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Effects of *Listeria monocytogenes* Hemolysin on Phagocytic Cells and Lysosomes

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The effects of *Listeria monocytogenes* hemolysin on lysosomes and phagocytic cells were investigated. Hemolysin caused release of β-glucuronidase and acid phosphatase from suspensions of rabbit and rat lysosomes prepared from liver homogenates. The degree of lysis was proportional to the concentration of hemolysin added. There appeared to be no significant difference between the sensitivities of rat and rabbit lysosomes to disruption. Studies on the effect of *p*H and temperature on lytic activity suggested that hemolysin could function under conditions which might exist within phagocytic cells. Peritoneal exudates from rabbits and mice were exposed to hemolysin and observed by phase microscopy. Hemolysin possessed leucocidal activity and caused degranulation of both rabbit and mouse cells. Optimal activity against lysosomes and peritoneal exudate cells required activation of hemolysin with a reducing agent and could be prevented if hemolysin was previously incubated with cholesterol.

A number of workers have partially characterized the physical and chemical properties of soluble hemolysin produced by the facultative intracellular parasite *Listeria monocytogenes* (3, 5, 7; M. Rogul and A. D. Alexander, Bacteriol. Proc., p. 82, 1964). The lysin is nondializable, and susceptible to irreversible inactivation by trypsin or cholesterol. Hemolytic activity drops upon standing or after filtration through a sintered-glass filter (3, 7). Nearly complete activity can be restored by additon of reducing agents such as cysteine or hydrosulfite.

The influence of soluble hemolysin in the establishment and progress of listeric infection has not been elucidated. 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 (3, 7). Recent reports in the literature suggest the presence of a lytic factor, possibly hemolysin, during intracellular growth of the organism.

Njoku-Obi and Osebold (8) proposed that a cytolytic factor was operative in the in vitro interaction of sheep peritoneal exudate cells with *L. monocytogenes*. They suggested that the factor may be elaborated by the bacterium within phagocytes to provoke lysis of cells. Preliminary evidence indicated that *Listeria* hemolysin exerted a possible toxicity of a lytic nature on mouse peritoneal exudate cells obtained by glycogen stimulation (7).

Armstrong and Sword (1) obtained electron micrographs of *Listeria*-infected mouse spleen which indicated that a lytic factor was involved in the interaction of the bacterium and the phagocyte. They suggested that hemolysin may function to disrupt the membrane of the phagocytic vacuole or phagosome, allowing the phagocytized organism to escape the hydrolytic action of lysosomal enzymes.

The purpose of this study was to examine the activity of *Listeria* hemolysin on isolated lysosomes and peritoneal exudate cells. Subsequent reports will describe the toxic and lethal effects of *Listeria* hemolysin on experimental animals.

MATERIALS AND METHODS

Organism. *L. monocytogenes* strain 9-125 (also known as Potel no. 3 and 54 xxviii Murray) was employed for production of hemolysin. This strain, originally isolated by J. Potel from a human case of listeriosis in Halle, East Germany, was obtained from K. F. Girard, Massachusetts Public Health Department, Jamaica Plains, Mass. *L. monocytogenes* strain A 4413, serotype 4b, was used for immunization.

Cultivation. Stock cultures of *L. monocytogenes* were maintained on frozen tryptose agar (Difco) slants. Cultivation for production of hemolysin was in Brain Heart Infusion broth (BHI, Difco) at 37 C for 24 hr. Cultivation for immunization was in tryptose broth (Difco) at 37 C for 18 hr.

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Animals. Female New Zealand White rabbits (1 to 1.5 kg) were purchased locally. Female Sprague-Dawley rats (140 to 160 g) and white female mice (CD-1 strain, pathogen-free, 12 to 18 g) were purchased from Charles River Mouse Farms, North Wilmington, Mass.

Hemolysin purification. *L. monocytogenes* strain 9-125 was grown in 2 liter volumes for 24 hr in BHI. The bacteria were removed by centrifugation, and the supernatant fluid was sterilized by filtration through membrane filters of pore size 0.45 and 0.20 μm (Millipore Corp., Bedford, Mass.). Ammonium sulfate was added to 60% saturation followed by stirring for 18 hr at 8 C. The heavy brown precipitate was harvested by centrifugation and dissolved in a minimal amount of phosphate-buffered saline (PBS), 0.1 M, *p*H 6.6. The dissolved precipitate was dialyzed against distilled water containing 0.5% ethanol overnight at 8 C, and the euglobulin fraction was obtained by centrifugation. The precipitate was dissolved in 10 to 20 ml of PBS and stored at −20 C prior to use. This purification generally yielded 10 to 20 ml of hemolysin with a minimum specific activity of 8,500 complete hemolytic units (CHU) per mg of protein. For use, hemolysin was reactivated by addition of 0.006 M cysteine and incubated for 30 min at 37 C. Protein values for hemolysin were determined by the method of Lowry et al. (6).

Hemolysin titration. Hemolysin (0.5 ml) was added to 0.5 ml of PBS containing cysteine and incubated at 37 C for 30 min to allow reactivation. Doubling dilutions were made by using 0.5 ml of PBS as diluent. A washed 1% rabbit erythrocyte suspension (0.5 ml) in PBS was added to each tube and incubated at 37 C for 1 hr and observed for hemolysis. The titer was recorded as CHU, i.e., the inverse of the dilution of the last tube showing complete lysis of the rabbit erythrocytes.

Lysosome-containing large granule fraction (LGF). The LGF containing lysosomes was obtained by a modification of the method of Weissmann and Thomas (13). Livers removed immediately after the animals were killed were placed in phosphate-buffered sucrose (*p*H 6.6; 0.25 M). The tissue was weighed, minced, and washed to remove gross blood. Homogenates were made with a motor-driven Teflon homogenizer with buffered sucrose as diluent and centrifuged for 10 min at 800 $\times$ *g* to remove cell debris and nuclei. The supernatant fluid was decanted and centrifuged for 20 min at 15,000 $\times$ *g* to sediment the large granule fraction. This fraction was washed twice with buffered sucrose, resuspended in buffered sucrose to final concentration of 2 ml/g of tissue, and employed as a course of lysosomes. All procedures were carried out at 4 C.

Assay for lysis of lysosomes. Various amounts of reactivated hemolysin in 1 ml of PBS were added to 3 ml of a suspension of lysosome-containing fraction. The suspension was incubated at varying temperatures, *p*H values or periods of time, and centrifuged at 20,000 $\times$ *g* for 25 min to sediment intact lysosomes and disrupted membranes. The supernatant fluid was tested for β-glucuronidase and acid phosphatase activity as indicators of lysis.

Acid phosphatase. Acid phosphatase was determined by hydrolysis of *p*-nitrophenyl phosphate as described in Sigma technical bulletin 104 (Sigma Chemical Co., St. Louis, Mo.).

β-Glucuronidase. β-Glucuronidase was determined by the method of Fishman et al. (2), as described in Worthington Technical Bulletin (Worthington Biochemical Corp., Freehold, N.J.).

Buffers for pH experiments. Citrate (0.05 M)-phosphate (0.1 M) buffers were employed for *p*H values from 5.5 to 6.8, and phosphate buffers (0.1 M) were used for *p*H values from 6.3 to 8.0. The hemolysin and the LGF were suspended in the various buffers containing 0.25 M sucrose and 0.006 M cysteine.

Cholesterol suspension. Freshly recrystallized cholesterol (10 mg) was dissolved in 5.0 ml of absolute methanol. This was slowly poured into 50.0 ml of boiling distilled water to make a final concentration of 5.2×10^{-4} M. The suspension was cooled slowly and filtered.

Cholesterol-inhibition studies. The hemolysin was reactivated and 0.5 ml was added to 0.5 ml of the cholesterol suspension and allowed to react for 30 min at 37 C. A 3-ml amount of the LGF preparation was added, and the suspension was incubated for 1 hr at 37 C. Intact lysosomes and membranes were removed by centrifugation at 20,000 $\times$ *g* for 25 min, and the supernatant fluid was assayed for β-glucuronidase and acid phosphatase activity.

Comparison of sensitivities of rabbit and rat lysosomes. LGF preparations from rat and rabbit liver were standardized to contain β-glucuronidase activity which would hydrolyze 1.5 μmoles of phenolphthalein glucuronide per ml per 30 min. Doubling dilutions of the standardized preparations were made, a sample of each dilution was disrupted with Triton X100 (Rohm and Haas Co., Philadelphia, Pa.), and the enzyme activity was determined (Fig. 5, Total enzyme). Another 3.0-ml sample of each dilution was treated with 40 CHU (1.0 ml), incubated at 37 C for 80 min, and assayed for free β-glucuronidase activity (Fig. 5, Released by hemolysin).

Peritoneal exudate cells. Mice were killed by cervical dislocation, and the peritoneal cavity was washed with Hanks balanced salt solution containing 0.2% heat-inactivated fetal calf serum (BSS-S). The cells were washed by low-speed centrifugation (450 $\times$ *g*) and resuspended in BSS-S to a final concentration of 20,000 cells per ml. Mice given a single intraperitoneal injection of approximately 10^4 *L. monocytogenes* strain A4413 10 days prior to use were employed as a source of immune cells. Exudates were stimulated in rabbits by intraperitoneal injection of 50 ml of 0.1% glycogen in 0.85% NaCl. Animals were killed by exsanguination, and the peritoneal cavity was washed with BSS-S. The exudate cells were treated as described for mouse cells. Hemolysin (0.1 ml) was added to 1.0 ml of cell suspension, and degranulation of cells was observed with phase contrast optics.

RESULTS

Lytic activity of hemolysin on isolated lysosomes. *L. monocytogenes* hemolysin caused re-

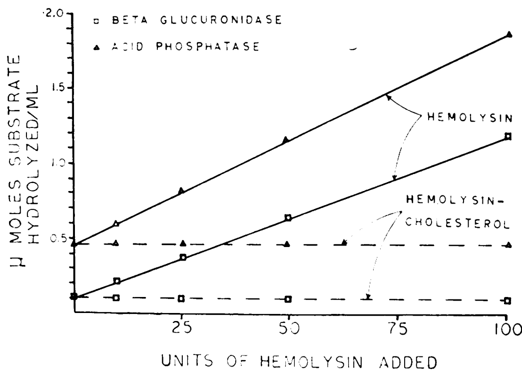

FIG. 1. *Effect of hemolysin on lysosomes from rabbit liver. Enzyme activities were assayed after 1 hr at 37 C.*

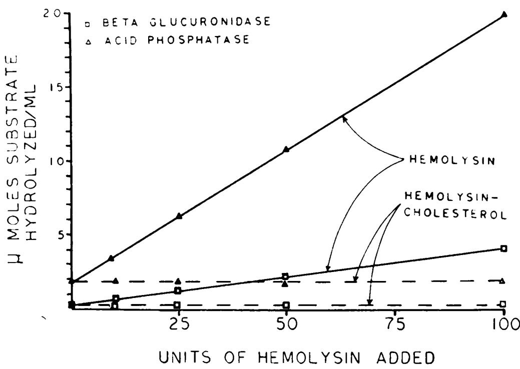

FIG. 2. *Effect of hemolysin on lysosomes from rat liver. Enzyme activities assayed after 1 hr of incubation at 37 C.*

lease of hydrolytic enzymes from isolated lysosomes as reflected by free acid phosphatase and β-glucuronidase activity proportional to the concentration of hemolysin employed (Fig. 1 and 2). The free enzyme activities of both acid phosphatase and β-glucuronidase were approximately fourfold greater at 100 than at 25 CHU. Lysis was prevented when hemolysin was first inhibited by cholesterol, or when not first reactivated with cysteine. The amount of enzyme released from rat lysosomes (Fig. 2) appeared to be approximately 10-fold greater than from rabbit lysosomes (Fig. 1) at each concentration of hemolysin. This would suggest that lysosomes from the rat, a species quite resistant to listeric infection, are more sensitive to disruption by hemolysin than lysosomes from rabbits. Subsequent data suggested that this was not the case. Rate of lysis studies on both rat and rabbit lysosomes with the use of a constant amount of hemolysin indicated optimal release of enzymes from rabbit lysosomes occurred after an 80-min incubation period (Fig. 3), whereas optimal release from rat lysosomes occurred after 60 min (Fig. 4).

Comparison of sensitivities of rabbit and rat lysosomes. A direct comparison of sensitivities of rabbit and rat lysosomes is shown in Fig. 5. The total enzyme activity of each dilution follows a predictable curve. Both the rat and rabbit preparations reached zero at a dilution of 1:64, as determined by the assay procedure. The straight slope of the lytic curve, representing those lysosome dilutions which contain sufficient numbers of lysosomes to allow a constant amount of hemolysin to exert full lytic activity, changed at a dilution of 1:4 for both rabbit and rat preparations. This suggests that there is no significant difference between the amount of β-glucuronidase released from either preparation after an 80-min

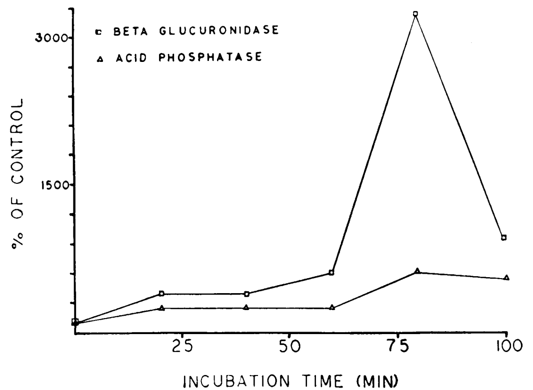

FIG. 3. *Effect of incubation time on lytic activity of hemolysin on lysosomes from rabbit liver. Fifty hemolytic units was used throughout, and enzyme activity was assayed after incubation at 37 C.*

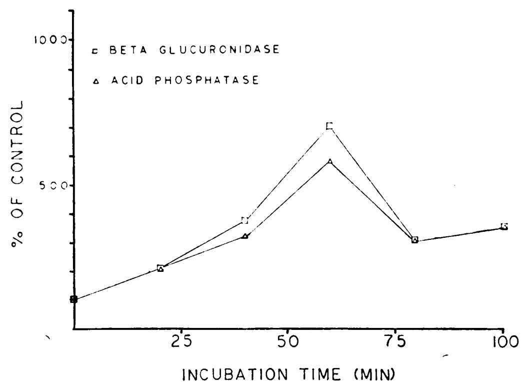

FIG. 4. *Effect of incubation time on lytic activity of hemolysin on lysosomes from rat liver. Twenty-five CHU were used throughout, and enzyme activity was determined after incubation at 37 C.*

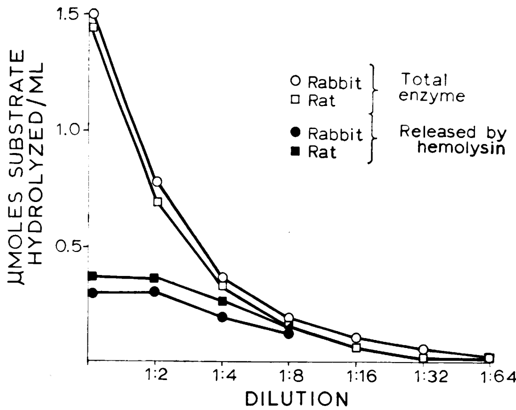

FIG. 5. *Comparison of sensitivities of rabbit and rat lysosomes to hemolysin. Forty CHU was used throughout, and β-glucuronidase activity was assayed after 80 min at 37 C.*

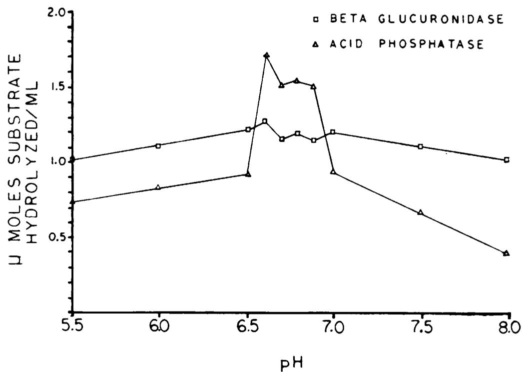

FIG. 6. *Effect of pH on lytic activity of hemolysin on lysosomes. One-hundred CHU was used throughout, and enzyme activity (experimental values minus control values) was assayed after 1 hr at 37 C.*

incubation period, by using a constant concentration of hemolysin.

Effect of pH and temperature on lytic activity of hemolysin. The results of overlapping *p*H studies are shown in Fig. 6. Lytic activity was generally increased through *p*H 6.6 to 6.9. Optimum lysis occurred at *p*H 6.6. The activity dropped off sharply below *p*H 6.6 and above *p*H 6.9; however, release of enzymes was significant at both extremes of the *p*H range tested. Activity below *p*H 5.5 and above *p*H 8.0 was not determined, since lysosomes are unstable and lyse spontaneously at these *p*H values.

There was no lytic activity when hemolysin was incubated with lysosomes at 0 C (Fig. 7). Lysis increased as the temperature was raised to 45 C. Lysis at 38 C was nearly twice as great as at 20 C. The differential between 38 and 45 C was less, although significant.

Degranulation of rabbit peritoneal exudate cells. The sequence of events leading to destruction of rabbit peritoneal exudate cells after treatment with hemolysin is shown in Fig. 8. Approximately 4 min after addition of hemolysin, there was increased movement of intracellular particles and the cytoplasm appeared to liquify. The cytoplasmic membrane, although appearing intact, seemed to be damaged, with pseudopods in evidence by 7.5 min (Fig. 8B). The phase-dense granules lysed "explosively" and were replaced with phase-clear areas or coalesced into slightly phase-dense patches. The density of the cytoplasm decreased, and the nucleus began to round up and lose density (Fig. 8C, 8D, and 8E). By 30 min (Fig. 8F), the cells showed marked cytological changes due, presumably, to the hydrolytic lysosomal enzymes released into the cytoplasm. Vacuolation of the cytoplasm was evident. A few cells did not degranulate, or degranulated only partially when lower concentrations of hemolysin were employed. When high concentrations of hemolysin were used, all cells rapidly degranulated. Degranulation did not occur when hemolysin was preincubated with cholesterol, or when not first reactivated by cysteine.

Degranulation of mouse peritoneal exudate cells. The effect of hemolysin on the phase-dense intracellular particles of mouse peritoneal exudate cells is shown in Fig. 9. Approximately 6 min after exposure to hemolysin, the cytoplasmic membrane showed evidence of injury (Fig. 9B). Numerous pseudopods formed, but degranulation was not observed. Degranulation began slowly after about 8 min (Fig. 9C) but occurred more slowly and was less pronounced than observed with rabbit cells. Lysis appeared more like a "fading effect" as the particles slowly lost phase

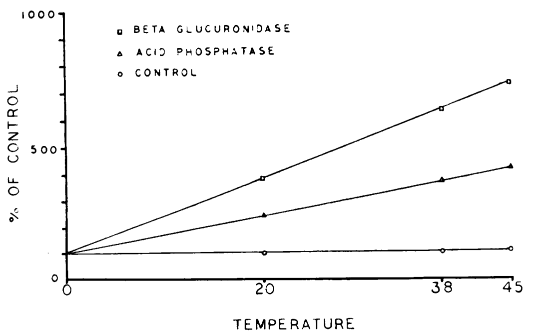

FIG. 7. *Effect of incubation temperature on lytic activity of hemolysin on lysosomes. Fifty CHU was used throughout, and enzyme activity was determined after 1 hr of incubation.*

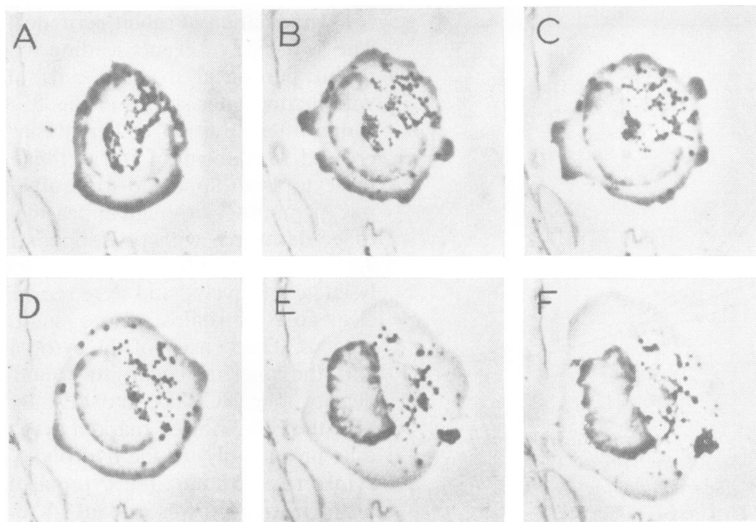

FIG. 8. *Rabbit peritoneal exudate cell showing the sequence of events leading to destruction of the cell after treatment with 50 CHU/ml. × 1,500. (A) 4.5 min; (B) 7.5 min; (C) 8 min; (D) 9.5 min; (E) 11 min; (F) 30 min.*

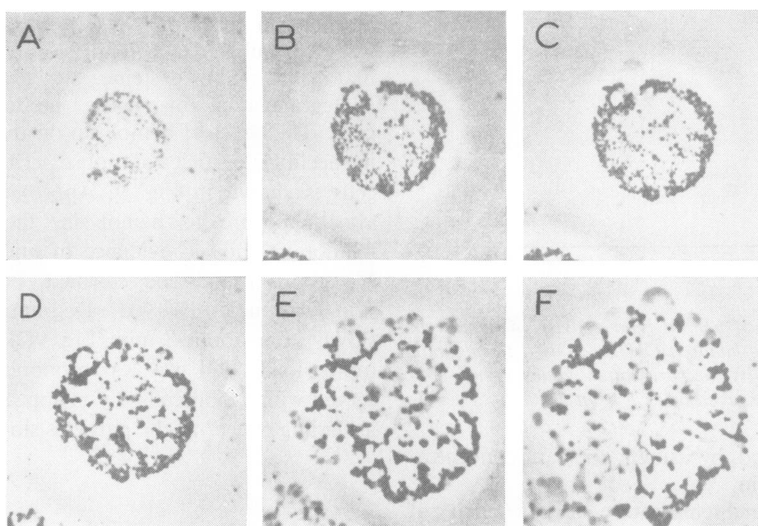

FIG. 9. *Mouse peritoneal exudate cell showing the sequence of events leading to cell destruction after treatment with 100 CHU/ml. × 1,350. (A) 5 min; (B) 6.5 min; (C) 8 min; (D) 9.5 min; (E) 11 min; (F) 30 min.*

density and were replaced by slightly phase-dense patches. As degranulation progressed, the lysis took on the explosive character seen in the rabbit cells. The cytoplasm appeared to liquify only after degranulation had begun. The nucleus, although visible, appeared to be vacuolated (Fig. 9E). By 30 min after exposure, the degranulation was almost complete and the nucleus no longer visable. A few cells did not degranulate, or degranulated only partially when lower concentrations of hemolysin were employed. Cells from immunized mice showed degranulation after exposure to hemolysin; however, increased amounts of hemolysin (up to 150 CHU/ml) were frequently required to initiate degranulation. Degranulation did not occur when hemolysin was preincubated with cholesterol or when not first reactivated with cysteine.

DISCUSSION

Listeria hemolysin caused the release of lysosome-associated enzymes from the isolated LGF of rabbit and rat liver. This suggests that hemolysin may play a role as a cytolytic or granu-

lolytic factor in *Listeria* infections. There is evidence that a lytic factor may be involved in the interaction of *Listeria* and the phagocyte. Njoku-Obi and Osebold (8) noted a cytolytic factor in the interaction of normal sheep peritoneal exudate cells with *L. monocytogenes* in vitro. Armstrong and Sword (1) observed phagosomal membrane disruption in electron micrographs of *Listeria*-infected mouse spleen and suggested that hemolysin may be the lytic factor.

The present data do not bear directly on hemolysin activity toward phagosomal membrane; however, it appears possible that hemolysin could be involved in such a function. The kinetics of lysis and the effect of temperature and *p*H on the lytic activity tend to support the view that hemolysin is an enzyme, possibly a lipase or phospholipase (5, 9) and that hemolysin could disrupt phagosomes or lysosomes by acting on the lipoprotein or phospholipid components of the membrane. The wide *p*H range for lytic activity suggests that hemolysin could function at the reduced *p*H levels commonly associated with the host cell during phagocytosis.

Sword and Wilder (12) have shown that several lysosome-associated enzymes increased in the plasma of *Listeria*-infected mice, but not when actively immune animals are challenged. The present data suggest that this may be due to a direct interaction between lysosomes and soluble hemolysin produced by the intracellular bacterium. Hemolysin produced within the cytoplasm of the phagocyte would be free to act on lysosomal membrane, causing the release of hydrolytic enzymes in the active state. Should this occur, the usual result is cellular death and autolysis (4). The lytic factor observed by Njoku-Obi and Osebold (8) may function in this manner.

The agent responsible for phagosomal membrane disruption would almost certainly need to be produced within the phagocytic vacuole. However, the changes observed by Sword and Wilder (12) and Njoku-Obi and Osebold (8) could also be initiated extracellularly.

Listeria hemolysin caused degranulation of both rabbit and mouse peritoneal cells; however, differences seemed to exist between the rate and nature of degranulation for the two types of cells. Phase-dense particles of the rabbit cells lysed "explosively," whereas the granules of the mouse cells gradually lost phase density and were replaced by a slightly phase-dense patch. The site of injury to both types of cells may be the cytoplasmic membrane rather than the lysosomal membrane as reported for streptolysin O by Hirsch et al. (4). Both rabbit and mouse cells showed the formation of numerous pseudopods prior to initiation of degranulation. Damage to the plasma membrane could result in altered ability to maintain osmotic differences, and the disruption of phase-dense particles could be a secondary event due to osmotic changes within the cytoplasm. This interpretation is in line with evidence offered by Zucker-Franklin (14) that the primary site of injury with streptolysin O was at the plasma membrane, causing increased membrane permeability which eventually resulted in osmotic lysis of the cells. Leakage of lysosomal enzymes from damaged and dead leukocytes may contribute to the increased plasma levels of lysosome-associated enzymes observed in *Listeria*-infected mice.

The demonstrated granulolytic and leucocidal activity of *Listeria* hemolysin suggests a role in listeric infection. Phagocytosis and subsequent intracellular digestion of bacteria are generally regarded as one of the main defense mechanisms of the host. The progress of listeric infection may depend, in part, on the capacity of the bacterium to influence the rate of ingestion or intracellular digestion by normal phagocytes. Hemolysin-induced damage to the plasma membrane of phagocytic cells could reduce the rate of ingestion and increase the bacterium to healthy phagocyte ratio.

The interaction between hemolysin and intracellular structures of the phagocyte would seem to be more complex. Disruption of the phagocytic membrane and escape of the bacterium into the cytoplasm of the host cell could effectively reduce the function of lysosome-associated antibacterial components. The intracytoplasmic organism would have a preferred position, being reasonably well protected from further phagocytosis during its intracellular multiplication.

The mechanism by which increased numbers of organisms are released from the host cell is not well understood. This might occur through hemolysin-mediated release of acid hydrolases from lysosomes and the subsequent dissolution of the host cell. The breakdown of lysosomes may, in addition, stimulate the growth of the pathogen. Sword (10, 11) reported that both in vitro and in vivo growth of *L. monocytogenes* and mortality from experimental infection appeared to be correlated with availability of iron to the bacteria. Sword (*unpublished data*) has also shown that in vitro growth of *L. monocytogenes* can be enhanced by inclusion of organic iron compounds such as ferritin in the growth medium. Ferritin iron and possibly other storage forms of iron commonly found within lysosomes and phagosomes may become available to the invading pathogen as a result of lysosomal damage and cell destruction (12). If these iron sources are utilized and enhance bacterial growth, it seems

likely that the majority of *Listeria* cells would be capable of producing more hemolysin and that this in turn would lead to further destruction of cells in the liver, spleen, and other tissues invaded by the organism.

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