Purification, Characterization, and Toxicity of the Sulfhydryl-Activated Hemolysin Listeriolysin O from Listeria monocytogenes

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We purified and characterized an extracellular hemolysin produced by Listeria monocytogenes. Hemolysin production was greatly enhanced by growing bacteria in resin (Chelex)-treated medium. This hemolysin was separated as a homogeneous protein of 60,000 daltons by using thiol-disulfide exchange affinity chromatography. This protein was a sulfhydryl-activated toxin, termed listeriolysin O, which shared the classical properties of other bacterial sulfhydryl-activated toxins: (i) inhibition by very low amounts of cholesterol; (ii) activation by reducing agents and suppression of the lytic activity by oxidation; (iii) antigenic cross-reactivity with streptolysin O. However, listeriolysin O differed remarkably from the other sulfhydryl-activated toxins in that its cytolytic activity towards erythrocytes from various animal species was maximum at low pH (≈5.5) and was undetectable at pH 7.0. This suggests that the lytic activity of the toxin in host tissues might be better expressed in the acidic microenvironment, including macrophage phagosomes where bacteria presumably replicate. Listeriolysin O was lethal to mice (50% lethal dose of ca. 0.8 μg) and induced a rapid inflammatory reaction when injected intradermally. These results favor the view that listeriolysin O might play a major role during intracellular replication of L. monocytogenes, ultimately promoting death of infected macrophages.

Listeria monocytogenes (sensu stricto) is a facultative intracellular bacterium responsible for severe infections of humans and animals. Virtually all strains isolated from natural infections produce a zone of hemolysis on blood agar medium (18, 35, 36, 39). However, the nature of the factor(s) responsible for this phenomenon remains controversial. After the demonstration of a soluble extracellular hemolysin by Harvey and Faber (20), a number of workers attempted to isolate it (17, 22–24, 26, 30, 38). The hemolysin (listeriolysin) was apparently a heat-labile antigenic protein, the lytic activity of which was enhanced by reducing agents and was suppressed by oxidation (22, 30) and by exposure to cholesterol (26) or to antistreptolysin O (anti-SLO) (22). These properties and the cardiotoxic effects of the hemolytic material (27, 37), which were apparently similar to those elicited by SLO (19), suggested that listeriolysin might belong to the group of the sulfhydryl (SH)-activated bacterial toxins (2, 40), the prototype of which is SLO (1). However, other reports were not in agreement with this view. In the first place, the hemolysin appeared to have a molecular weight (M<sub>r</sub>) of 170,000 (23), whereas SLO and related purified toxins have molecular weights in the range of 60,000 (26). Other studies indicated an M<sub>r</sub> of 10,000 for a material activatable by reducing agents (38). On the other hand, a lipolytic material of 52,000 M<sub>r</sub> was associated with the 170,000-M<sub>r</sub> hemolysin (23). A hemolytic lecithinase was also described (24). Recently, a partially purified SLO-like hemolysin of M<sub>r</sub> 55,000 to 60,000 released by Listeria ivanovii has been reported (32). Rabbit antisera against this hemolysin have been used for the detection of an M<sub>r</sub> 60,000 hemolysin, called α-listeriolysin, in L. monocytogenes (32). Another unrelated hemolysin(s) not detectable by these antisera was termed β-listeriolysin (32).

The pathogenic role of the hemolytic material during infection by L. monocytogenes is poorly understood. This material is cardiotoxic, lethal to mice (27, 37), and cytolytic for many eucaryotic cells, including macrophages (5, 25, 42). It also lyases isolated organelles such as lysosomes (25). These findings suggested that the hemolysin might be implicated in the mechanisms of the intracellular growth of L. monocytogenes in macrophages, as inferred by electron microscopic study of infected mouse spleen (5). This view has been questioned (6), but received recent support from a work showing a close relationship between virulence and hemolysin secretion (15). A nonhemolytic L. monocytogenes mutant obtained by transposon insertion was found to have lost the capacity to grow in host tissues of infected mice, and virulence was restored in its hemolytic revertant strain (15). The present study was undertaken to further characterize and to purify to homogeneity the putative SH-activated listeriolysin as a step toward the elucidation of the mechanisms of virulence of L. monocytogenes. Since toxin production is restricted in high iron concentration (13), bacteria were grown in a medium treated with Chelex, a procedure which has been described to remove free iron (31). This treatment allowed us to obtain high levels of hemolysin. The toxin was then separated from the culture supernatant as a homogeneous protein of M<sub>r</sub> 60,000 by using thiol-disulfide exchange affinity chromatography, as previously used for the purification of three SH-activated toxins, SLO (34), alveolysin (16), and perfringolysin O (J. E. Alouf and C. Geoffroy, manuscript in preparation). The purified hemolysin from L. monocytogenes displayed the classical
properties of the SH-activated toxins and induced strong inflammatory reactions.

**MATERIALS AND METHODS**

**Bacterial strain and culture media.** The hemolysin was purified from the hemolytic *L. monocyctogenes* strain EGD serovar 1/2a, originally from the Trudeau Institute. The bacteria were grown in brain heart infusion (BHI) broth (Diagnostics Pasteur, Marnes la Coquette, France) or in a Chelex-treated medium prepared as follows. A 10-fold-concentrated solution was first prepared: proteose peptone no. 3 (Difco Laboratories, Detroit, Mich.), 200 g; yeast extract (Difco), 50 g; Na2HPO4 · 12H2O, 83 g; KH2PO4, 7 g; quartz-distilled water, 1,000 ml. This solution was stirred (100 rpm) for 8 h at room temperature (Incubator Shaker, model G25; New Brunswick Scientific Co., Edison, N.J.). Chelex 100 beads (100 to 200 mesh, sodium form; Bio-Rad Laboratories, Richmond, Calif.) were added to obtain a 2% final suspension. The resin was then removed by filtration through a 0.45-μm-pore size Nalgene filter (Nalgene Co., Rochester, N.Y.). The Chelex-treated concentrate was further diluted in quartz-distilled water (1:10), adjusted to pH 7.5, and autoclaved at 115°C for 20 min. Sterile glucose (2% final) and sodium bicarbonate (0.25% final) were added before inoculation.

**Hemolysin purification.** Ten milliliters of an overnight bacterial culture in BHI broth was grown in 500 ml of Chelex-treated medium for 18 h at 37°C and then used to inoculate 27 liters of the same medium, dispensed in 5-liter flasks. After a 24-h incubation at 37°C, the bacteria were removed by centrifugation at 10,000 × g for 20 min. The supernatant fluid was concentrated by ultrafiltration at 4°C in an Amicon DC-2 apparatus (Lexington, Mass.), using Amicon H1P30 hollow fibers, which eliminate material of below Mt 30,000. The crude concentrate, adjusted to pH 6.0, was then passed through two tandem columns of thiopropyl-Sepharose 6B (Pharmacia, Uppsala, Sweden) prepared as follows. Four grams of freeze-dried thiopropyl-Sepharose 6B, containing ca. 35 μmol of available SH per ml of gel, was used for each column. The wet powder was allowed to swell in phosphate-buffered saline (PBS) (pH 6.0) for 2 h. The slurry was poured into a Pharmacia column (2.5 by 30 cm) and equilibrated by washing with 100 ml of buffer. The first column was previously reduced with 20 mM cysteine, allowing reduction of hemolysin. The resulting effluent was passed directly through the second column, retaining the hemolysin and other reduced materials, which were then eluted with 5 mM dithiothreitol in PBS (pH 7.5). The eluate was concentrated by ultrafiltration in a stirred cell equipped with an Amicon PM-30 membrane and gel filtered on a Sephacryl S-200 (Pharmacia) column (2.5 by 100 cm). The eluted hemolytic effluent was concentrated as before and passed through a Bio-Gel P-100 (Bio-Rad) column (2.5 by 100 cm), and the appropriate effluent was finally gel filtered on a Fractogel HW-50 (Merck Laboratories, Darmstadt, Federal Republic of Germany) column (2.5 by 100 cm). All columns were equilibrated and eluted with PBS (pH 6.0) containing 10% glycerol.

**Hemolysin assay.** The hemolysin assay is based on the estimation of the hemolytic activity of the toxin (activated with 20 mM cysteine) towards erythrocytes from humans and various animal species (sheep, horse, rabbit), as previously described (4). Measurement was made of the optical absorbance at 541 nm of hemoglobin released from erythrocytes (6 × 10⁸ cells per ml) incubated (at 37°C for 45 min) with 1 ml of appropriately diluted toxin in PBS (pH 6.0) containing 0.1% bovine albumin (Sigma Chemical Co., St. Louis, Mo.). One hemolytic unit (HU) is the amount of toxin needed to release half the hemoglobin (50% lysis) of the erythrocytes. It is estimated graphically by plotting percent lysis versus toxin volume on a log-probit graph (4).

**SDS-polyacrylamide gel electrophoresis.** Proteins were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described (28). Samples of 25 to 50 μl were boiled for 90 s in 2% (wt/vol) SDS–5% (vol/vol) 2-mercaptoethanol–10% (vol/vol) glycerol–0.002% (wt/vol) bromophenol blue in 0.1 M Tris hydrochloride buffer (pH 6.8). Electrophoresis was performed in a linear gradient of 7.5 to 25% (wt/vol) acrylamide at 5 mA for 15 h. The proteins were visualized by staining with Coomassie brilliant blue or silver nitrate.

**Purified SH-activated toxins and anti-SLO serum.** SLO and alveolysin were obtained by a procedure previously described (16, 34). Perfringolysin O was prepared similarly (unpublished data). Pneumolysin was kindly provided by Mary K. Johnson (Tulane University, New Orleans, La.). Hyperimmune horse anti-SLO (serum no. 525), previously described (3), and nonimmune horse serum (GIBCO, Paisley, Scotland) were used in immunoprecipitation and neutralization experiments.

**Hemolytic activity after incubation with steroids.** Steroids were dissolved in double-distilled absolute ethanol at a concentration of 1 mg of cholesterol per ml, or 10 mg/ml for epicholesterol and dehydroepiandrosterone (Sigma). One-milliliter samples of toxin at a concentration of 30 HU/ml in PBS (pH 6.0) supplemented with 0.1% ovalbumin (Sigma) were incubated for 30 min at 22°C with 10 μl of various dilutions of the tested steroids in ethanol. The controls included 1 ml of toxin and 10 μl of ethanol. The hemolytic activity of the mixture was then measured, and the concentration of sterol was determined as that inhibiting the hemolytic activity of 1 HU of hemolysin.

**Hemolytic activity after incubation with SH-group reagents.** Reagents used were HgCl₂, iodoacetic acid, N-ethylmaleimide, iodoacetamide, p-chloromercuribenzoate, tosyllysine chloromethyl ketone, and tosylphenylalanine chloromethyl ketone, provided by Sigma. All reagents were dissolved (10 mM) in PBS (pH 6.0), except for the last two, which were dissolved (100 mM) in absolute ethanol, and then incubated for 30 min at 22°C with toxin samples. The hemolytic activity was determined with the appropriate controls as described above for steroids.

**Mouse toxicity tests.** Specific-pathogen-free IC FR female Swiss mice (Charles River, St. Aubin les Elbeuf, France) 6 to 8 weeks old were used. Mice were injected intravenously (i.v.) or intraperitoneally with 0.5 ml of PBS (20 mM cysteine) at various pHs, containing increasing amounts of hemolysin. The 50% lethal dose (LD₅₀) was determined by the probit method. Neutralization experiments were carried out with pure toxin preincubated in vitro with cholesterol (0.2, wt/wt; 30 min at 37°C), with 1 mM of N-ethylmaleimide (3 h at 37°C), or with horse anti-SLO (no. 525) or nonimmune horse serum (appropriately diluted in PBS, pH 6.8, for 30 min at 37°C) and injected i.v. in a volume of 0.25 ml. To estimate the inflammatory potential of the hemolysin, various amounts of the toxin (diluted in PBS, pH 6.8) were injected into the right hind footpad of mice in a volume of 50 μl. Footpads were measured 30 min later with dial calipers (Schnittlaster, Hessen, Federal Republic of Germany) that are capable of measuring 0.05-mm increments in thickness.

**Other methods.** Agarose double-immunodiffusion assays...
were performed on glass slides in 1% agarose in PBS (pH 6.8). Wells of 4 mm were cut in the agar gel plates and filled with appropriate antigens and sera. Proteins were assayed according to the method of Bradford (9) with Bio-Rad reagents. Histological studies were performed on 1-μm-thick sections of the footpad fixed in Bouin solution, embedded in paraffin, and stained with hematoxylin and eosin.

RESULTS

Purification of the SH-activated hemolysin. Bacteria were grown for 18 h at 37°C in Chelex-treated medium, which allowed a remarkably greater hemolysin production. The hemolytic titer was about 1,500 HU/ml in the eluted medium (10⁶ bacteria per ml), as compared to 64 HU/ml in BHI (10¹⁰ bacteria per ml). A summary of the purification steps, all carried out at 4°C, is presented in Table 1. Culture supernatant (27 liters) was concentrated to 650 ml by ultrafiltration. The concentrate was applied to thiopropyl-Sepharose 6B columns, and the bound toxin was then eluted, concentrated to 16 ml, and applied to a Sephacryl S-200 column. The pooled effluents that contained the peak of hemolytic activity were sequentially passed through Bio-Gel P-100 and Fractogel HW-50 columns, and the eluate was concentrated to 8 ml. This fraction exhibited a specific activity of ca. 10⁶ HU/mg and appeared as a single polypeptide chain of Mr 60,000, visualized as one sharp band by SDS-polyacrylamide gel electrophoresis after staining with Coomassie brilliant blue (Fig. 1) or silver nitrate (not shown). Increasing the load of pure toxin up to 5 μg of protein did not reveal any additional band.

Characteristics of the purified hemolysin. The purified hemolysin displayed the usual properties of the SH-activated cytolysins. The hemolytic activity of the toxin was inhibited by cholesterol. In the presence of ovalbumin as protein protectant, 1 HU was inhibited by ca. 0.2 ng of cholesterol. Epicholesterol was a very weak inhibitor (15 μg/HU), although it did not inhibit alveolysin or SLO under the same conditions. Dehydroepiandrosterone, which has the same steroid nucleus as cholesterol but lacks the aliphatic side chain, was inactive even at 100 μg/HU, as previously found for other SH-activated toxins (33). The hemolytic activity of the toxin was totally inhibited by 1 mM HgCl₂ or ρ-chloromercuribenzoate. Inhibition by the mercurials was reversed by 2 mM dithiothreitol or cysteine. Iodoacetic acid at 2 mM, 2 mM iodoacetamide, 1 mM tosyllysine chloromethyl ketone, and 1 mM tosylphenylalanine chloromethyl ketone were not inhibitory. Under the same conditions, alveolysin activity was inhibited by 50 and 15% with the latter two reagents, respectively. The toxin was antigenically related to SLO, since the pure toxin gave a single immunoprecipitate line by gel diffusion versus horse anti-SLO (Fig. 2), but did not immunoprecipitate with nonimmune horse serum at all (not shown).

Effect of pH on hemolytic activity. The hemolytic activity of the purified hemolysin from L. monocytogenes as a function of pH was compared with those of SLO, perfringolysin O, pneumolysin, and alveolysin. The results observed on sheep erythrocytes are illustrated in Fig. 3. The activity of lyster hemolysin had the lowest optimum pH (pH 5.5), as compared to those of pneumolysin (pH 6.0), perfringolysin O and alveolysin (pH 6.5), and SLO (pH 7.0). In contrast to the other four hemolysins, the listerial hemolysin exhibited a narrow pH range of activity, and hemolysis was not observed at pH 7.0. The hemolytic activity was fully

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TABLE 1. Purification of SH-activated toxin from L. monocytogenes

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Vol (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (10⁶ HU)</th>
<th>Sp act (HU/mg of protein)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Culture supernatant fluid</td>
<td>27,000</td>
<td>7,290</td>
<td>43.00</td>
<td>5.925</td>
<td>100.0</td>
</tr>
<tr>
<td>2. Concentrated supernatant</td>
<td>650</td>
<td>2,145</td>
<td>42.00</td>
<td>21,666</td>
<td>98.0</td>
</tr>
<tr>
<td>3. Thiopropyl-Sepharose 6B chromatography</td>
<td>16</td>
<td>56</td>
<td>4.80</td>
<td>85.714</td>
<td>11.4</td>
</tr>
<tr>
<td>4. Sephacryl S-200 gel filtration</td>
<td>20</td>
<td>33</td>
<td>2.80</td>
<td>227,692</td>
<td>5.8</td>
</tr>
<tr>
<td>5. Bio-Gel P-100 gel filtration</td>
<td>4.5</td>
<td>3</td>
<td>1.35</td>
<td>437,782</td>
<td>3.1</td>
</tr>
<tr>
<td>6. Fractogel HW-50 gel filtration</td>
<td>8.0</td>
<td>0.60</td>
<td>0.60</td>
<td>1,000,000.000</td>
<td>1.3</td>
</tr>
</tbody>
</table>

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FIG. 1. SDS-polyacrylamide gel electrophoresis of Table 1 fractions (30 μl, 2,000 to 9,000 HU). Lanes: 1, culture supernatant (8 μg); 2, step 2 (10 μg); 3, step 3 (100 μg); 4, step 4 (50 μg); 5, step 5 (20 μg); 6, step 6 (2.2 μg); 7, molecular weight markers. Gels were stained with Coomassie brilliant blue. Purified hemolysin (lane 6) migrates as a single band of M, ca. 60,000.

FIG. 2. Immunological cross-reactivity between L. monocytogenes hemolysin and SLO. Upper wells: right, purified hemolysin (10 μg); left, purified SLO (10 μg). Bottom well: 20 μl of undiluted equine anti-SLO no. 525. No immunoprecipitation was observed with nonimmune horse serum used as controls (not shown).
restored by lowering the pH from 7.0 to 5.5. Similar results were observed with human, horse, and rabbit erythrocytes.

**Mouse toxicity.** The LD$_{50}$ of purified hemolysin was determined by i.v. injection into mice. The LD$_{50}$ was estimated at about 0.8 µg per mouse (Table 2). Mice died with convulsions and opisthotonos. When toxin was administered in PBS at pH 6.8, 6.0, or 5.5, the mice died within 1 to 2 min, as opposed to control mice, which received the same volume (0.5 ml) of PBS at various pH levels but did not display any clinical symptom. At pH 7.2, the mice died after a delay of 30 to 60 min, but the LD$_{50}$ was the same. When toxin was injected by the intraperitoneal route, animals died several hours later, and the LD$_{50}$ was assessed to be about 1.7 µg per mouse. No mortality was observed from injecting intradermally up to 5 µg of toxin.

The hemolysin lost all its lethal potential when treated with 1 mM N-ethylmaleimide (3 h at 37ºC) with cholesterol (0.2, wt/wt; 30 min at 37ºC), or with heat (1 h at 60ºC) (Table 2). In addition, a lethal dose of toxin (1 µg) was neutralized by incubation (30 min at 37ºC) with equine anti-SLO before i.v. injection (Table 2). In contrast to control mice receiving the same amount of toxin preincubated with nonimmune horse serum, which died within minutes, the animals injected with neutralized toxin survived either indefinitely (undiluted anti-SLO) or only for 5 to 6 h (anti-SLO 1:10 in PBS, pH 6.8).

The toxin diluted in PBS (pH 6.8) was also injected intradermally into mouse footpads. It induced a rapid inflammatory response that peaked in 0.5 h and slowly faded over the next 24 h. Histological examination showed an exudate mostly constituted of polymorphonuclear cells. The dose dependence of this inflammatory reaction is shown in Fig. 4. One microgram of hemolysin induced an inflammatory swelling of about 20 U (1 U = 0.1 mm) of thickness. Even minute doses (0.01 µg) were capable of inducing a significant reaction (~6 U). When the toxin was previously incubated with cholesterol, the inflammatory reaction was almost completely abrogated.

**DISCUSSION**

Although it has generally been thought that *L. monocytogenes* produces an SH-activated hemolysin, this hemolysin had not previously been available in a sufficiently homogeneous form to assess its nature unambiguously. The failure of previous attempts to purify it is very likely due to several factors: (i) the low levels of listeriolysin released in conventional culture media (20 to 200 HU/ml), which also vary from strain to strain (32); (ii) inappropriate purification methods; (iii) the presence of more than one hemolysin in culture fluids (23, 24, 32, 42). These difficulties were overcome in the present work, which, for the first time allowed the purification of the hemolysin to homogeneity and its clear-cut characterization as an SH-activated toxin. A high level of extracellular toxin (1,500 HU/ml) was obtained in a resin (Chelex)-treated broth, as opposed to the amount of hemolysin produced in BHI broth (64 HU/ml). This confirmed previous observations indicating that toxin production is optimum at low iron levels and declines as a function of increasing iron concentration (13). Similar iron control is known for diphtheria toxin, *Pseudomonas aeruginosa* exotoxin A, and other toxins (21). The purification of the hemolysin was initiated with thiol-disulfide exchange columns, which selectively retain SH-containing proteins. Any other crucial technical improvement which allowed us to obtain homogeneous hemolysin was the final gel filtration step through Fractogel HW-50, a new hydrophilic matrix. The yield of toxin purification was very low, due to important losses at stage 3 of the purification (Table 1). An improvement of the purification procedure is currently in progress.

**TABLE 2.** Toxicity of hemolysin in mice

<table>
<thead>
<tr>
<th>Hemolysin (µg)</th>
<th>Treatment before injection</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td></td>
<td>10/10</td>
</tr>
<tr>
<td>2.5</td>
<td></td>
<td>10/10</td>
</tr>
<tr>
<td>1.2</td>
<td></td>
<td>10/10</td>
</tr>
<tr>
<td>0.6</td>
<td></td>
<td>0/10</td>
</tr>
<tr>
<td>5.0</td>
<td>Cholesterol*</td>
<td>0/10</td>
</tr>
<tr>
<td>5.0</td>
<td>N-ethylmaleimide*</td>
<td>0/10</td>
</tr>
<tr>
<td>5.0</td>
<td>Heating*</td>
<td>0/10</td>
</tr>
<tr>
<td>1.0</td>
<td>Anti-SLO (1:1)</td>
<td>0/5</td>
</tr>
<tr>
<td>1.0</td>
<td>Anti-SLO (1:10)</td>
<td>5/5</td>
</tr>
<tr>
<td>1.0</td>
<td>Normal serum (1:1)</td>
<td>5/5</td>
</tr>
<tr>
<td>1.0</td>
<td>Normal serum (1:10)</td>
<td>5/5</td>
</tr>
</tbody>
</table>

* Hemolysin was injected intravenously in a volume of 0.5 ml of PBS (pH 6.8).

* Mortality in 6- to 8-week-old Swiss mice: number of dead mice/total of injected mice; except in the group receiving anti-SLO 1:10, in which mice survived only for 5 to 6 h, all mice died within minutes after i.v. injection.

* Incubation with cholesterol (0.2, wt/wt; 30 min at 37ºC).

* Incubation with 1 mM N-ethylmaleimide (3 h at 37ºC).

* For 1 h at 60ºC.
The purified toxin displayed a specific activity of the same order (10^6 HU/mg) as that reported for other purified SH-activated toxins (3). Its molecular weight was also similar (ca. 60,000). The toxin was lethal to mice at a level (LD50 of 0.8 µg) comparable to that reported for SLO (0.2 µg) (1). It displayed the classical properties of the SH-activated toxins: (i) it is inhibited by very low amounts of cholesterol; (ii) it is activated by reducing agents and its toxicity is suppressed by reagents which modify SH groups; (iii) it is antigenically related to SLO, as assessed by immunoprecipitation and neutralization tests with horse anti-SLO. On the basis of these similarities, we propose that this hemolysin be termed listeriolyisin O, according to the nomenclature recommended by Bernheimer when other cytolysins(s) than the SH-activated one are produced by a microorganism, as is the case for Streptococcus sp. and Clostridium sp. (7). Whether α-listeriolysin (32) and listeriolyisin O are identical remains to be clarified in spite of apparent common properties as regards molecular weight and cross-reactivity with SLO. Moreover, by using immunoblotting with a rabbit immune serum raised against highly purified listeriolyisin O, we detected this toxin in culture supernatants of bacteria, grown under adequate conditions as described in this work, from all L. monocytogenes strains currently tested and obtained from human clinical isolates (manuscript in preparation).

Several of our findings concerning the properties and the conditions of production of the SH-activated toxin might be of critical importance in terms of pathogenicity of L. monocytogenes. The purified toxin was cytolytic at very low doses: 1 HU corresponds to 1 ng, equivalent to ca. 10^10 molecules. It can be calculated that only 30 to 40 molecules of listeriolyisin O is needed to lyse a single erythrocyte. This number is similar to that found with SLO (1). Likewise, Bhatki et al. (8) estimated that the presence of approximately 100 SLO monomers per cell is required to generate one functional lesion on a cell. These results can be compared to the amounts of listeriolyisin O produced in vitro after 18-h incubation at 37°C. It can be calculated that listeriolyisin O production reached about 15,000 molecules per bacterial cell in Chelex-treated medium (10^9 bacteria per ml produce 1,500 HU/ml). Even in inappropriate medium for hemolysin production (BHI broth), the amount of listeriolyisin O still reached about 64 molecules per bacterial cell (10^8 bacteria per ml produce 64 HU/ml). Thus it is quite reasonable to suppose that the amount of listeriolyisin O produced by one or several bacterial cells inside phagosomes is sufficient to damage macrophage membranes. In this connection, it is known that the amount of free iron in host tissues is very low, 10^-18 M (12), due to the high association constant of the host iron-binding proteins (12), suggesting that a significant increase of toxin production could occur in the cellular microenvironment during infection (13).

The most interesting finding concerns the optimum pH of listeriolyisin O activity (Fig. 3), which is significantly lower than that of all other SH-activated toxins purified so far. The cytolytic activity was maximum at pH ~5.5 and undetectable at pH 7.0, whereas the other toxins were mostly active at pH ≥ 6.5 to 7.0. This finding is apparently at variance with the observation of hemolysis zones on blood agar prepared at neutral pH. It should be kept in mind that growing bacteria acidify the surrounding medium, thus favoring binding of toxin molecules onto cell membrane cholesterol. This suggests that listeriolyisin O would display better activity in the acidic environment in vivo, including in phagosomes where bacteria presumably replicate. In this connection, it is tempting to relate this finding to the fact that L. monocytogenes is the only intracellular facultative microorganism among the bacterial pathogens secreting SH-activated toxins.

Altogether, these results support the model of intracellular growth of L. monocytogenes proposed by Armstrong and Sword in 1966 (5, 41) and substantiated by a recent report (15). The sequence of events following bacterial invasion of the host tissues during infection starts with a very rapid phagocytosis of invasive bacteria (29) in an iron-deprived environment (12), which stimulates hemolysin secretion (13). Internalization of bacteria inside acidic phagosomes of resident macrophages would favor local accumulation of listeriolyisin O in the cellular microenvironment and subsequent irreversible binding to the membrane cholesterol. This leads to disruption of intracellular membranes and toxin export in the macrophage cytoplasm, rendering the cytoplasmic Fe^{3+} available for bacterial growth through a nonspecific mechanism of iron acquisition (14) and ultimately stimulating rapid bacterial replication (41). Moreover, it might be speculated that the local production of listeriolyisin O associated with bacterial multiplication in host tissues is also implicated in the development of multiple discrete foci, which appear in the spleen and liver during the initial phase of infection and are mainly populated with polymorphonuclear cells (29). This is supported by the observation that the cytolytic toxin can induce an inflammatory reaction even at very low doses (0.01 µg) when inoculated in the footpad (Fig. 4). On the view of recent reports on other SH-dependent toxins (10, 11), listeriolyisin O might similarly initiate the generation of leukotrienes from granulocytes and macrophages upon binding on their cytoplasmic membrane in infectious foci, and thereby potentiate inflammatory reaction during the early phase of infection. Finally, since there is evidence that the process of intracellular bacterial growth is a prerequisite for in vivo induction of T-cell-mediated immunity (6a), we are currently studying the role of listeriolyisin O during the initiation of acquired immunity to L. monocytogenes.

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LITERATURE CITED