INFECTION AND IMMUNITY, July 1974, p. 152-156

Vol. 10, No. 1
Printed in U.S.A.

Slime of *Pseudomonas aeruginosa*: In Vivo Production

GEORGE DIMITRACOPOULOS, JOHN W. SENSAKOVIC, AND PASQUALE F. BARTELL

Department of Microbiology, College of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, New Jersey 07103

Received for publication 25 February 1974

Indirect hemagglutination inhibition tests were employed to detect slime in concentrations as low as 1 μg/ml. Increasing concentrations of slime resulted in proportionately greater inhibition of hemagglutination. Peritoneal aspirates and plasma of mice injected with slime were shown to exhibit the inhibitory activity of slime. The rapid dissemination of slime into the peripheral circulation was also indicated by the hemagglutination of mouse erythrocytes by specific anti-slime serum. By similar methods, the inhibitory activity of slime was also detected in peritoneal aspirates and plasma of mice infected with lethal doses of viable *Pseudomonas aeruginosa*. Furthermore, the inhibitory activity was found to increase with time after viable cell infection, whereas such increases were not detected after the injection of heat-killed organisms. Ferritin-labeled slime antibodies were found to completely surround cells of *Pseudomonas aeruginosa* obtained from the peritoneal cavity of mice 5 h postinfection.

The control of *Pseudomonas aeruginosa* infection continues to pose a serious problem in many clinical conditions. Despite intense efforts, the exact mechanism of pathogenicity remains unknown.

A previous report from this laboratory (2) indicated that lethality in experimental infection was due to the in vivo elaboration of a lethal factor(s) produced early in the infectious process, preceding increases in the microbial population which appeared to occur as a terminal event. The extracellular slime of *P. aeruginosa* has received serious attention and possesses the characteristics of a virulence factor and acts as a protective antigen (4, 10, 11, 14, 15). More recently, highly purified preparations of slime have been shown to produce toxic manifestations, leucopenia, and death of mice (12). These events were found to be indistinguishable from those observed during lethal infection produced with viable bacilli. Furthermore, purified slime was also shown to markedly inhibit phagocytosis.

The production of extracellular slime is considered to be a species characteristic of *P. aeruginosa* (9), and in vitro production has been reported under various cultural conditions (5, 6, 8, 9). However, the in vivo production of extracellular slime has not been reported.

The purpose of this study was to demonstrate the in vivo production of slime and its appearance in the peripheral circulation of mice infected with *P. aeruginosa* as further support for the possible role of slime in experimental infection.

Although its use is well established in the *Pseudomonas* literature, in this paper the term "slime" specifically refers to the refined polysaccharide-containing component isolated from the extracellular slime layer.

MATERIALS AND METHODS

Microorganisms and extracellular slime polysaccharides. The organisms used for this study, *P. aeruginosa* strains BI and EI, were originally isolated from human clinical specimens and have been previously described (3).

The extracellular slime polysaccharides used, slime polysaccharide B (SPB) from strain BI and slime polysaccharide A (SPA) from strain EI, were extracted and purified by previously described procedures (1, 12), to the extent that column chromatography and immunodiffusion indicated homogeneity.

Animal challenge. Male, white Swiss mice, 20 g, were used in groups of five, and supplied water and Purina mouse chow ad libitum. Each experiment was repeated at least three times. All injections were 0.2 ml by the intraperitoneal route. Various concentrations of slime to be injected were suspended in sterile 0.1 M sodium phosphate-buffered saline (PBS; pH 7.5). Organisms were grown on Trypticase soy agar (Difco) slants for 18 h at 37 C, then suspended in PBS to a turbidity of 750 U in a Klett-Summerson photoelectric colorimeter with a no. 54 green filter. Prior to injection, bacterial cells were washed in 10 ml of PBS and sedimented at 3,500 × *g*, 10 min. After a second

washing, the organisms were resuspended to the original volume in PBS. The number of viable cells was determined by bacterial colony count and was approximately 10^{10} to 5×10^{10}/ml. Heat-killed cells were also prepared in the manner described above, followed by exposure to 100 C for 15 min.

Peritoneal aspirates, plasma and erythrocyte samples. After the appropriate injections, samples were collected from mice at various time intervals and assayed. Peripheral blood samples were obtained retro-ocularly with micro-Natelson heparinized blood-collecting tubes and pooled. Erythrocytes and plasma were separated by centrifugation at $840 \times g$ for 15 min. After cervical fracture, peritoneal cavities were carefully washed with 1.0 ml of PBS containing 100 U of heparin per ml, and the fluids were aspirated. The pooled aspirates were centrifuged at $840 \times g$ for 15 min, and the supernatant fluids were assayed.

HA. Formalinized sheep red blood cells (Difco) were sensitized with purified slime by incubating a 5% suspension of cells in PBS with an equal volume of purified slime (200 μg/ml in PBS) at room temperature for 30 min. The sensitized cells were then washed three times in PBS and resuspended to a 5% concentration in PBS. Sensitized cells (0.05 ml) were added to 0.5-ml serial dilutions of the appropriate standard anti-slime serum, mixed well, allowed to settle at room temperature for 2 h, and read. The titer was expressed as the reciprocal of the highest serum dilution resulting in a positive hemagglutination (HA) pattern.

Mice erythrocytes were collected as peripheral blood samples and sedimented at $100 \times g$ for 5 min. The sedimented erythrocytes were then suspended to final concentration of 5% in PBS, 0.05 ml was added to serial dilutions of the appropriate standard anti-slime serum, and the hemagglutination titer was determined.

HAI. The inhibitory activity of slime, peritoneal aspirates, or plasma was determined by testing these samples as inhibitor in indirect HA inhibition tests (HAI). The sample (0.1 ml) was added to 0.5-ml serial dilutions of the appropriate standard anti-slime serum. After incubation at room temperature for 60 min, 0.05 ml of the sensitized sheep cells was added to each tube and the hemagglutination titer was determined after a 2-h incubation period at room temperature.

The standard rabbit anti-slime sera employed in these tests were produced with purified slimes of strains BI and EI, and demonstrated hemagglutination titers of 640. These sera have been shown to produce a single band when reacted with crude cellular extracts in immunodiffusion tests.

Indirect visualization of slime. Five hours after the injection of viable organisms, peritoneal aspirates were obtained and the bacterial cells were sedimented at $3,500 \times g$ for 10 min. The cells were then resuspended to the original volume in PBS and treated with ferritin-labeled rabbit anti-slime serum or ferritin-labeled rabbit normal serum, conjugated as described by Sri Ram et al. (13). After incubation for 30 min at room temperature, the cells were washed three times in PBS, negatively stained with 2% phosphotungstic acid in distilled water containing 0.01% glycerol, and examined with a Hitachi HU-12 electron microscope.

RESULTS

The ability of slime, obtained from *P. aeruginosa* strain BI, to act as inhibitor in indirect HAI tests is indicated in Table 1. The hemagglutination titer was reduced when as little as 1 μg of slime per ml was reacted with the standard anti-slime serum used in these tests. Increasing concentrations of slime resulted in a proportionately greater inhibition of HA. Identical results were also observed in a system consisting of the slime of strain EI and its specific antiserum. The inhibitory activity of slime is unaffected by heat treatment at 100 C for 15 min (Table 1). Previous results also indicated that the lethal activity of slime is unaffected by heat treatment at 100 C for 15 min (J. W. Sensakovic and P. F. Bartell, unpublished results).

Mice peritoneal aspirates and plasma obtained 1 h after intraperitoneal injection of slime possessed HAI activity, whereas no inhibitory activity was detected in PBS-injected controls (Table 2). No difference in inhibitory activity was detected between mice injected

TABLE 1. *Indirect hemagglutination inhibition of slime-coated sheep erythrocytes with various concentrations of slime and heat-treated slime from Pseudomonas aeruginosa strain BI*

Inhibitor	Concn (μg/ml)	Hemagglutination titer[a]	Hemagglutination inhibition[b]
None		640	
PBS[c]		640	1
Slime	1	320	2
Slime	10	80	8
Slime	100	10	64
Slime (100 C, 15 min)	1	320	2
Slime (100 C, 15 min)	10	80	8
Slime (100 C, 15 min)	100	10	64

[a] Reciprocal of highest dilution of anti-slime serum resulting in hemagglutination of slime-coated sheep erythrocytes.

[b] Hemagglutination titer in absence of inhibitor divided by hemagglutination titer obtained when inhibitor was added to serial dilutions of anti-slime serum prior to addition of slime-coated sheep erythrocytes.

[c] Phosphate-buffered saline, pH 7.5.

with slime at concentrations of 12.5 or 25 μg/g of mouse. However, when 50 μg/g of mouse was injected, there was a greater than twofold increase of inhibitory activity in both peritoneal aspirates and plasma samples compared to the lower doses.

The indication that slime is rapidly disseminated into the peripheral circulation was further confirmed when mice erythrocytes, obtained after the intraperitoneal injection of slime, were found to hemagglutinate when reacted with rabbit anti-slime serum (Table 3).

It was then of interest to ascertain whether HA-inhibitory activity was associated with peritoneal aspirates and plasma samples obtained after infection with *P. aeruginosa* strain EI. It can be seen that HA-inhibitory activity was detectable in both peritoneal aspirates and plasma samples (Fig. 1). The inhibitory activity of peritoneal aspirates was observed by 2 h after infection, increasing to high levels by h 12. However, by h 15, there appeared to be a decrease in the HA-inhibitory activity of the peritoneal aspirates. Plasma samples showed no inhibitory activity in samples obtained during the first 4 h after infection. However, by h 12 the level of activity was comparable to that of the peritoneal samples, persisting until the h 15 and death of the mice. When samples were available at later hours in one or two surviving mice, there appeared to be lower levels of inhibitory activity. On the other hand, the injection of heat-killed organisms did not provoke increased levels of inhibitory activity in either the peritoneal aspirates or the plasma. The low level of activity detected in peritoneal aspirates obtained 2 h after injection of heat-killed organisms is presumed to reflect the introduction of preformed slime which was undetectable by the h 12, and produced no observable manifestations in the mice. However, plasma samples taken after the introduction of heat-killed bacteria did not exhibit any detectable HA-inhibitory activity during the observation period. The injection of PBS (pH 7.5) did not evoke HA-

TABLE 2. *Indirect hemagglutination inhibition of slime-coated sheep erythrocytes with mouse peritoneal aspirates and plasma obtained 1 h after i.p.[a] injection of slime from Pseudomonas aeruginosa strain EI*

Slime injected i.p. (μg/g)	Hemagglutination inhibition[b]	
	Peritoneal aspirate[c]	Plasma[c]
0	1	1
12.5	8	8
25.0	8	8
50.0	>16	16

[a] i.p., Intraperitoneal.

[b] Hemagglutination titer in absence of inhibitor divided by hemagglutination titer obtained when peritoneal aspirate or plasma was added to serial dilutions of anti-slime serum prior to addition of slime-coated sheep erythrocytes. Hemagglutination titer taken as reciprocal of highest serum dilution resulting in hemagglutination of slime-coated sheep erythrocytes.

[c] Samples obtained 1 h after injection.

TABLE 3. *Hemagglutination of mouse erythrocytes obtained at various time intervals after i.p.[a] injection of slime from Pseudomonas aeruginosa strain EI*

Slime injected i.p. (μg/g)	Hemagglutination titer[b]		
	1 h[c]	3 h	5 h
0	0	0	0
12.5	10	20	20
25.0	20	20	20

[a] Intraperitoneal.

[b] Expressed as reciprocal of highest dilution of standard anti-slime serum resulting in hemagglutination after addition of mouse erythrocytes.

[c] Time that mice erythrocytes were obtained.

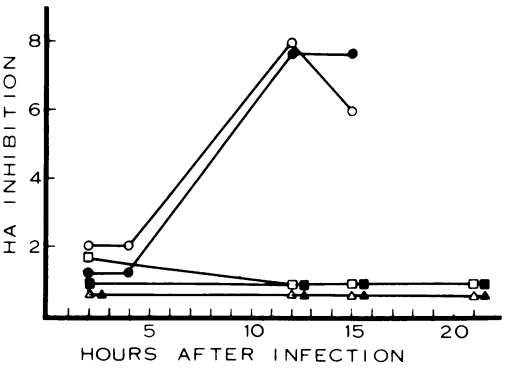

FIG. 1. *Indirect hemagglutination inhibition of slime-coated sheep erythrocytes with mouse peritoneal aspirates (○) and plasma (●) obtained at various time intervals after intraperitoneal injection of Pseudomonas aeruginosa strain EI viable cells; peritoneal aspirates (□) and plasma (■) after the intraperitoneal injection of heat-killed cells; peritoneal aspirates (△) and plasma (▲) after the intraperitoneal injection of phosphate-buffered saline, pH 7.5. HA inhibition, hemagglutination titer in absence of inhibitor divided by hemagglutination titer obtained when peritoneal aspirates or plasma were added to serial dilutions of anti-slime serum prior to the addition of slime-coated erythrocytes. Titers taken as reciprocal of the highest serum dilution resulting in hemagglutination.*

inhibitory activity in either peritoneal aspirates or plasma samples.

Figure 2 depicts the appearance of HA-inhibitory activity in mice peritoneal aspirates obtained at hourly intervals after the intraperitoneal injection of viable cells of *P. aeruginosa* strain BI. Inhibitory activity was detectable in the aspirates almost immediately after injection and showed a twofold increase within 1 h. In this case, further increases were not detectable after the h 1, and the activity remained at a constant level during the 5-h observation period. The mice usually succumbed by 15 to 18 h after infection. The injection of heat-killed cells did not result in increased inhibitory activity but rather fell to undetectable levels by 4 h after injection. These mice uniformly survived as did those injected with PBS (pH 7.5).

Figure 3A presents an electron micrograph showing the dense localization of ferritin-labeled rabbit anti-slime serum along the entire periphery of *P. aeruginosa* strain BI, extending outward a considerable distance in an irregular manner. In contrast, the negative control cells reacted with ferritin-labeled normal rabbit serum did not exhibit this reaction (Fig. 3B). The organisms observed were obtained from the mouse peritoneum 5 h after injection.

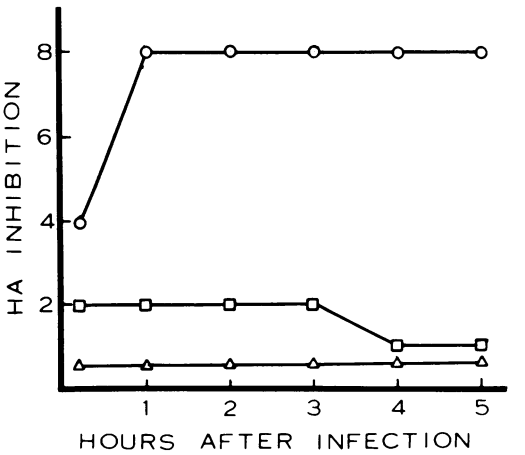

FIG. 2. *Indirect hemagglutination inhibition of slime-coated sheep erythrocytes with mouse peritoneal aspirates obtained at various time intervals following intraperitoneal injection of Pseudomonas aeruginosa strain BI. Symbols: O, viable cells; □, heat-killed cells; Δ, phosphate-buffered saline, pH 7.5. HA inhibition, hemagglutination titer in absence of inhibitor divided by hemagglutination titer obtained when peritoneal aspirates were added to serial dilutions of anti-slime serum prior to addition of slime coated sheep erythrocytes. Titers taken as reciprocal of the highest serum dilution resulting in hemagglutination.*

DISCUSSION

The purified extracellular slime polysaccharides SPA and SPB, extracted from *P. aeruginosa* strains EI and BI, respectively, are known to be antigenically distinct. This distinction is demonstrable by indirect HA and by mice protection tests (12). Both active and passive immunization of mice against purified slime have been shown to prevent the toxic manifestations leucopenia and death, produced by injection of slime. More importantly, similar immunizations protected against the identical manifestations observed during lethal infection initiated with viable bacilli (12). In the same experiments, protection was shown to be strain specific.

Through the use of indirect HAI tests and highly specific antibodies produced against purified slime, the results presented in this paper show that slime can be detected in vitro in concentrations as low as 1 μg/ml. Increased concentrations of slime resulted in proportionately greater HAI activity.

Based on the specific interaction between slime and its antibody, the HAI test was capable of detecting slime in concentrations of 10 μg/ml in the peripheral circulation as well as in peritoneal aspirates after the injection of slime (12.5 μg/g mouse). Furthermore, peripheral erythrocytes obtained from mice injected with slime (12.5 μg/g) were agglutinated when mixed with specific antibody against slime. These results indicate the presence of slime on the surface of mice erythrocytes which could only have occurred by coating in vivo after the rapid dissemination of slime.

Perhaps more significant was the fact that, after intraperitoneal infection of mice with viable cells, the increasing inhibitory activity of slime was detected in the peripheral circulation, as well as in peritoneal aspirates prior to eventual death of the mice. Conversely, the injection of heat-killed cells or PBS did not result in increasing levels of HA-inhibitory activity. Although inhibitory activity could be detected in mice injected with heat-killed cells, this activity was ascribed to the injection of small amounts of preformed slime. In all cases, this small amount of slime fell to undetectable levels and all mice survived. These results are taken as an indication of the in vivo production of slime during lethal infection of mice. Through the use of ferritin-labeled antibody to SPB, slime antibodies were demonstrated to completely sur-

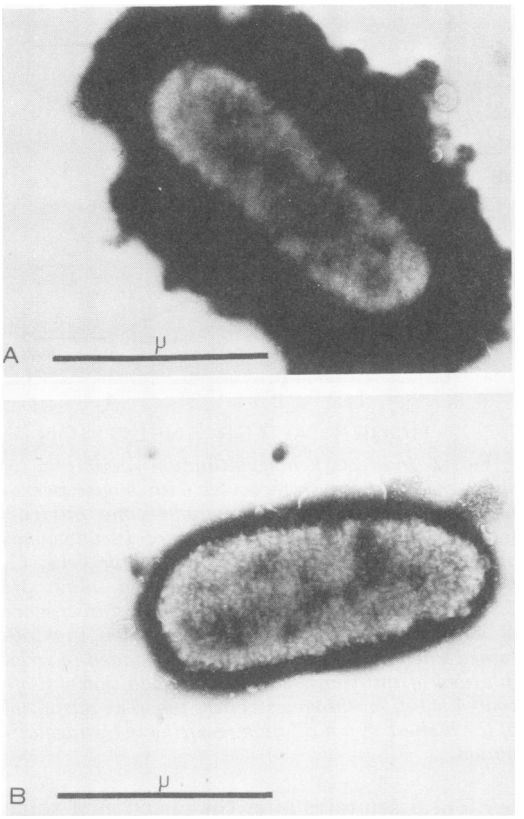

FIG. 3. *Electron micrograph of Pseudomonas aeruginosa strain BI recovered from mouse peritoneal cavity 5 h after infection and reacted with ferritin-labeled rabbit anti-slime serum (A) or ferritin-labeled normal rabbit serum (B).*

round *P. aeruginosa* strain BI obtained from the peritoneal cavity of mice 5 h postinfection.

The results presented here are consistent with other studies dealing with experimental infection (2, 12) and clinical observations (7, 16, 17), which suggest that death in *P. aeruginosa* infection is related to the liberation of toxic products from the site of primary infection and that septicemia occurs as a terminal event. With this in mind, specimens obtained from certain clinical cases involving *P. aeruginosa* infection are being screened for specific HAI activity.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service grant AI-08504 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. Bartell, P. F., T. E. Orr, and B. Chudio. 1970. Purification and chemical composition of the protective slime antigen of *Pseudomonas aeruginosa*. Infect. Immunity **2**:543-548.
2. Bartell, P. F., T. E. Orr, and M. Garcia. 1968. The lethal events in experimental *Pseudomonas aeruginosa* infection of mice. J. Infect. Dis. **118**:165-172.
3. Bartell, P. F., T. E. Orr, and G. K. H. Lam. 1966. Polysaccharide depolymerase associated with bacteriophage infection. J. Bacteriol. **92**:56-62.
4. Bass, J. A., and J. C. McCoy. 1971. Passive immunization against experimental *Pseudomonas* infection: correlation of protection to Verder and Evans "O" serotypes. Infect. Immunity **3**:51-58.
5. Brown, M. R. W., J. H. Scott Foster, and J. R. Clamp. 1969. Composition of *Pseudomonas aeruginosa* slime. Biochem. J. **112**:521-525.
6. Callahan, W. S., B. Beyerlein, and J. D. Mull. 1964. Toxicity of *Pseudomonas aeruginosa* slime. J. Bacteriol. **88**:805-806.
7. Foley, F. D., K. A. Greenwald, G. Nash, and B. A. Pruitt. 1969. Pathology of pseudomonas infection. Tex Med. **65**:36-39.
8. Goto, S., S. Enomoto, Y. Takahashi, and R. Motomatsu. 1971. Slime production by *Pseudomonas aeruginosa*. I. Conditions for slime production by the cellophane plate method. Jap. J. Microbiol. **15**:317-324.
9. Haynes, W. C. 1951. *Pseudomonas aeruginosa*—its characterization and identification. J. Gen. Microbiol. **5**:939-950.
10. Liu, P., Y. Abe, and J. Bates. 1961. The roles of various fractions of *Pseudomonas aeruginosa* in its pathogenesis. J. Infect. Dis. **108**:218-228.
11. Schwartzmann, S. and J. Boring. 1971. Antiphagocytic effect of slime from a mucoid strain of *Pseudomonas aeruginosa*. Infect. Immunity **3**:762-767.
12. Sensakovic, J. W., and P. F. Bartell. 1974. The slime of *Pseudomonas aeruginosa*: biological characterization and possible role in experimental infection. J. Infect. Dis. **129**:101-109.
13. Sri Ram, J., S. S. Tawde, G. B. Pierce, Jr., and A. R. Midgley, Jr. 1963. Preparation of antibody—ferritin conjugates for immuno-electron microscopy. J. Cell Biol. **17**:673-675.
14. Stone, H. H. 1966. Review of pseudomonas sepsis in thermal burns: verdoglobin determination and gentamicin therapy. Ann. Surg. **163**:297-305.
15. Stone, H. H., K. S. Given, and J. D. Martin, Jr. 1967. Delayed rejection of skin homografts in pseudomonas sepsis. Surg. Gynecol. Obstet. **124**:1067-1070.
16. Teplitz, C. 1965. Pathogenesis of pseudomonas vasculitis and septic lesions. Arch. Pathol. **80**:297-307.
17. Teplitz, C. 1969. Pathology of burns, p. 22-88. *In* C. Artz and J. Moncrief (ed.), The treatment of burns. Saunders, Philadelphia.