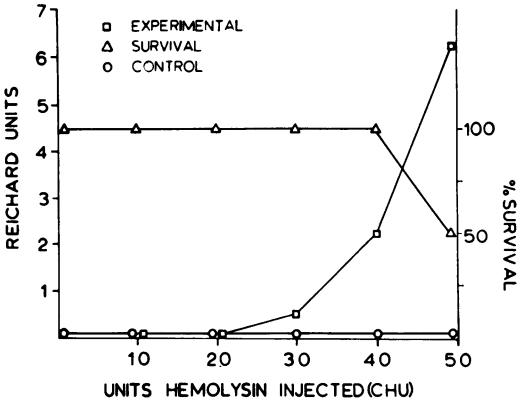

FIG. 7. *Plasma ornithine carbamyltransferase and per cent survival of mice receiving graded doses of hemolysin intravenously. Each point represents mean values of 20 to 30 mice.*

Ornithine carbamyltransferase. Depression of the phagocytic index suggested damage to the reticuloendothelial system of the liver, since small amounts of intravenously injected colloids are found in the Kupffer cells surrounding the hepatic portal tracts (2). To evaluate the possibility that hemolysin also caused liver damage, changes in plasma ornithine carbamyltransferase were determined (Fig. 6). This enzyme is specific for liver, having been reported to be localized in mitochondria of hepatocytes (20). Hepatic injury was indicated by an initial two- to fivefold increase in

enzyme levels, occurring up to 4 hr after intravenous injection of 50 CHU, followed by a marked increase at 6 hr. There is a progressive increase in values from 6 to at least 24 hr, with a plasma enzyme level approximately 25-fold greater than normal at 24 hr.

Administration of relatively small amounts of hemolysin caused increased plasma ornithine carbamyltransferase levels (Fig. 7). Initial release was observed when animals were challenged with 30 CHU, a slightly greater amount of hemolysin than was required to initiate increased plasma β-glucuronidase levels and depression of the phagocytic index. A marked increase in enzyme levels occurred at 40 and 50 CHU, with 50 units being lethal for some animals (50%).

Effects on liver carbohydrate. The previous experiments suggested that hemolysin caused extensive damage to hepatocytes, the major glycogen synthesizing and storage cell type. In addition, liver carbohydrate changes during infection with *L. monocytogenes* have been demonstrated (29). Experiments were performed by using both fasted and fed CD-1 mice to determine whether hepatocyte damage was extensive enough to result in a major metabolic derangement of the liver.

Liver carbohydrate values for fasted mice are shown in Fig. 8. Mice received 50 CHU intravenously either at the beginning of the fasting period or 12 hr after the fast began. Total liver carbohydrate declined in all groups including normal controls, reaching a minimum of 4 to 6 mg (wet weight) per g of liver at 24 hr. The peak level of liver carbohydrate for normal animals was at 36 hr. A slight difference between normal and experimental values was observed at 30 hr. Both experimental groups showed significantly different values at 36 hr, either remaining the same as the value at 30 hr or declining.

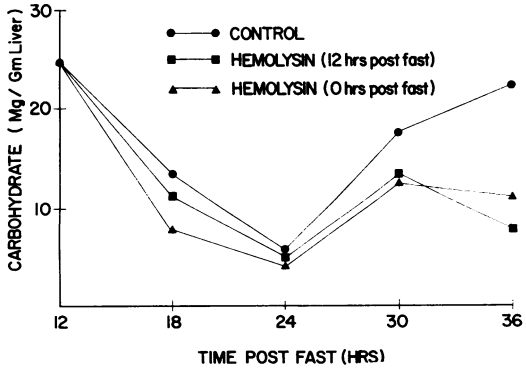

FIG. 8. *Total liver carbohydrate of fasted mice receiving 50 hemolytic units intravenously. Each point represents mean values of 25 to 40 mice.*

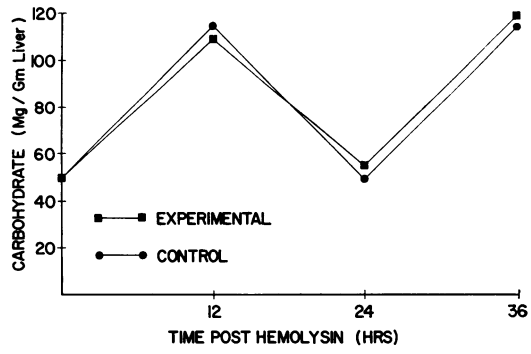

FIG. 9. *Total liver carbohydrate of fed mice receiving 50 hemolytic units intravenously. Each point represents mean values of 15 to 20 mice.*

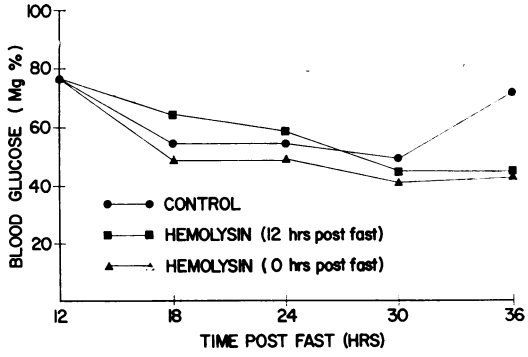

FIG. 10. *Blood glucose levels in fasted mice receiving 50 hemolytic units intravenously. Each point represents mean values of 25 to 40 mice.*

Total liver carbohydrate of fed animals is shown in Fig. 9. Hemolysin (50 CHU) was injected intravenously at 9:00 PM and total liver carbohydrate was determined every 12 hr. Liver carbohydrate values fluctuated in both the normal and experimental groups, giving the appearance of a diurnal rhythm in carbohydrate levels. No significant differences were observed between the normal and experimental values.

Blood glucose. Blood glucose levels are maintained throughout long periods of fasting at the expense of the major source, liver carbohydrate. During fasting, the blood glucose supplied by the liver must come from stored carbohydrate or noncarbohydrate precursors (28). To determine whether carbohydrate levels observed in the liver were reflected in blood, blood glucose levels were determined.

Blood glucose levels in fasted mice are shown in Fig. 10. There were no significant differences among the groups in the early phase of fasting. Blood glucose levels showed a slow progressive decline, reaching a minimum at 30 hr. Glucose

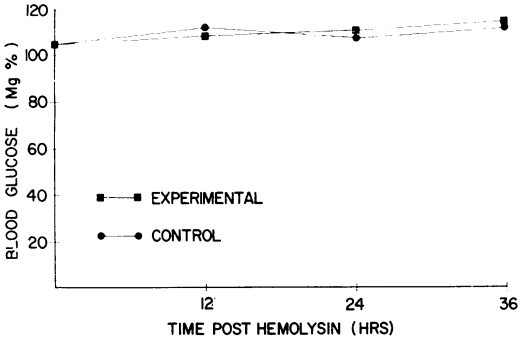

FIG. 11. *Blood glucose levels in fed mice receiving 50 hemolytic units intravenously. Each point represents mean values of 15 to 20 mice.*

TABLE 1. *Blood lactate and pyruvate levels in fasted mice receiving 50 hemolytic units intravenously*

Time post-fasting (hr)	Lactate[a]		Pyruvate[a]	
	Normal	Hemolysin	Normal	Hemolysin
12	19.7	19.0	1.49	1.50
24	17.8	18.7	1.57	1.70
36	19.1	24.7	1.75	1.73

[a] Each value represents the mean of 20 mice.

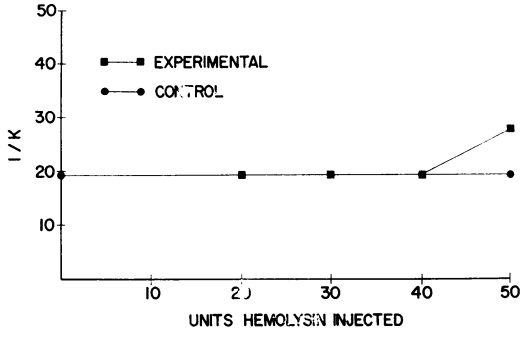

FIG. 12. *Carbon clearance in immune mice (group I) receiving graded doses of hemolysin intravenously.*

levels increased at 36 hr in the normal animals, reflecting the increased availability of liver carbohydrate. In contrast, blood glucose levels in the experimental hemolysin-treated groups remained the same as the 30-hr values, reflecting the lower liver carbohydrate values seen in the previous experiments.

Blood glucose levels of fed animals showed no significant differences between normal and experimental groups (Fig. 11). The fluctuation shown in liver carbohydrate of fed animals was not apparent in blood glucose levels.

Blood lactate and pyruvate. To determine whether the reduced capacity of hemolysin-treated mice to synthesize liver carbohydrate resulted from a diminished availability of glucose precursors, blood lactate and pyruvate levels in fasted animals were examined (Table 1). Blood pyruvate levels increased during the period of fasting in both normal and hemolysin-treated mice, but there was no significant difference between the two groups. Lactate levels remained fairly constant in both groups of animals with hemolysin-treated mice showing a slight increase at 36 hr. This increase was not considered to be significantly different than normal values.

Immune protection studies. Since the data indicated toxic and lethal properties for *Listeria* hemolysin, immune protection studies were undertaken to evaluate their participation in immunity to listeriosis. Carbon clearance in hemolysin immunized mice receiving graded doses of hemolysin was studied. Mice immunized by multiple intraperitoneal injection of 200 CHU nonreactivated hemolysin (group I) showed a greatly increased resistance to RES depression after intravenous administration of activated hemolysin (Fig. 12). The phagocytic index showed no reduction except at 50 CHU, with a carbon clearance value similar to that observed for normal mice at 20 CHU. Immunization with a single

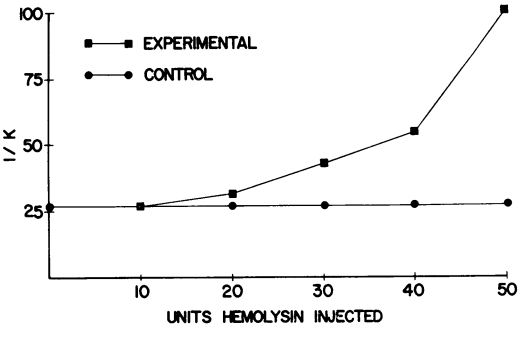

FIG. 13. *Carbon clearance in immune mice (group III) receiving graded doses of hemolysin intravenously.*

TABLE 2. *LD_{50} values in immunized mice*[a]

Group	LD_{50}
I	3.35×10^5
II	5.5×10^4
III	9.76×10^4
IV	1.89×10^8
V	1.4×10^4

[a] Data are from a representative experiment.

TABLE 3. *Hemolysin neutralization titers and protection against challenge with a lethal dose of hemolysin in immunized mice*[a]

Group	Neutralization titer	S/T[b] at 1 hr	Per cent survival
			%
I	1,280	6/10	60
II	320	1/10	10
III	320	1/10	10
IV	320	3/10	30
V	80	0/10	0

[a] Data are from a representative experiment.
[b] Survival/total.

intravenous injection of 50 CHU of activated hemolysin (group III) did not increase resistance to carbon clearance depression when compared to normal animals (Fig. 13).

Slight increases in LD_{50} values were observed when hemolysin-immunized mice were challenged with *L. monocytogenes* strain A4413, a strain of high virulence (Table 2). Nonimmunized mice (group V) showed an LD_{50} of 1.4×10^4. Mice immunized by multiple intraperitoneal injections of 200 CHU nonreactivated hemolysin showed an LD_{50} value of 3.35×10^5, whereas multiple (group II) and single (group I) intravenous injection of 50 CHU activated hemolysin showed LD_{50} values of 5.5×10^4 and 9.76×10^4, respectively.

Residual foot pad thickening was increased in hemolysin and actively immunized groups. Actively immunized mice showed a residual thickness of 0.7 mm. Group I and group II showed residual thickness of 0.26 and 0.43 mm, respectively, whereas normal animals showed 0.14 mm thickening. Group II was not tested.

Immunized mice possessed varying degrees of resistance to lethal challenge with intravenously administered hemolysin and increased serum neutralization titers (Table 3). Group I showed the highest neutralization titer, 1,280, as well as the greatest resistance (60% survival) after intravenous challenge with hemolysin. Mice immunized with live *L. monocytogenes* had a neutralization titer of 320 compared to a natural inhibitory titer of 80 in normal mouse sera. Group IV mice showed a 30% survival after hemolysin administration. Immunization with either single or multiple intravenous injection of activated hemolysin elicited a neutralization titer of 320 and slightly increased resistance to hemolysin (10%).

DISCUSSION

It is generally agreed that increased plasma or serum enzyme activity is due to cellular damage and that there is a correlation between the increase of individual enzymes and the dose of a toxin, or the size of the area supplied by a ligated vessel (21, 23, 30). Certain enzyme patterns are characteristic for a particular organ and allow identification without recourse to morphological methods. Severe cell damage with an acute onset leads to the rapid breakdown of cell membranes and a concomitant rapid increase in plasma enzyme levels (3).

Intraperitoneal administration of hemolysin resulted in a rapid increase in plasma β-glucuronidase levels, suggesting that peritoneal cells were damaged. The mechanism of hemolysin-initiated damage in vivo is presumably similar to that suggested for in vitro leucocidal activity (14). Rapid and marked increase in plasma β-glucuronidase after intravenous injection of hemolysin was observed, suggesting a severe cell damage and breakdown of cell membranes. Although it is not certain that hemolysin is the toxic factor responsible for increased lysosomal enzyme levels shown to occur during *Listeria* infection in mice (25), the present data strongly suggest that hemolysin is capable of initiating the increase.

The rapid return of plasma enzyme levels to near normal indicated that toxic activity was of short duration, suggesting that the lysin was rapidly detoxified, eliminated from the animal, or bound to cells prior to exerting its toxic activity. The latter alternative is probable, since it can be demonstrated that hemolysin sediments with cell stroma after lysis of erythrocytes (*unpublished data*). The first step in toxic injury by *Listeria* hemolysin may be a specific and irreversible binding of lysin to the cell membrane.

Intravenous administration of hemolysin in mice caused a pronounced depression in the clearance of colloidal carbon particles. This is indicative of damage to the reticuloendothelial system of the liver, since small amounts of colloids are cleared by the liver, being found in the Kupffer cells surrounding the portal tracts (2). It is probable that much of the increase in plasma β-glucuronidase observed after intravenous administration of hemolysin originated in the liver and in the Kupffer cells of the reticuloendothelial system. Dose response data showed good correlation between the minimal amount of hemolysin required to initiate increased plasma β-glucuronidase levels and depression of reticuloendothelial function.

Leucocidal properties of *Listeria* hemolysin have been demonstrated in vitro (14). The present data strongly indicate that leucocidal activity occurs in vivo as well. This suggests that hemolysin may function as an aggressin during the establishment and progress of listeric infection.

Phagocytosis by cells of the reticuloendothelial

system and subsequent intracellular digestion of bacteria is one of the main defense mechanisms of the host. Hemolysin-initiated depression of the RES could result in a decreased response of the host to the invading pathogen. Thus, the relationship between host phagocyte and *Listeria*, a facultative intracellular parasite, could be significantly altered in favor of infection, even with very small depression of RES function.

There is evidence that the ratio of healthy phagocytic cells to bacteria may be important in the progress of listeriosis. Within mouse macrophages in vitro, *L. monocytogenes* appeared to have a mean generation time of 4 to 8 hr and to kill the host cell when the intracellular population increased to approximately 16 bacteria per cell (18). Macrophages initially infected with a single organism released their content of pathogens at 20 hr (17). The kinetics of the second intracellular growth cycle were dependent on the number of organisms ingested by macrophages in the immediate environment. This relationship might exist in the host as well. *Listeria* hemolysin may tend to accelerate the progress of listeriosis by causing an alteration of the healthy phagocyte-to-bacterium ratio.

Listeria hemolysin appears to possess several other toxic properties in addition to its leucocidal activity. Intravenous but not intraperitoneal administration of small amounts of hemolysin results in the death of a variable percentage of the animals. Slightly larger doses uniformly caused a convulsive, rapidly fatal reaction in all mice. The mechanism of the lethal reaction is the subject of a separate report.

Jenkins and Watson (Bacteriol. Proc., p. 103, 1968) reported a decrease of lipoprotein in serum from rabbits which had received an intravenous injection of a hemolytic preparation. This was interpreted as indicating impaired liver function. The present experiments suggest that hemolysin may cause not only impairment of function but may be involved in primary or secondary damage to the liver parenchyma.

Ornithine carbamyltransferase, an enzyme found specifically in liver, increased after intravenous administration of hemolysin, indicating the presence of hepatocyte damage. The continued and progressive increases in plasma levels of the enzyme suggest that there is, in addition to a primary toxic effect, a secondary damage with possible extension of the damaged area into the surrounding healthy cells.

Listeria hemolysin did not appear to interfere with glycogenolysis or glycogenesis, since total liver carbohydrate and blood glucose of fed animals showed no significant difference between normal controls and hemolysin-treated animals. In addition, there were no significant differences in the rate of glycogen utilization between fasted hemolysin-treated mice and fasted control animals. However, there was a diminished gluconeogenic response in fasted mice receiving sublethal doses of hemolysin. This damage could occur at several levels, since the rate-limiting steps of the overall process may reside either at the site of substrate mobilization or at the liver itself (12). In the fasted animal, it is the hypoglycemia which normally initiates the primary response, activation of glycogenolytic enzymes. Glycogen stores are usually sufficient to maintain blood glucose levels for the period of time required to induce the gluconeogenic enzymes. Release of glucocorticoids causes the mobilization of glucose precursors which saturates the existing gluconeogenic enzymes and induces de novo synthesis of the rate-limiting enzymes (27). Decreased protein synthesis by the hepatocytes might also account for differences in liver carbohydrate and blood glucose between the fasted hemolysin-treated animals and control mice. A reduced capacity for de novo synthesis of rate-limiting gluconeogenic enzymes could account for the reduced gluconeogenic response. Failure to mobilize starting material or failure of these precursors to gain entrance into the cytoplasm of the liver cell could also account for a reduced synthetic capability. This would not appear to be the case, however, since there were no significant differences in blood lactate or pyruvate between experimental and normal fasted animals. The slight increase in lactate level at 36 hr in hemolysin-treated mice might be the result of a reduced gluconeogenic activity rather than the cause. The possibility of changes in glucocorticosteroid levels sufficient to effect rate-limiting enzymes or mobilization of precursor material was not included in this study. However, macroscopic changes in the adrenal glands were not noted in hemolysin-treated mice.

Preliminary histological examination of liver sections showed large, discrete patches of coagulation necrosis, whereas the parenchymal cells outside of the necrotic areas appeared normal (*unpublished data*). Part of the damage to hepatocytes could conceivably result from release of lysosome-associated acid hydrolases into the surrounding tissue. Several reports suggest that lysosomal enzymes can alter extracellular materials and induce inflammation. Protease released from lysosomes caused dissolution of chondromucoprotein of chick and rabbit cartilage in vitro and in vivo (7, 26). Degradation of collagen could also be effected by enzymes concentrated in the lysosomal fractions of rat liver (10). Presumably, hemolysin-initiated release of lysosomal acid hydrolases into the surrounding environment could

account for part of the histological alteration. However, it is not likely that the damage is a secondary result of this mechanism, since the data do not show correlation between lysosomal damage and the release of ornithine carbamyltransferase. It seems more probable that hemolysin causes direct damage to hepatocyte plasma membrane, resulting in hepatocyte destruction or changes in membrane permeability.

Mice previously exposed to *Listeria* hemolysin preparations or immunized with a live, highly virulent strain of *L. monocytogenes* were more resistant to several of the toxic parameters studied. Sera from all immunized groups showed an increased capability to neutralize toxic effects of hemolysin both in vitro and in vivo. In vitro neutralization titers increased in the sera of all groups of immunized mice. The increased resistance to depression of the RES and the reduced lethality after intravenous administration of hemolysin suggest that the lysin is neutralized in vivo. The in vivo neutralization presumably occurs through production of an antihemolysin antibody.

The increased serum neutralization titers and resistance to intravenous challenge with hemolysin in *Listeria*-immunized mice indicate that hemolysin is produced during listeriosis and is antigenic, but not necessarily a protective immunogen.

It is generally agreed that live vaccines are required to elicit a significant degree of resistance to listeric infection. In addition, resistance can be passively transferred by cells from immune donors, but not with hyperimmune sera. It is quite probable that there may be several factors involved in immunity to listeric infection, since it has been shown that immune macrophages more readily ingest and digest *Listeria* than do normal macrophages (1, 18, 19). Acquired cellular immunity in the mouse may also be due, in part, to the interaction of antigen and a specific antibody absorbed to the surface of macrophages (17).

Hemolysin acting as a single immunogen was not capable of increasing resistance to challenge with a highly virulent strain of *L. monocytogenes*. Increased residual foot pad thickening of immunized mice suggests that a delayed-type hypersensitivity might be elicited by hemolysin. It is possible that macrophage-bound antihemolysin antibody could be involved in immunity. It should be noted that there was a slight increase in resistance (up to 20-fold) in the hemolysin-immunized mice. Specific antibody bound to macrophage membrane could conceivably function to neutralize hemolysin and interfere with membrane disruption; however, in the absence of prior exposure to other factors in listeric infection, the bound antibody might not be significantly protective. Therefore, hemolysin cannot be ruled out as one of the protective antigens or agents on the basis of the present data.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI-04343 from the National Institute of Allergy and Infectious Diseases and by Public Health Service Training Grant 5 T1 GM-703 from the National Institute of General Medical Sciences.

G. Charles Kingdon was supported by Public Health Service predoctoral fellowship 5-F1-GM-32,329-03 from the National Institute of General Medical Sciences.

We thank R. E. McCallum, Louise Utt, and D. Thomas for their able assistance.

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