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Mechanism for Nonspecific Immunity to *Listeria monocytogenes* in Rats Mediated by Platelets and the Clotting System

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A proposed mechanism for nonspecific immunity to *Listeria monocytogenes* in rats based on the existence of an activatable lysin is described. Using a deoxyribonucleic acid release assay, we found lysin activity in serum made from whole blood but not in serum made from platelet-free plasma. Washed platelets and platelet lysates exhibited only partial activity as compared with that in serum. This activity was amplified by the addition of platelet-free plasma serum. The activity of the lysin was unaffected by heparin, dialysis, a serine esterase inhibitor, or heating to 56°C for 30 min. Effective inhibitors were ethylenediaminetetraacetic acid and stronger heating (to 65°C). *Listeria* organisms were found to reduce the recalcified clotting time of platelet-rich plasma in a dose-dependent fashion, indicating that the organisms can exhibit procoagulant activity. The susceptibility of rats to *Listeria* infection was enhanced by anticoagulant treatment. Rats were infected with *Listeria* organisms with and without administration of heparin. Heparin-treated rats developed bacteremia, and some died. None of the control rats developed bacteremia or died. These results suggest that natural immunity to *Listeria* infection is partly due to a platelet-dependent lysin which is activated during clotting and is, in turn, promoted by the *Listeria* organisms themselves.

Acquired resistance to *Listeria monocytogenes* is generally regarded as an expression of cell-mediated immunity in which the specific reaction of committed lymphocytes with antigen activates macrophages to exert a nonspecific bactericidal effect (9). Whereas initially susceptible animals such as mice can develop such immunity during the course of infection, rats are quite resistant without the benefit of prior exposure (4). We report here that a powerful lysin for *L. monocytogenes* exists in rat serum which is produced during clotting of blood in the presence of platelets. We propose a mechanism for natural resistance to *L. monocytogenes* in rats.

MATERIALS AND METHODS

Animals. Breeding stocks of DA rats were obtained from the Blackburn Animal House, University of Sydney, Sydney, New South Wales, and breeding stocks of Wistar rats were obtained from the Australian Atomic Energy Commission, Lucas Heights, New South Wales. They were bred and maintained at the Gore Hill Research Laboratories of the New South Wales Institute of Technology.

Bacteria. *L. monocytogenes* (strain NB62), *Escherichia coli* (Seattle strain, ATCC 25922), *Staphylococcus aureus* (Seattle strain, ATCC 25923), *Salmonella enteritidis* (strain 11RX), *Bacillus subtilis* (soil isolate), and *Klebsiella pneumoniae* (clinical isolate) were obtained from the Microbiology Department of The Royal North Shore Hospital of Sydney, St. Leonards, New South Wales.

Reagents. Phenylmethylsulfonyl fluoride, thymidine, and zymosan were obtained from Sigma Chemical Co., St. Louis, Mo. Human dried thrombin and antiseptic-free sodium heparin were obtained from Commonwealth Serum Laboratories, Melbourne, Australia. Triton X-100 was a generous gift from Rohm and Haas Co., Philadelphia, Pa. Ethylenediaminetetraacetic acid and all other chemicals were analytical grade reagents. Dulbecco modified Eagle medium (hereafter referred to as medium) was obtained from GIBCO Laboratories, Grand Island, N.Y.

Serum, plasma, and platelet preparations. Whole rat serum was obtained by clotting blood taken by cardiac puncture from etherized rats of either sex. Platelet-rich plasma was obtained similarly by adding blood to 0.13 M sodium citrate to give a final concentration of 10 mM and removing the platelet-rich plasma supernatant after sedimenting erythrocytes and leukocytes (centrifugation at 800 × *g* for 10 min). Platelet-free plasma was prepared by centrifuging platelet-rich plasma (4,000 × *g*, 10 min, 4°C). Platelets in the pellet were removed and washed twice by centrifugation (4,000 × *g*, 10 min, 4°C) and suspension in medium supplemented with 10 mM sodium citrate. Platelet concentration was adjusted to 6×10^8 to 8 ×

10^8/ml, approximately the same as that in the platelet-rich plasma. Platelet-free plasma serum was made by adding calcium to a final concentration of 10 mM to the platelet-free plasma and pelleting the resulting fibrin clot (centrifugation at 10,000 × *g* for 5 min) after incubation at 37°C for 2 h. Platelet-rich plasma serum was made similarly from platelet-rich plasma.

Labeling of bacteria. Bacteria were grown from stock suspensions stored at −70°C in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.). The deoxyribonucleic acid of the organism was labeled by a modification of the method of Friedlander (2) by growing a 1/100 dilution of stock in broth containing 10 μCi of tritiated thymidine (New England Nuclear Corp., Boston, Mass.) per ml overnight at 37°C. Carrier thymidine (50 μg/ml) was added, and the bacteria were incubated for a further 15 min at room temperature. Unincorporated label was removed by washing the bacteria by centrifugation (4,000 × *g*, 10 min, 4°C) and gentle suspension in 0.15 M NaCl. The concentration of bacteria was determined by visual counting with a Petroff-Hauser bacteria counter (Clay Adams Inc., New York, N.Y.) under the phase-contrast microscope and adjusted to 10^9/ml. Radioactivity was determined with a water-miscible scintillant (Picofluor 15; Packard Industries, Downers Grove, Ill.), in a Packard 2425 scintillation counter. Raw data as counts per minute were converted to disintegrations per minute with the automatic external standard and quench correction curve. The final preparations of bacteria had specific activities of 100,000 to 150,000 dpm/10^8 organisms, with the exception of *E. coli* and *S. aureus*, which had specific activities of 10,000 to 20,000 dpm/10^8 organisms.

Bactericidal assay. Bactericidal activity was determined by the method established by Friedlander (2) in which the release of radioactively labeled deoxyribonucleic acid from the organism is taken as evidence for its death. The assay for *L. monocytogenes* lysin activity was carried out with 1.5-ml microcentrifuge tubes (Eppendorf Gerätebau, Hamburg, West Germany) containing 15 μl of serum, plasma, platelet suspension, or supernatant diluted to 0.5 ml with medium. Labeled *L. monocytogenes* suspension (50 μl) was added, and the tubes were incubated for 2 h at 37°C in a moist CO_2 atmosphere (5% in air). The tubes were centrifuged with a Beckman Microfuge B (Beckman Instruments, Inc., Fullerton, Calif.) (10,000 × *g*, 5 min), and a portion of the supernatant was removed for scintillation counting. This "nonpelletable" radioactivity was taken to represent the degree of lysis of *Listeria* organisms. Concentrations of various added reagents refer to the total volume of the assay.

Determination of viable bacteria. Suspensions of bacteria were enumerated initially by visual counting of organisms as described above. Viable counts were determined by decimally diluting bacterial suspensions to ca. 10^3 to 10^4 organisms per ml with Trypticase soy broth and applying 20-μl aliquots in duplicate to partly dried blood agar plates. After incubation overnight at 37°C, the numbers of bacteria expressed as colony-forming units were found by multiplying the number of colonies by the dilution factor. Bacteremia was determined similarly by diluting freshly drawn blood directly into broth.

Studies of infection in vivo. *L. monocytogenes* was grown overnight in Trypticase soy broth at room temperature, sedimented by centrifugation (400 × g, 10 min), and suspended in 0.15 M NaCl. The concentration of bacteria was adjusted to ca. 10^9 organisms per ml by visual counting, and the colony-forming units were determined as described above. Groups of rats received various doses of *L. monocytogenes* as intraperitoneal injections. Some rats also received heparin (100 U, intraperitoneally) simultaneously with the infecting dose and again 24 h later. The degree of bacteremia was determined at 48 h in one group, and another group was used to determine the number of deaths occurring up to 2 weeks after infection.

RESULTS

Nonpelletable radioactivity as a measure of bacterial death. To verify the method, we compared killing determined by colony counts with that determined by nonpelletable radioactivity released from labeled bacteria. Labeled *Listeria* organisms (ca. 10^8 organisms in 1-ml aliquots) were sonicated for various periods up to 60 s. Portions were removed for colony counts; the remainder was centrifuged, and the nonpelletable radioactivity in the supernatant was removed for scintillation counting. The results, expressed as a percentage of the total colony count (for colony counts) or of the total radioactivity (for scintillation counting) in the unsonicated preparation of bacteria, are shown in Fig. 1. A linear relationship with a good degree of correlation (determination coefficient, 0.81) was found between the loss of colony-forming units from the sonicated bacteria and the amount of nonpelletable radioactivity released.

Properties and origin of the lysin for *L.*

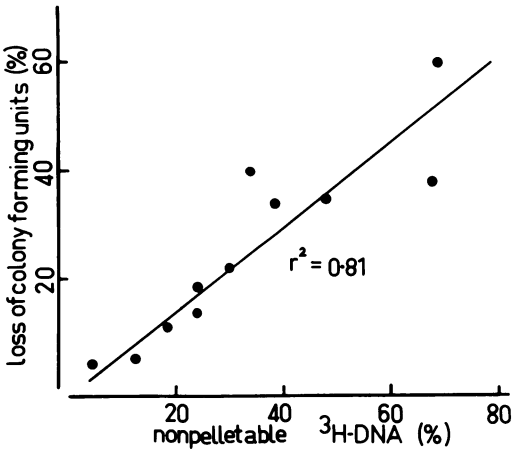

FIG. 1. *Correlation between assessment of bacterial killing as determined by colony counting and by release of nonpelletable radioactivity. The results are expressed as a percentage of the initial number of colony-forming units and of the total radioactivity, respectively. DNA, Deoxyribonucleic acid.*

monocytogenes. Serum made from whole rat blood but not from platelet-free plasma exhibited potent lytic activity for *L. monocytogenes*. Rat serum at a concentration of 1% in 0.5 ml of medium lysed ca. 10^7 organisms in 30 min, whereas platelet-free plasma serum had no effect (Fig. 2). This lysin activity, subsequently determined as the percentage of the labeled deoxyribonucleic acid released from ca. 5×10^7 labeled bacteria in a 2-h period (Table 1), was unaffected by heparin, dialysis, a serine esterase inhibitor (phenylmethylsulfonyl fluoride), heating to 56°C for 30 min, additional calcium, or sodium citrate, whereas 2 mM ethylenediaminetetraacetic acid or stronger heating (to 65°C) for 30 min were inhibitory. The addition of serum heated to 65°C to normal serum did not appreciably diminish the activity of the normal serum, showing that loss of activity rather than production of inhibitors was the result of such heating. By means of the same assay, rat serum was also found to have potent lytic activity for *S. enteritidis* and lower lytic activity for *S. aureus* and *B. subtilis*, whereas *K. pneumoniae* and *E. coli* were unaffected (Table 2).

The appearance of the lysin was apparently dependent on the clotting of plasma in the presence of a platelets because whole serum and serum made from platelet-rich plasma contained high levels of activity, but serum made from platelet-free plasma did not (Table 1, experiment 2). The lysin was not necessarily contained in a totally active form within the platelets, however, because washed platelets exhibited only moderate levels of activity which were marginally increased when platelets were lysed with the surfactant Triton X-100 or by sonication. Similar levels of lysin activity were released from platelets by thrombin, which is known to cause platelet degranulation. High levels of activity

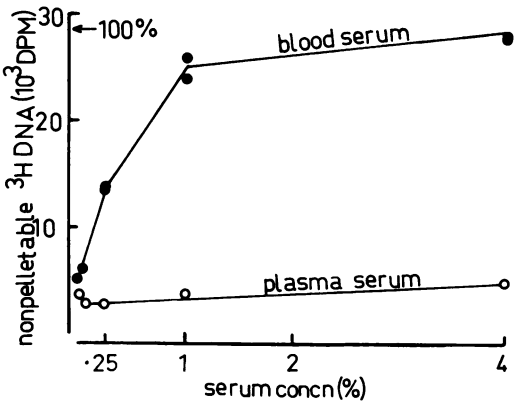

FIG. 2. *Release of nonpelletable radioactivity from labeled L. monocytogenes incubated in serum made from whole rat blood or in serum made from platelet-free plasma. Ca. 10^7 organisms were incubated for 30 min in 0.5 ml of medium. The point of 100% release is indicated. DNA, Deoxyribonucleic acid. DPM, Disintegrations per minute.*

TABLE 1. *Properties of L. monocytogenes lysin activity in rats*

Expt	Added substance and experimental conditions[a]	Net release (% ± SD) of nonpelletable radioactivity[b] (n = 3)
1	Whole serum	80 ± 1
	Whole serum + heparin (10 U/ml)	70 ± 1
	Whole serum + ethylenediaminetetraacetic acid (2 mM)	24 ± 5
	Whole serum, dialyzed (16 h)	69 ± 3
	Whole serum + PMSF (10 mM), dialyzed (16 h)	63 ± 10
	Whole serum + Ca^{2+} (1 mM)	82 ± 1
	Whole serum + sodium citrate (10 mM)	77 ± 7
	Whole serum, heated (56°C, 30 min)	68 ± 4
	Whole serum, heated (65°C, 30 min)	0 ± 0
	Whole serum + heated (65°C, 30 min) serum	52 ± 5
2	Whole serum	101 ± 25
	PFPS	8 ± 2
	Platelet-rich plasma serum	99 ± 9
	Washed platelets	36 ± 3
	Sonicated washed platelets	49 ± 5
	Platelets + Triton X-100 (0.1%)	50 ± 13
	SN_T	47 ± 2
	SN_Z	17 ± 2
	PFPS + SN_T	78 ± 7
	PFPS + SN_Z	32 ± 3
	PFPS + washed platelets	87 ± 7
3	Whole serum	93 ± 2
	Washed platelets	27 ± 6
	Washed platelets + PFPS	53 ± 7
	Washed platelets + heated (56°C, 30 min) PFPS	4 ± 1
	Washed platelets + PMSF (10 mM)-treated PFPS, dialyzed (16 h)	2 ± 1

[a] PMSF, Phenylmethylsulfonyl fluoride. PFPS, Platelet-free plasma serum. SN_T and SN_Z, Supernatants from platelet suspension incubated for 30 min at 37°C with thrombin (10 U/ml) and zymosan (1 mg/ml), respectively.

[b] The amounts released in control experiments with no additions were 8 ± 2%, 9 ± 0%, and 9 ± 1%, respectively. SD, Standard deviation.

TABLE 2. *Effect of rat serum on release of nonpelletable radioactivity from bacteria*

Bacteria	Net release (% ± SD[a]/2 h) of nonpelletable radioactivity
E. coli	0
S. aureus	28 ± 1
S. enteritidis	102 ± 5
K. pneumoniae	1 ± 0
L. monocytogenes	86 ± 1
B. subtilis	20 ± 1

[a] SD, Standard deviation.

were seen when platelet-free plasma serum, which had no activity of its own, was incubated together with either whole washed platelets or the supernatant from thrombin-treated platelets. This augmented activity obtained by adding platelet-free plasma serum to washed platelets was almost totally abolished by heating the former to 56°C for 30 min before adding it to the assay or by the addition of phenylmethylsulfonyl fluoride (Table 1, experiment 3). These changes in lysin activity were not due to variations in the calcium content of the assay. Several of the added substances contained excess citrate (platelets and platelet extracts), and others contained excess free calcium ion (platelet-free and platelet-rich plasma serum). These substances, 15 μl of which was diluted to a final volume of 500 ml with medium whose calcium content was 1.8 mM, altered the final calcium concentration by no more than 0.3 mM. Much higher levels than this of either calcium or citrate had no effect on lysin activity (Table 1, experiment 1). The ability of heating to abolish lysin augmentation by platelet-free plasma serum also shows that ionic effects were not responsible (Table 1, experiment 3).

Involvement of the clotting system in immunity. These results suggested a mechanism for nonspecific immunity to *L. monocytogenes* which would require the organisms themselves to initiate clotting in the presence of platelets. We tested this possible mechanism in two ways. First, the effect of incubating *L. monocytogenes* on the recalcified clotting time of citrated platelet-rich plasma serum was determined. Citrated platelet-rich plasma (100 μl) was incubated with an equal volume of 0.15 M NaCl containing various numbers of *L. monocytogenes* organisms for 30 min at 37°C. Calcium chloride (10 μl, 0.1 M) was added to initiate clotting, the endpoint of which was observed by tilting the test tube. This treatment led to a dose-dependent reduction in clotting time from 55 to 35 s, indicating that *L. monocytogenes* can exhibit procoagulant activity. Second, the effect of anticoagulant treatment on the course of infection with *L. monocytogenes* was studied. Groups of rats were infected with 1.3×10^9 colony-forming units with and without the administration of heparin. After 48 h, all three rats in the heparin group were found to have intense bacteremia. The number of colony-forming units per ml of blood was 9,680, 36,800, and 17,400, respectively. Bacteremia in the three rats of the control group was undetectable. Other groups of rats were infected similarly to determine mortality. In the heparin group receiving at least 1.3×10^9 colony-forming units of *L. monocytogenes*, three of three and three of five rats died in two separate experiments. None of the control rats died.

DISCUSSION

The method used in this study of determining bactericidal activity as the release of nonpelletable radioactivity is efficient and specific. Because a linear relationship with a good degree of correlation was found between this method and the traditional method of colony counting, the two are quite comparable. In theory, the radioactivity release method should be more precise. The statistics of radioactivity counting provide a high degree of counting accuracy, and the measured parameter increases from 0 with increasing numbers of lysed bacteria. In colony counting, clumping may result in reduced apparent counts, and because the correlate of killing, i.e., loss of colony-forming units, is derived from the difference between two large numbers of limited precision, the result itself is not precise.

The lysin that is present in rat serum and that acts against *L. monocytogenes* is unlikely to be related to complement because neither phenylmethylsulfonyl fluoride nor heating to 56°C inhibited it. It is at least partly dependent on divalent cations and is destroyed by heating to 65°C. These properties are almost identical to those of the "listericidal factor," previously found in rabbit serum (10), which itself resembles beta-lysins, a class of potent bactericidal agents of both platelet and nonplatelet origin. The beta-lysins of platelet origin are released from platelets upon blood clotting, tend to accumulate at sites of infection, and are thought to be of major importance in the control of certain infections (1). They are toxic for *B. subtilis* (14) and *S. aureus* (8). In the present work, rat serum was also found to be lytic for these bacteria, but there is no evidence that this lytic activity was due to the same platelet-dependent lysin.

The appearance of the lysin in serum is clearly dependent on the presence of platelets because only sera made in the presence of platelets contained lysin activity. However, the lysin does not appear to be contained in a totally active form within the platelets because whole platelets and platelet extracts exhibited significantly less activity than whole serum. In the absence of complement, thrombin, but not zymosan, has been shown to damage the platelets, causing release of various effector substances, including histamine, serotonin (11), and mediators of the clotting pathway (7). In the present work, high levels of lysin activity approaching those of whole serum were achieved by the admixture of washed

platelets or the supernatant from thrombin-treated, but not zymosan-treated, platelets with platelet-free plasma serum. This suggests that a platelet substance released under conditions in which clotting occurs and in which thrombin is present reacts with the platelet-free plasma serum to produce the active form of the lysin. Heating platelet-free plasma serum to 56°C or treating it with phenylmethylsulfonylfluoride before adding it to platelets severely inhibited the expression of lysin activity. Because neither of these treatments affected the active form of the lysin present in serum, this result is further evidence that the lysin can be generated from a latent form.

The property of being converted from a latent to an active form suggests a means by which the lysin could participate in natural resistance. The procoagulant activity generated by *L. monocytogenes* and demonstrated in vitro shows that infection would tend to promote clotting, although it does not demonstrate that infection actually initiated it. Clotting would tend to damage platelets in the vicinity, causing release and generation of the active form of the lysin, which would then attack the infecting organisms. The enhancing effect of heparin treatment on *Listeria* infection could be due to a variety of causes other than its anticoagulant activity, but because this is its most obvious property, we have given it greatest consideration as a possible mechanism. We feel, therefore, that the clotting pathway is a major contributor to resistance to *L. monocytogenes* in rats.

This proposed involvement of platelets and the clotting system in nonspecific immunity to *L. monocytogenes* may have wider implications for acquired immunity and resistance to bacterial infections in general. The present platelet-dependent lysin may be toxic to a number of different bacteria, both gram positive and gram negative. Platelet aggregation and clotting can be promoted by bacteria in vitro (5) and similarly are thought to be caused by bacterial infections in vivo (12, 16). Thrombocytopenia has been correlated with poor prognosis in severe bacterial infections (13), and platelets have been seen to associate with infected macrophages (6) and to enhance their microbicidal activity (15). Recently it has been shown that macrophages themsleves can promote clotting (3). This occurs when macrophages are activated by products from lymphocytes treated with antigen to which they have been sensitized. It is conceivable that such activated macrophages could provide the procoagulant activity necessary to produce a platelet-dependent lysin in response to challenge in an immune host. Such cooperation between platelet and macrophage could provide an effective bactericidal mechanism.

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LITERATURE CITED

1. **Donaldson, D. M., and J. G. Tew.** 1977. Beta-lysin of platelet origin. Bacteriol. Rev. **41**:501–513.
2. **Friedlander, A. M.** 1975. DNA release as a direct measure of microbial killing. I. Serum bactericidal activity. J. Immunol. **115**:1404–1408.
3. **Geczy, C. L., and K. E. Hopper.** 1981. A mechanism of migration inhibition in delayed-type hypersensitivity reactions. II. Lymphokines promote procoagulant activity of macrophages *in vitro*. J. Immunol. **126**:1059–1075.
4. **Gray, M. L., and A. H. Killinger.** 1966. *Listeria monocytogenes* and listeric infections. Bacteriol. Rev. **30**: 309–382.
5. **Guckian, J. C.** 1975. Effect of pneumococci on blood clotting, platelets, and polymorphonuclear leukocytes. Infect. Immun. **12**:910–918.
6. **Horn, R. G., M. G. Kienig, J. S. Goodman, and R. D. Colling.** 1969. Phagocytosis of *Staphylococcus aureus* by hepatic reticuloendothelial cells. Lab. Invest. **21**: 406–414.
7. **Horowitz, H. I., and T. H. Spaet.** 1961. Generation of coagulation product I and its interaction with platelets and phospholipids. J. Appl. Physiol. **16**:112–114.
8. **Johnson, F. B., and D. M. Donaldson.** 1968. Purification of staphylococcal lysin from rabbit serum. J. Bacteriol. **96**:589–595.
9. **Mackaness, G. B.** 1970. The monocyte in cellular immunity. Semin. Hematol. **7**:172–184.
10. **Schultz, L. D., and M. S. Wilder.** 1971. Cytotoxicity of rabbit blood for *Listeria monocytogenes*. Infect. Immun. **4**:703–708.
11. **Siraganian, R. P.** 1972. Platelet requirement in the interaction of the complement and clotting systems. Nature (London) New Biol. **239**:208–210.
12. **Veenhoven, W. A., M. R. Halie, P. J. Stijnen, and H. O. Nieweg.** 1979. Bacterial infections and thrombocytopenia in chronic idiopathic thrombocytopenia purpura. Acta Haematol. (Basel) **62**:259–266.
13. **Vik-Mo, H., K. Lote, and A. Nordy.** 1979. Disseminated intravascular coagulation in patients with meningococcal infection: laboratory diagnosis and prognostic factors. Scand. J. Infect. Dis. **10**:187–191.
14. **Weksler, B. B., and R. L. Nachman.** 1971. Rabbit platelet bactericidal protein. J. Exp. Med. **134**:1114–1130.
15. **Wilder, M. S., and D. J. Lubin.** 1973. Platelet enhancement of postphagocytic destruction of *Listeria monocytogenes* in cultured mouse peritoneal macrophages. Infect. Immun. **8**:843–845.
16. **Zipursky, A., J. Palki, R. Milner, and G. I. Akenzua.** 1976. The hematology of bacterial infections in premature infants. Pediatrics **57**:839–853.