JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1990, p. 2331–2334
0095-1137/90/102331–04$02.00/0

Vol. 28, No. 10

Production of Hemolysin and Other Extracellular Enzymes by Clinical Isolates of *Pseudomonas pseudomallei*

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Received 14 February 1990/Accepted 24 July 1990

One hundred clinical isolates of *Pseudomonas pseudomallei* from humans were tested for their ability to produce extracellular, biologically active substances which are thought to contribute to the virulence of *Pseudomonas* species. All isolates produced at least one extracellular enzyme; 91 strains were positive for lecithinase, lipase, and protease; but none was positive for elastase. Ninety-three strains produced a hemolysin which was detectable around the heavy growth on saline-washed sheep erythrocyte brain heart infusion agar but not demonstrable around individual colonies or in broth culture filtrate. In contrast, a hemolysin which was cytolytic around individual colonies of *P. pseudomallei* on the assay plate and in broth culture filtrate was exhibited by four strains. By using one of these four isolates as the test strain, the latter hemolysin was characterized further. It was heat labile, most active in an acid environment (pH 5.5), and cytolytic in broth culture filtrate for a variety of animal and human erythrocytes. Sterols, particularly cholesterol and 7-dehydrocholesterol, inhibited its hemolytic activity, but the activity was not enhanced by reducing agents or suppressed by reagents which modify sulfhydryl-activated hemolysins. A nonhemolytic mutant of the test strain of *P. psuedomallei* retained the extracellular enzymes of its parent, indicating that the hemolysin was not a lecithinase, lipase, or protease.

Melioidosis, a disease of animals and humans caused by the soil saprophyte *Pseudomonas pseudomallei*, is endemic primarily in Southeast Asia and northern Australia. The disease in humans varies greatly in its clinical presentation, ranging from an asymptomatic state manifested only by the presence of specific antibodies; to a benign pneumonitis; to an acute, subacute, or chronic pneumonia; to an acute, subacute, or chronic suppurative process; to an overwhelming, toxemic, and rapidly fatal illness. Furthermore, *P. pseudomallei* may remain dormant in asymptomatic individuals only to recrudesce, if conditions are favorable and in many cases years after initial exposure, as an acute exacerbation. Although melioidosis is frequently seen in patients with impaired host defense mechanisms, it can occur as a single independent illness in persons who were previously well and without any underlying disease (2, 13).

Little is known about the virulence factors associated with infection by *P. pseudomallei*. Histopathological studies of tissue from animals with experimental pulmonary melioidosis showed that subjects which died with peracute infection had no gross or microscopic lesions but had evidence of profound toxemia; animals with acute infection had nodules of caseation or coagulation, with hemorrhage primarily in the lungs; while animals with chronic infection had metastatic areas of caseous necrosis and granulation, large progressing abscesses, and evidence of an enhanced allergic tissue reaction, a picture similar to that seen in subjects with tuberculosis (8). Endotoxin (17), antigens producing a tuberculin-type allergy (8), exotoxin (11, 12), a hemolytic lysolipoid endotoxin (18), and protease (12) are some biologically active substances that have been investigated previously, but because these studies were usually performed with only a single strain of *P. pseudomallei*, the overall extent to which these substances are involved in the pathogenesis of melioidosis is not known.

Also, relatively little is known concerning the pattern of extracellular products of *P. pseudomallei* populations of clinical origin. This study was undertaken to determine the prevalence of virulence factors of this bacterium by screening 100 strains isolated from clinical specimens for biologically active extracellular substances. A hemolysin which was found to occur infrequently was also characterized.

MATERIALS AND METHODS

Bacterial strains. One hundred strains of *P. pseudomallei* were available for testing. All strains were distinct clinical isolates from humans residing in northern Australia. The strains were isolated from 78 men and 22 women with the mean age of the patients being 53.4 years (range, 16 to 74 years). A total of 42 isolates were recovered from specimens of blood, 29 from sputum, 12 from wound pus, and 8 from urine, and 9 were cultured from other clinical specimens. All strains were identified as *P. pseudomallei* by colonial morphology, biochemical reactions, and agglutination with specific antiserum (1). Strains were propagated on nutrient agar slants and stored at 4°C.

Detection of biologically active substances. Strains were screened by agar plate assay for the ability to produce protease by the method described by Sokol et al. (21) and for the ability to produce lecithinase by the method of Esselmann and Liu (9). Elastase activity was detected by a zone of clearing extending from the edge of a 15-mm streak of bacterial growth after incubation at 30°C on brain heart infusion (BHI) agar (Difco Laboratories, Detroit, Mich.) plates containing 0.3% elastin (Sigma Chemical Co., St. Louis, Mo.). Lipase activity was measured by the hydrolysis of the polyoxyethylene sorbitan ester of oleic acid (Tween 80; Sigma) as the substrate by the method of Tirunarayanan and Lundbeck (22). Hemolysin production was assessed in BHI agar with 5% thrice-saline-washed sheep erythrocytes. A hemolytic zone larger than the crowded colonies and confluent growth or larger than the overlying individual colony after 48 h of incubation at 30°C was considered

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positive (14). To monitor the tests for extracellular enzymes, a laboratory strain of *Staphylococcus epidermidis* was used as the negative control and a laboratory strain of *Pseudomonas aeruginosa*, isolated from a corneal ulcer in a male patient, was used as the positive control.

Preparation of crude culture filtrate and hemolysin concentrate. A strain of *P. pseudomallei* that showed hemolysis around individual colonies and was isolated from a septicemic male patient who died of melioidosis was selected as the test strain for characterization. In addition to hemolysin, the strain was found to produce protease, lecithinase, and lipase.

Two 2-liter flasks, each containing 500 ml of BHI broth supplemented with 0.2% dextrose (14) were inoculated with 5 ml of an 18-h broth culture and incubated at 30°C for 48 h. The cultures were centrifuged (10,000 × *g*, 20 min) at 4°C, and the supernatant was membrane filtered (pore size, 0.22 μm; Millipore Corp., Bedford, Mass.) to remove any remaining cells. Crude hemolysin was prepared by ammonium sulfate fractionation (20 to 60% saturation) and then dialysis of the fraction against 10 mM Tris hydrochloride (pH 7.4). Protein concentrations were measured by a microassay method (4) with bovine serum albumin (Sigma) as the standard. The hemolysin and culture filtrate were stored at 4°C until use.

Assay for hemolytic activity. A preliminary titration with 50% hemolysis as the endpoint (as judged visually) was performed. A quantitative assay was then done by adding increments of the highest dilution of culture filtrate or crude hemolysin that caused 50% lysis in the preliminary test to 2% thrice-saline-washed sheep erythrocyte suspension in 10 mM Tris hydrochloride (pH 7.4)–160 mM NaCl buffer (SES) in a total volume of 2 ml. After incubating the mixtures for 1 h at 30°C, the unlysed erythrocytes were removed by centrifugation and the optical density of the supernatants at 545 nm was determined. Controls with SES alone as a negative control and 0.2% sodium dodecyl sulfate instead of hemolysin as a positive control for spontaneous and complete hemolysis were also included in the test. One hemolytic unit (HU) was defined as the activity of hemolysin resulting in 50% hemolysis of the SES.

Culture filtrate and hemolysin activity studies. The cytolytic activity of the hemolysin was further characterized by the assay for hemolytic activity described above, but with 2% suspensions of thrice-saline-washed erythrocytes from human and various animal species in assay buffer and culture filtrate. To determine the effects of heat treatment and changes in pH on the activity of the hemolysin, the crude hemolysin was incubated in assay buffer under various temperature-time conditions before the 2% sheep erythrocyte suspension was added and the culture filtrate was incubated with 2% suspensions of sheep erythrocytes in various buffers (pH range, 5.0 to 9.0), respectively. To establish whether certain substances would affect the activity of the hemolysin, the culture filtrate was preincubated at 4°C for 4 h with disodium EDTA (1 and 10 mM), dithiothreitol (1 mM), 2-mercaptoethanol (1 mM), *N*-ethylmaleimide (1 mM), or *p*-chloromercuribenzoate (1 mM), obtained from Sigma, and then the residual hemolytic activity in SES was measured. Calcium chloride (1 and 10 mM) was also added to the SES directly to determine its effect on hemolytic activity. Studies on the inhibition of hemolysin activity by sterols were performed by diluting sterols with SES to obtain various concentrations, adding crude hemolysin (2 HU), incubating the mixtures at 4°C for 1 h, and determining the hemolytic activity in SES by standard assay. The sterols used were cholesterol, 7-dehydrocholesterol, dihydrocholesterol, ergosterol, estradiol, pregnenolone, and stigmasterol (Sigma) and were dissolved in ethanol to form an initial 10 mM solution.

TABLE 1. Production of biologically active substances by 100 human clinical isolates of *P. pseudomallei*

Extracellular enzyme	No. of positive strains
Elastase	0
Hemolysin (weakly active)[a]	93
Hemolysin (strongly active)[b]	4
Lecithinase	97
Lipase	96
Protease	94

[a] Hemolysin was active around confluent growth only, as described in the text.

[b] Hemolysin was active around individual colonies, as described in the text.

Isolation of a nonhemolytic mutant. The hemolytic strain of *P. pseudomallei* was inoculated into 10 ml of BHI broth containing mitomycin C (0.4 mg/liter; ICN Pharmaceuticals Inc., Cleveland, Ohio) and incubated at 30°C. After 24 and 48 h, samples were cultured on sheep blood agar plates at 30°C overnight, and nonhemolytic colonies were selected and characterized as *P. pseudomallei* by biochemical tests.

RESULTS

Detection of biologically active substances. The results of testing 100 clinical isolates of *P. pseudomallei* for the production of elastase, hemolysin, lecithinase, lipase, and protease are given in Table 1. The overwhelming majority of strains produced lecithinase, lipase, and protease, while none of the strains produced elastase. Only four isolates showed hemolytic activity as determined by a clear zone of alpha hemolysis around individual colonies on sheep blood agar plates; these strains were also positive for lecithinase, lipase, and protease. However, 93 other isolates produced small zones of alpha hemolysis around crowded colonies or confluent growth but not around individual colonies, and these zones became more marked after incubation for an additional 48 h. All strains produced one or more of the extracellular substances described above.

Activity of *P. pseudomallei* hemolysin. Under test conditions, no hemolytic activity could be demonstrated in culture filtrates of strains that showed hemolysis around confluent growth but not around individual colonies on sheep blood agar. The activity of the culture filtrate of the hemolytic test strain was 10.4 HU of hemolysin per ml, while approximately 70% of the hemolytic activity of the culture supernatant was recovered by ammonium sulfate fractionation (20 to 60% saturation). The culture filtrate was also cytolytic against chicken, guinea pig, horse, human, and rabbit erythrocytes, with human erythrocytes being the most susceptible to the activity of the hemolysin (Table 2). The hemolysin was considerably more active in an acid environment than under alkaline conditions. At pH 5.5, the hemolysin was most active against sheep erythrocytes and the activity was almost three times greater than that at pH 7.5 (Table 3).

Inactivation studies. The hemolysin was markedly affected by heat treatment. Incubating the crude hemolysin (15 HU) at 56°C for 30 and 10 min reduced the cytolytic activity by 96 and 90%, respectively, while heating in boiling water for 15 and 5 min resulted in a loss of 98 and 95%, respectively, of the activity shown by the unheated control. No loss of

TABLE 2. Hemolytic spectrum of *P. pseudomallei* hemolysin[a]

Erythrocyte species	Relative activity (%)[b]
Human (AB)	180
Horse	140
Sheep	100
Chicken	95
Guinea pig	90
Rabbit	80

[a] Broth culture filtrate (10.4 HU/ml) was used.
[b] Sheep erythrocytes were used as the 100% standard.

hemolytic activity against sheep erythrocytes was detected when the culture filtrate was incubated with calcium chloride, disodium EDTA, *N*-ethylmaleimide, or *p*-chloromercuribenzoate nor was the hemolytic activity enhanced by dithiothreitol or 2-mercaptoethanol.

Inhibition studies. The effect of incubating various concentrations of sterols with crude hemolysin (2 HU) on the cytolytic activity was variable (Table 4). The cytolytic activity of the hemolysin was not affected by 20 μM estradiol or 20 μM pregnenolone, while there was 50% inhibition of activity at a concentration of 0.5 μM and complete inhibition at a concentration of 3.5 μM by both cholesterol and 7-dehydrocholesterol. A 50% loss of activity of hemolysin was seen with other sterols at concentrations between 3.5 and 6 μM.

***P. pseudomallei* mutant.** A nonhemolytic mutant of *P. pseudomallei*, as judged by the plate assay and obtained by treatment of the hemolytic parent with mitomycin C, was identical to the parent in all biochemical characteristics tested. The culture filtrate prepared with the mutant strain failed to show any hemolytic activity when incubated in SES under standard conditions. The mutant strain retained the ability of its parent to produce lecithinase, lipase, and protease.

DISCUSSION

Both *Pseudomonas cepacia* and *P. pseudomallei* are members of *Pseudomonas* rRNA homology group II (the pseudomallei group). They are both soil saprophytes and opportunistic pathogens and are similar in genetic background, colonial morphology, ability to catabolize a wide variety of organic compounds, and resistance to the aminoglycoside and polymyxin groups of antibiotics (5, 10). In the laboratory, because of their similarity, it is easy for those who are not familiar with the bacteria to mistake the identity of one for the other (5). The pattern of extracellular products detected in the 100 strains of *P. pseudomallei* described in this study (Table 1) is similar to the pattern of extracellular substances reported in studies on populations of clinical isolates of *P. cepacia* (15, 16), further illustrating the close biological relationship between these two species. However, the role of these extracellular enzymes in the pathogenesis of disease caused by either of these two species remains unclear.

TABLE 3. Hemolytic activity of *P. pseudomallei* hemolysin[a] after incubation at different pH values

Buffer[b]	pH	% of maximum hemolytic activity[c]
Sodium acetate	4.5	70
	5.0	88
	5.5	100
Sodium phosphate	6.0	80
	6.5	68
	7.0	55
Tris hydrochloride	7.5	38
	8.0	45
	8.5	50
	9.0	58

[a] Broth culture filtrate (10.4 HU/ml) was used.
[b] Buffers were made to 10 mM and incorporated 160 mM NaCl.
[c] Hemolytic activity was measured at A_{545}.

TABLE 4. Inhibition of hemolytic activity of *P. pseudomallei* hemolysin[a] by various concentrations of sterols

Sterol	Concn (μM) of sterol needed to give a residual hemolytic activity of:		
	0%	50%	100%
Cholesterol	3.5	0.5	0
7-Dehydrocholesterol	3.5	0.5	0
Dihydrocholesterol	>10	3.5	<0.5
Ergosterol	>10	4.0	<0.5
Estradiol			>20
Pregnenolone			>20
Stigmasterol	>10	6.0	<0.5

[a] Crude hemolysin (2 HU) was used.

Because there is contention concerning the hemolytic status of *P. pseudomallei* and because hemolysins produced by various bacteria are known to contribute to their virulence, we characterized the more active *P. pseudomallei* hemolysin. From results of earlier studies, the organism was considered to be nonhemolytic on blood agar (6, 23), but strains producing distinct alpha hemolysis have been reported (7, 19). By using a cellophane plate technique and cultures of *P. pseudomallei*, Liu (14) detected a heat-stable hemolysin that was neutralized by animal and human sera. Liu also demonstrated hemolysis around crowded, but not individual, colonies of *P. pseudomallei* on BHI agar plates incorporating washed erythrocytes and suggested that one reason why the bacterium was considered to be nonhemolytic was that the hemolysin was neutralized by the serum in ordinary blood agar medium. However, Heckly and Nigg (12) were unable to demonstrate any hemolytic activity in broth culture filtrates of *P. pseudomallei* which they were using for exotoxin studies.

Our findings in the present study indicate that there are at least two hemolysins produced by *P. pseudomallei*. One is common, being found in the overwhelming majority of strains, weakly cytolytic because its action is seen only as small hemolytic zones around heavy growth on sheep blood agar and not detectable in broth culture filtrate under the present test conditions. This is most probably the heat-stable hemolysin isolated by Liu (14). The other occurs infrequently, is heat labile, and is capable of producing clear zones of alpha hemolysis around well-separated individual colonies on sheep blood agar, and its cytolytic activity can be observed in broth culture filtrate.

P. aeruginosa generates two hemolysins which may contribute to its virulence; one is a heat-labile phospholipase C (9), a lecithinase, and the other is a heat-stable glycolipid (20). It is unlikely that the heat-labile hemolysin of *P. pseudomallei* is associated with lecithinase or the other extracellular products because, after loss of hemolytic activ-

ity following mutagenesis, the strain of *P. pseudomallei* remained positive for these enzymes, including lecithinase. Although the heat-labile hemolysin was inhibited by low amounts of cholesterol and 7-dehydrocholesterol, it does not belong to the sulfhydryl-activated hemolysins, the prototype of which is streptolysin O, because the activity was not enhanced by reducing agents (dithiothreitol and 2-mercaptoethanol) or suppressed by reagents, such as *p*-chloromercuribenzoate, which modify sulfhydryl groups (3). The hemolysin produced by *P. cepacia* is heat labile, its activity is inhibited by cholesterol and 7-dehydrocholesterol but not estradiol, and it is not inactivated by *p*-chloromercuribenzoate or enhanced by sulfhydryl-reducing agents (16), features which bear a close resemblance to the characteristics of the heat-labile *P. pseudomallei* hemolysin.

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