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Killing of *Listeria monocytogenes* by Conventional and Germfree Rat Sera

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Serum from both germfree and conventional rats, but not plasma or plasma serum, killed *Listeria monocytogenes* in vitro by a calcium-dependent mechanism that was independent of either complement or lysozyme and was not inhibited by the addition of iron. The listericidin was purified by passing either rat serum or platelet lysate through a nitrocellulose filter (0.2 μm) and eluting the activity from the filter with 0.02 N HCl. The partially purified listericidin was heat stable (56°C for 30 min), removed by absorption with zymosan or bentonite, sensitive to treatment with trypsin or pronase, and inhibited by the addition of citrate (0.045 M), suggesting that the serum listericidin is a cationic protein. The development of serum listericidal activity, which could be important in the innate resistance of rats to *L. monocytogenes*, was dependent on both age and microbial status. Although some discrepancies exist between the serum listericidin and previous descriptions of serum β-lysin, we believe that the rat serum listericidin is a similar cationic protein.

Normal mammalian sera have bactericidal activity against both gram-negative and gram-positive bacteria. Heat killing of gram-negative, heat-labile bacteria (56°C for 30 min) requires binding to the bacterium of specific immunoglobulin classes which activate the complement cascade and disrupt the outer bacterial membrane, resulting in cell death (8). Serum killing of gram-positive bacteria is effected by a family of cationic proteins referred to as β-lysin. β-Lysin, which has been reviewed recently (7), is heat stable (56°C for 30 min) (13, 24, 28), released during blood clotting (10, 13), calcium dependent (12, 23), bactericidal in the absence of antibody and complement (5, 28), and separable from lysozyme (6, 14, 19, 24). Platelets have been implicated as being either the source of β-lysin or necessary for its production (7, 10, 13).

Acquired cellular resistance of rats to the gram-positive facultative intracellular pathogen *Listeria monocytogenes* requires sensitization of specific T-lymphocytes and subsequent macrophage activation (18, 22). Mechanisms of innate resistance to listeriosis may also be important in rats. Our preliminary observations indicated that sera from both conventional (CONV) and germfree (GF) rats were capable of killing *L. monocytogenes* in vitro. The purpose of this investigation was to characterize and purify the listericidal component of rat serum in the hope that such information could subsequently be used to evaluate the role of this bactericidal component in vivo.

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MATERIALS AND METHODS

Bacteria. *L. monocytogenes* (serotype 1) and all other bacterial strains used were obtained from the Wisconsin State Laboratory of Hygiene (Madison, Wis.) or from the culture collection of the Department of Medical Microbiology, University of Wisconsin Medical School, Madison. All bacterial strains were maintained as 1-ml aliquots of log-phase cells in brain heart infusion broth (BHI; Difco Laboratories, Detroit, Mich.) at −70°C. Before each experiment an aliquot was thawed, added to 50 ml of BHI, incubated overnight at 37°C with shaking, and then subcultured in BHI to obtain a 2- to 3-h log-phase culture.

Rats. Female CONV Sprague-Dawley rats (60 to 90 days old) were obtained from King Animal Laboratories (Oregon, Wis.). GF Sprague-Dawley rats were obtained from the University of Wisconsin Gnotobiotic Laboratory.

Serum and other blood components. Rats were given ether and exsanguinated by cardiac puncture; serum, plasma, plasma serum, or platelet-rich plasma were obtained from the heart blood using the methods of Shultz and Wilder (28). Platelets were harvested from platelet-rich plasma by centrifugation (2,000 × *g* for 20 min), washed three times in citrated saline (0.9 g of sodium chloride and 0.18 g of sodium citrate in 100 ml of deionized water), and suspended in phosphate-buffered saline (pH 7.2) at a platelet concentra-

tion of 10^9/ml. The platelet suspension was rapidly frozen in an ethanol-dry ice bath and thawed in tap water (22°C) three times. The platelet lysate was removed after centrifugation (2,000 × *g* for 15 min) to remove debris.

Lysozyme. Lysozyme activity was determined by a modification of the method of Jolles (15). Egg white lysozyme (grade I) was obtained from Sigma Chemical Co. (St. Louis, Mo.). Lysozyme activity was removed from rat serum by bentonite absorption (5 mg/ml for 10 min at 4°C).

Killing and lysis of *L. monocytogenes*. The bactericidal assay was done in triplicate with U-bottomed microtiter plates (Linbro, Hamden, Conn.); each well contained 100 μl of serum and 100 μl of a log-phase *L. monocytogenes* suspension (diluted in BHI to approximately 10^4/ml). The plate was shaken on a Micro mixer (Cooke Laboratories, Alexandria, Va.) for 10 s and incubated at 37°C. After 1 and 2 h of incubation 25 μl was removed from each well and plated on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.). At the same time points an additional 20 μl was removed from each well, diluted in phosphate-buffered saline, and plated on Trypticase soy agar which was then incubated at 37°C for 18 to 24 h. *L. monocytogenes* colonies were counted, and the results were expressed as the mean ± standard error $\log_{10}$ viable *L. monocytogenes* per 0.1 ml of reaction mixture. BHI and undiluted rat serum served as positive and negative controls, respectively, for the growth of *L. monocytogenes*.

To compare the listericidal and lytic activities of the partially purified listericidin, *L. monocytogenes* suspended in 1.5 ml of peptone-saline (7×10^8/ml) was added to sterile glass test tubes which contained 1.5 ml of listericidin. After 0, 30, 60, 90, 120, 180, and 240 min of incubation at 37°C the optical density (at 525 nm) of the *L. monocytogenes* suspension was measured (Spectronic 21 spectrophotometer; Bausch & Lomb, Inc., Rochester, N.Y.); 20-μl samples were removed, diluted in peptone-saline, and plated on Trypticase soy agar.

Zymosan. Zymosan A (Difco) was prepared by the method of Kabat and Mayer (16). Sera were depleted of complement by incubation with 15 mg of zymosan per ml of serum at 37°C for 1 h, centrifuged (2,000 × *g* for 20 min), and stored at −70°C.

Hemolytic complement activity. Hemolytic complement activity was determined by a microtiter assay. Each complement source tested was serially diluted (twofold) in 50 μl of phosphate-buffered saline. Each well then received 50 μl of heat-inactivated (56°C for 30 min) goat anti-sheep erythrocyte serum and 100 μl of 1% washed sheep erythrocytes. The endpoint for complement activity was chosen as the highest serum dilution which gave 100% hemolysis of the sheep erythrocytes. Untreated guinea pig serum had hemolytic activity at a 1:8 dilution, whereas neither zymosan-absorbed nor C4-deficient guinea pig serum had any hemolytic activity.

Preparation of rat anti-*L. monocytogenes* immunoglobulin. Hyperimmune sera from rats which had survived two intravenous challenges of 10^6 viable *L. monocytogenes* were pooled. The immunoglobulin G-rich fraction was precipitated with 18% sodium sulfate, suspended in phosphate-buffered saline, and dialyzed at 4°C against four changes of phosphate-buffered saline. This immunoglobulin preparation had an agglutination titer of 1:128 against formalinized *L. monocytogenes* (5×10^9/ml).

Partial purification of listericidal activity. Serum listericidal activity was partially purified by the procedure of Sharma and Middlebrook (27). Rat serum (8 ml) was passed twice through a Nalgene filter unit (0.2 μm; Nalgene Labware Div., Rochester, N.Y.) by using a negative pressure of 5 lb/in^2. The filter was washed first with 20 ml of 0.15 M NaCl and then with 100 ml of deionized water. The listericidal activity was eluted with 8 ml of 0.02 N HCl, and the eluate was adjusted to pH 7.5 with 1 N NaOH. The eluate from a separate filter unit identically treated with saline, deionized water, and 0.02 N HCl had no listericidal activity, indicating that a toxic substance was not being removed from the filter itself.

Titers of listericidal activity in both the rat serum and the partially purified listericidin were determined by diluting them in phosphate-buffered saline and testing each dilution in the bactericidal assay described above. The highest dilution capable of causing at least a 2 log reduction in viable *L. monocytogenes* after incubation for 2 h at 37°C was defined as 1 U of listericidal activity. The protein concentration of the listericidin was estimated by measuring absorbance at 280 nm with a Beckman DU spectrophotometer. Specific activity was defined as the number of listericidal units per unit of absorbance at 280 nm.

Listericidin stability. The partially purified listericidin was incubated at 37°C for 3 h with the following enzymes (100 μg/ml) obtained from Sigma: deoxyribonuclease, ribonuclease, pronase, trypsin, and lipase. Heparin (1 mg/ml; Sigma), ribonucleic acid (1 mg/ml; Calbiochem-Behring Corp., La Jolla, Calif.), and citrate (0.045 M) were also incubated with the listericidin at 4°C for 30 min before testing for listericidal activity. None of these reagents, by itself, adversely affected the growth of *L. monocytogenes*.

RESULTS

Listericidal activity of mammalian sera. New Zealand White and Cottontail rabbit sera were listericidal, confirming previous reports (28, 29). In addition, GF and CONV Sprague-Dawley rat sera also killed *L. monocytogenes* in vitro (Table 1). None of the other sera tested possessed bactericidal activity against *L. monocytogenes*. Subsequent experiments showed that listericidal activity was not unique to the Sprague-Dawley rat strain since sera from Harvard, Buffalo, Osborn-Mendel, Louvain, or Hooded (both normal and congenitally athymic) rat strains killed *L. monocytogenes* in vitro.

Listericidal activity in rat serum, plasma, and platelet lysate. Sera from both CONV and GF Sprague-Dawley rats, but not citrated or heparinized plasma or plasma serum, killed *L.*

TABLE 1. *Listericidal activity of mammalian sera*

Serum	Log_{10} change in bacteria[a]
New Zealand White rabbit	−3.61 ± 0.00
Cottontail rabbit	−3.61 ± 0.00
Sprague-Dawley rat	−2.28 ± 0.67
Germfree Sprague-Dawley rat	−2.54 ± 0.53
Human	+0.80 ± 0.04
Beagle	+0.33 ± 0.10
Germfree beagle	+0.03 ± 0.06
Guinea pig	+0.68 ± 0.01
BALB/c mouse	+0.32 ± 0.24
Nude mouse	+1.28 ± 0.08
Golden hamster	+1.36 ± 0.04

[a] Combined results from various experiments showing mean ± standard error change in viable *L. monocytogenes* after incubation in serum for 2 h at 37°C.

monocytogenes in vitro (Fig. 1). A rat platelet lysate also killed *L. monocytogenes* in vitro.

Bactericidal activity of rat serum against gram-positive bacteria. To determine whether susceptibility to rat serum was a unique property of our stock strain of *L. monocytogenes*, two clinical isolates of *Listeria* sp. and several other species of gram-positive bacteria were tested for their sensitivity to rat serum. All three strains of *L. monocytogenes* (all serotype 1) and a strain of *Bacillus subtilis* showed a >2 log reduction in viability in the presence of rat serum (Table 2). Rat serum caused a slight decrease in the viability of one strain of *Staphylococcus aureus*, and it inhibited the growth of *Streptococcus mutans*, but it did not affect the growth of the other bacterial species tested (Table 2). It is not clear what property, presumably shared by *L. monocytogenes*, *B. subtilis*, and *S. aureus*, is responsible for their sensitivity to rat serum.

Effect of temperature and bacterial concentration on killing of *L. monocytogenes* by serum. Serum listericidal activity was temperature dependent; a 2.3 log decrease in viable *L. monocytogenes* occurred when it was incubated in serum at 37°C for 2 h, whereas only a 0.5 log decrease occurred when it was incubated at 4°C for 2 h. Both CONV and GF rat sera killed *L. monocytogenes* at bacterial concentrations of 10^4 to 10^8 viable *L. monocytogenes* per ml.

Role of complement in the killing of *L. monocytogenes* by rat serum. Rat serum heated at 56°C for 30 min lost its serum listericidal activity. Although guinea pig serum itself possesses no listericidal activity, when it was added (50 μl) to heat-inactivated rat serum (100 μl) it restored killing activity (Table 3). This result suggests a possible role for complement in the listericidal activity of rat serum; however, heat-inactivated, zymosan-absorbed, and C4-deficient guinea pig sera, all of which are devoid of hemolytic complement activity, also restored listericidal activity to heat-inactivated rat serum (Table 3). These data, together with the inability of rat anti-*L. monocytogenes* immunoglobulin G plus fresh guinea pig serum to kill *L. monocytogenes* in vitro (Table 3), suggest that although rat serum listericidal activity is heat labile (56°C for 30 min), complement is not the heat-labile component.

Role of lysozyme. Both GF and CONV rat sera were found to have approximately 100 U of lysozyme activity per ml. Absorption of rat serum with bentonite removed both lysozyme activity and listericidal activity. The addition of

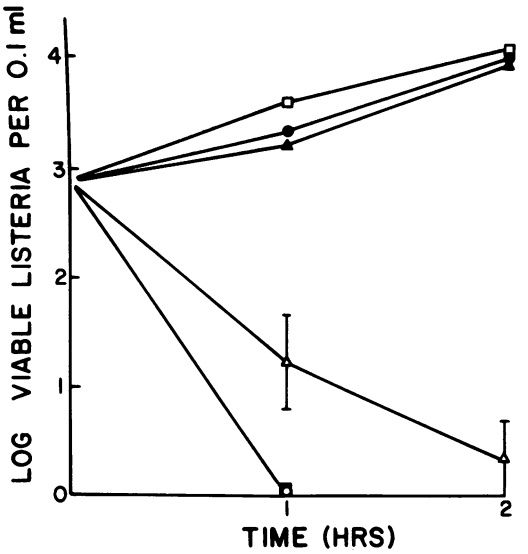

FIG. 1. *Survival of L. monocytogenes in the presence of rat serum, plasma, or platelet lysate. Symbols: ○, conventional rat serum; △, germfree rat serum; ■, platelet lysate; ●, conventional rat plasma; ▲, germfree rat plasma; □, BHI control.*

TABLE 2. *Bactericidal activity of rat serum against various gram-positive bacteria*

Organism	Log_{10} change in bacteria[a]
L. monocytogenes	−2.74 ± 0.00
L. monocytogenes (RC-282-29)	−2.71 ± 0.00
L. monocytogenes (RC-283-36)	−2.67 ± 0.00
Bacillus subtilis	−2.25 ± 0.00
Staphylococcus aureus	−0.13 ± 0.03
Streptococcus mutans	+0.15 ± 0.05
Streptococcus faecalis	+0.32 ± 0.03
Staphylococcus epidermidis	+0.65 ± 0.10
Streptococcus pyogenes	+0.79 ± 0.02
Erysipelothrix rhusiopathiae	+1.13 ± 0.02

[a] Mean ± standard error change in viable bacteria after incubation in rat serum for 2 h at 37°C.

TABLE 3. *Reconstitution of listericidal activity of heat-inactivated rat serum by guinea pig serum*

Treatment[a]	% Killed[b]
Unheated rat serum	100
HRS plus GPS	100
HRS plus zymosan-absorbed GPS	100
HRS plus C4-deficient GPS	100
HRS plus heated GPS	97
HRS plus bentonite-absorbed GPS	95
HRS	0
GPS	0
Rat anti-*L. monocytogenes* immunoglobulin G plus GPS	0
Zymosan-absorbed rat serum	0
Bentonite-absorbed rat serum	0

[a] Each well contained 100 μl of heat-inactivated rat serum (HRS) plus 50 μl of guinea pig serum (GPS). Control wells contained 100 μl of HRS plus 50 μl of phosphate-buffered saline.

[b] Percentage of *L. monocytogenes* killed after 2 h of incubation at 37°C.

egg white lysozyme (Sigma), in 20 μl of phosphate-buffered saline, at a concentration equal to or 10 times that originally present in rat serum, failed to restore listericidal activity to bentonite-absorbed rat serum (Fig. 2). Thus, lysozyme does not appear to be the listericidal component in rat serum.

Effect of iron on serum listericidal activity. Shultz and Wilder found that iron prevented the killing of *L. monocytogenes* by rabbit serum (28). Ferric ammonium citrate was added to rat serum at a concentration of 50, 100, or 300 μg per ml of serum and incubated at 22°C for 30 min before testing for listericidal activity. Although there was a decreased rate of killing in the presence of 300 μg of ferric ammonium citrate per ml all *L. monocytogenes* were killed within 2 h of incubation (Fig. 3), suggesting that rat serum listericidal activity is not abrogated by the addition of iron.

Inhibition of serum listericidal activity by citrate and restoration of activity of calcium. We observed that sodium citrate, when added to serum (in 10 μl of deionized water) at a final concentration of 0.013 M, did not significantly inhibit serum listericidal activity. Since this is the citrate concentration that is used to collect citrated plasma it implies that the lack of listericidal activity in plasma is not the result of inhibition by citrate. At a serum concentration of 0.045 M citrate, however, serum listericidal activity was completely inhibited. Citrate could have chelated a listericidal component, a required cationic cofactor, or both. The addition of $CaCl_2$ at a final concentration of 2×10^{-2} M to citrate-inactivated (0.045 M) rat serum maximally restored serum listericidal activity (Table 4). The effect of Ca^{2+} appeared to be specific since neither Mg^{2+} (10^{-2} M) nor arginine (10^{-2} M) restored serum listericidal activity when added to citrated rat serum (0.045 M).

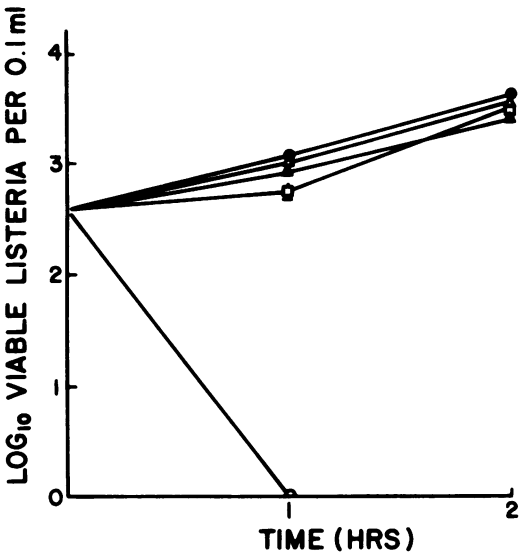

FIG. 2. *Failure of lysozyme to reconstitute the listericidal activity of bentonite-absorbed rat serum. Symbols: ○, unabsorbed rat serum; △, bentonite-absorbed serum alone; ●, bentonite-absorbed serum plus 100 U of lysozyme; ▲, bentonite-absorbed serum plus 1,000 U of lysozyme; □, BHI control.*

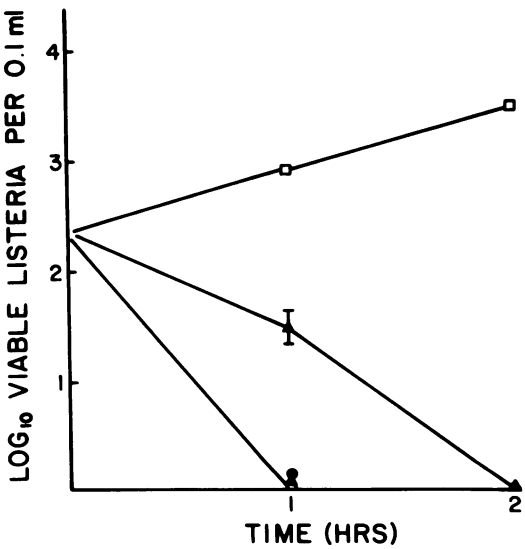

FIG. 3. *Resistance of rat serum listericidal activity to the addition of ferric ammonium citrate. Symbols: ○, serum alone; △, serum plus 50 μg of ferric ammonium citrate per ml; ●, serum plus 100 μg of ferric ammonium citrate per ml; ▲, serum plus 300 μg of ferric ammonium citrate per ml; □, BHI control.*

The addition of $CaCl_2$ at a concentration of 4×10^{-2} M or greater caused a decrease in listericidal activity, possibly by precipitation of the listericidal protein.

Serum listericidal activity in infant rats. The development of serum listericidal activity by infant rats was dependent on both their age and microbial status. The serum of 10-day-old, conventionally reared rat pups had listericidal activity nearly equal to that of adult rat serum, whereas *L. monocytogenes*-monoassociated rat pups and GF rat pups developed listericidal activity at 12 and 20 days of age, respectively (data not shown). The development of serum listericidal activity did not correlate with platelet numbers in CONV and GF rat pups.

Partial purification and stability of the serum listericidin. Rat serum listericidal activity could not be purified by the Seitz filtration technique described previously for β-lysin purification (14, 28); however, passage of rat serum through a nitrocellulose filter (0.2 μm) and elution with 0.02 N HCl by the technique of Sharma and Middlebrook (27) resulted in a 176-fold purification of listericidal activity. The same technique also partially purified the listericidal activity from a rat platelet lysate, suggesting that the serum and platelet listericidins are similar, if not identical. Although it killed and lysed *L. monocytogenes* (Fig. 4), the partially purified listericidin had no detectable lysozyme activity.

The serum listericidin was unaffected by deoxyribonuclease, ribonuclease, lipase, heating at 56°C for 30 min, or the addition of iron (300 μg of ferric ammonium citrate per ml); however, trypsin or boiling (100°C for 5 min) completely eliminated but pronase reduced by 80% the killing activity of the partially purified listericidin. Absorption of the partially purified listericidin with either bentonite or zymosan removed the killing activity, whereas the addition of the polyanions heparin (1 mg/ml) or ribonucleic acid (1 mg/ml) did not inhibit the listericidin. The addition of citrate (0.045 M) to the partially purified listericidin inhibited listericidal activity which could be restored by the addition of calcium (optimal listericidal activity at 6×10^{-2} M calcium). These data suggest that the listericidin is a cationic protein.

TABLE 4. *Effect of $CaCl_2$ on the listericidal activity of citrated rat serum*

Treatment	Log_{10} change in bacteria[a]
Rat serum plus 0.045 M citrate (CS)	+0.84 ± 0.01
CS plus 10^{-2} M $CaCl_2$	−0.71 ± 0.06
CS plus 2×10^{-2} M $CaCl_2$	−2.66 ± 0.00
CS plus 4×10^{-2} M $CaCl_2$	−0.19 ± 0.06
CS plus 6×10^{-2} M $CaCl_2$	+0.34 ± 0.03

[a] Mean ± standard error change in viable *L. monocytogenes* after 2 h of incubation at 37°C.

DISCUSSION

Sera from both GF and CONV rats, which are reported to contain equivalent amounts of β-lysin activity against *B. subtilis* (11), also kill *L. monocytogenes* in vitro. Rat serum listericidal

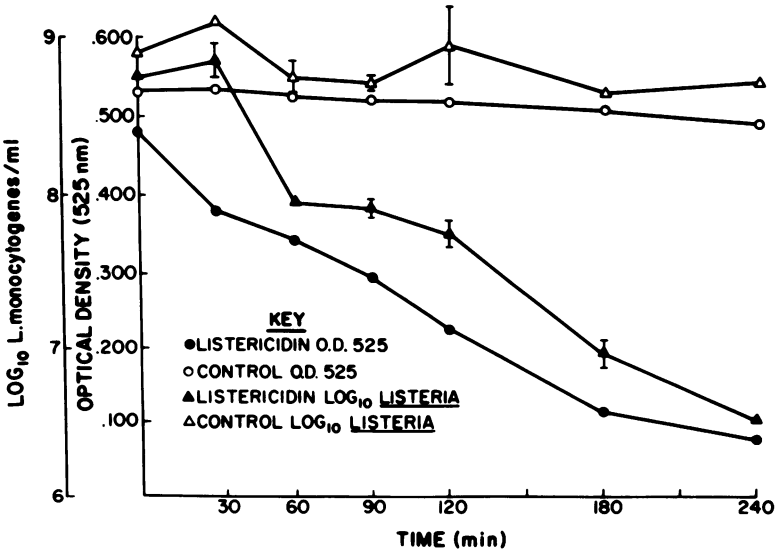

FIG. 4. *Killing and lysis of L. monocytogenes by the partially purified serum listericidin (closed symbols). Open symbols indicate a control eluate from a nitrocellulose filter unit (0.2 μm) through which no rat serum was passed.*

activity does not involve complement, as evidenced by the heat stability (56°C for 30 min) of the partially purified listericidin, the inability of rat anti-*L. monocytogenes* immunoglobulin G plus guinea pig complement to kill *L. monocytogenes*, and the ability of heated (56°C for 30 min), C4-deficient, or zymosan-absorbed guinea pig sera to reconstitute heat-inactivated (56°C for 30 min) rat serum. The increased heat stability of the partially purified listericidin is a curious phenomenon; however, purification of β-lysin is also reported to increase the heat stability of the bactericidal activity (8). Perhaps guinea pig serum, which is reported to enhance the β-lysin activity of rat serum against *B. subtilis* (21), contains a component which can produce the listericidin from some other component present in heat-inactivated rat serum. Ethanol fractionation of rat serum by the method of Myrvik and Leake (23), however, did not indicate that multiple components were responsible for rat serum listericidal activity. The presence of listericidal activity in rat serum or platelet lysate, but not in plasma or plasma serum, suggest that platelets, which have been reported to contain bactericidal components (2, 7, 9, 13, 30), release the listericidin during the clotting of rat blood. The activity of the partially purified listericidin could be removed by absorption with bentonite or zymosan, by treatment with proteolytic enzymes, or by the addition of citrate (0.045 M), implying that the listericidin is a cationic protein.

In contrast to the results of Shultz and Wilder (28, 29), rat serum listericidal activity could not be purified by the Seitz filtration method for the purification of β-lysin as described by Johnson and Donaldson (14). In addition, Shultz and Wilder reported that the rabbit serum listericidin is heat stable (56°C for 30 min) and is not present in a rabbit platelet lysate (28). Based on the similarity of other properties such as the increased heat stability of the partially purified rat listericidin, its cationic protein nature, and its lack of complement or lysozyme activity, we believe that the rat serum listericidin is related to both the rabbit serum listericidin and β-lysin. Other evidence suggests that β-lysin may actually be a family of similar bactericidal serum cationic proteins (14, 28).

The purification of both the rat serum and platelet listericidins by the nitrocellulose filter method described by Sharma and Middlebrook (27) for the purification of a listericidin from guinea pig peritoneal exudate cells suggests that rat and guinea pig listericidins share certain physical properties despite the differences in species and cellular origin. Cationic proteins from serum (1, 14, 28), semen (25), peritoneal exudate cells (27), polymorphonuclear leukocytes (31), platelets (7, 9, 13, 30), and synthetic cationic polyamines (17) have been shown to be bactericidal in vitro. These bactericidal cationic proteins are low-molecular-weight molecules that kill by disrupting the bacterial cytoplasmic membrane (7, 20, 29), possibly via disruption of the plasma membrane chemiosmotic gradient (26). The widespread distribution and similar properties of the naturally occurring cationic proteins suggest that they may be important antimicrobial agents in vivo; however, no conclusive evidence exists for the hypothesis.

Serum listericidal activity could be an important innate resistance mechanism against *L. monocytogenes* in rats. The intravenous 50% lethal dose of *L. monocytogenes* is 2×10^6 for adult Sprague-Dawley rats (180 to 200 g), whereas the intravenous 50% lethal dose is 6×10^3 for adult BALB/c mice (25 to 30 g) which lack serum listericidal activity. Acquired cellular resistance to *L. monocytogenes* in both rats and mice develops approximately 48 to 72 h after a primary challenge with *L. monocytogenes* (18, 22). Before acquired cellular resistance to *L. monocytogenes* develops, both cellular and humoral innate resistance mechanisms, including possible serum listericidal activity, would be of foremost importance in reducing the *L. monocytogenes* burden, especially if the portal of entry is through the gastrointestinal tract (3). The role of rat platelets, which appear to be the source of serum listericidin, in resistance to *L. monocytogenes* infection was described previously (4).

Further investigation of innate host defense mechanisms, such as the listericidin described in the present study, not only will enhance our basic understanding of the pathogenesis of facultative intracellular parasites, but might also prove to be clinically useful to augment the resistance of immunocompetent patients to facultative intracellular parasites.

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