Microbiol. Immunol.
Vol. 32 (7), 645–652, 1988

Resistance of *Pseudomonas pseudomallei* to Normal Human Serum Bactericidal Action

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(Accepted for publication, March 5, 1988)

Abstract The effect of human normal serum (HNS) on *Pseudomonas pseudomallei* was determined. It is apparent from our data that the organism is resistant to the normal serum bactericidal mechanism. Ancillary experiments to confirm this serum-resistant property of *P. pseudomallei* were done by examining the effects of growth phase conditions of the bacteria (i.e., logarithmic and stationary phases) and different buffered systems used as diluent in our bactericidal assay. Results obtained showed similar degree of resistance to serum bactericidal killing by 5 strains of the organisms tested. The possible survival advantage of serum-resistant property to *P. pseudomallei* as bacterial pathogens known to invade the blood stream is discussed.

Melioidosis caused by *Pseudomonas pseudomallei* can manifest itself as an acute, subacute, or chronic illness (15). The former variety of the disease may be of the acute pulmonary infection or acute septicemic infection. The septicemic form of melioidosis is often associated with high mortality. For disease-producing bacteria their initial contact with the host defense mechanism upon penetration of tissue barrier is the normal serum which is believed to play a significant role in protection against infection (6, 8, 12). It is known that a wide variety of bacteria are susceptible to the bactericidal effects of normal mammalian serum (2, 8, 9, 11, 22). Invasion of an animal host by *P. pseudomallei* has been implicated to be via cuts, skin abrasions, or scarification (15). Hence one of the first lines of host defense that it encounters will be the normal serum present in blood circulation. It has been suggested that resistance to serum bactericidal killing is an important virulence determinant in gram-negative bacteria (17). There appears to be a strong correlation between serum resistance and the ability of a variety of gram-negative bacteria to invade and survive in the human blood stream (5, 12, 19, 23). Since *P. pseudomallei* may be directly introduced into the blood via cuts, skin abrasions, and scarification and subsequently can cause septicemic form of infection, the question arises as to whether the ability of *P. pseudomallei* to multiply in the *in vivo* situation and to produce septi-

cemia is due to its serum resistance. This study was carried out to determine the effect of normal human serum on *P. pseudomallei* in an attempt to investigate the susceptibility of the organism to normal human serum (NHS).

MATERIALS AND METHODS

Bacteria. All *P. pseudomallei* strains used in the study were kindly supplied by R.A. Finkelstein, University of Missouri-Columbia. Each strain represents organisms from different sources and of different colony-types: *P. pseudomallei* UKMKL was isolated from a male patient who died of *P. pseudomallei* infection (smooth colony-type); *P. pseudomallei* 13 smooth was isolated from the rice fields in Krabi, Thailand (smooth colony-type); *P. pseudomallei* 25 donut was isolated from soil in Ubol, Thailand (donut-appearance colonies); *P. pseudomallei* 1188 HP-R was isolated from soiled water in Prachuab, Thailand (rough colony-type); and *P. pseudomallei* USAMRU Strain 4 was isolated from soiled water in Pangna, Thailand (smooth colony-type).

An enteropathogenic strain of *Escherichia coli* (SUKM-18) obtained from the Microbiology Department, Universiti Kebangsaan Malaysia, was included in certain assays as an indicator bacteria. This strain has been routinely shown in our laboratory to be readily killed by human normal serum.

Sera. Pooled human sera from 6 healthy male adult volunteers were prepared as follows: blood was obtained by venipuncture, and the blood was allowed to clot at room temperature overnight; the sera were then separated from clot by low speed centrifugation, pooled, filter-sterilized through 0.22 μm filters, distributed in 0.5 ml aliquots, and stored at -70 C until further use.

Serum bactericidal assay. An 18 hr culture of *P. pseudomallei* grown in brain-heart infusion broth (BHIB) was harvested by low speed (3,000 rpm) centrifugation (Janetzki T32). The bacterial pellet was resuspended in phosphate-buffered saline (PBS), pH 7.2, to give the desired concentration of organisms for each experiment.

Serum bactericidal assay was performed as follows: A hundred microliter volume of normal serum was added to a tube containing 0.8 ml of PBS and 0.1 ml of bacterial suspension (10^7 cells/ml). The mixture was incubated at 37 C in a shaking water bath for 2 hr. A negative control tube containing organisms suspended in PBS alone was included. Triplicate 0.1 ml samples were withdrawn at 0, 30, 60, and 120 min, and plated on brain-heart infusion agar (BHIA). The colony-forming units (CFU) were enumerated after 18 hr of incubation. A concurrent experiment using *E. coli* which is known to be serum sensitive was performed, to indicate the presence of bactericidal activity of NHS during the incubation period.

In cases where bactericidal activity was not evident, further tests were carried out as follows: The bacteria were (2) incubated with higher concentrations of normal sera (i.e., 20% and 30% normal sera); (1) incubated with 10% normal serum using different buffer system, i.e., Tris buffer, (10 mM, pH 7.2) and gelatin veronal buffer saline, (pH 7.2); (3) incubated with 10% normal serum using organisms obtained from different growth phases (i.e., after 8 hr and 24 hr incubation in

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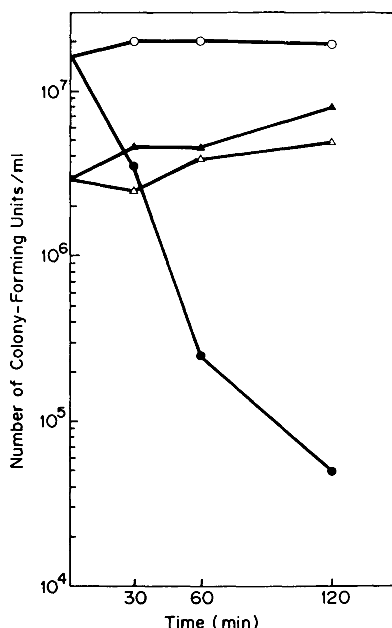

Fig. 1. Serum bactericidal activity against *P. pseudomallei* following incubation with (▲) or without (△) 10% normal human serum (NHS) for a period of 2 hr. The bactericidal activity of NHS throughout the incubation period was concurrently assayed by performing similar experiments using *E. coli* with (●) or without (○) 10% NHS. Mean of 4 replicates.

the growth medium). The 8 hr, 18 hr, and 24 hr cultures represent organisms in their early log phase, late log phase culture, and stationary phase, respectively.

RESULTS

Effect of Normal Human Serum on P. pseudomallei

NHS did not display any bactericidal activity on all the 5 strains of *P. pseudomallei* tested when incubated for 2 hr *in vitro*. The viability count of the bacteria incubated with 10% NHS was the same as that of the negative control, i.e., bacteria without serum (Fig. 1 and Table 1). In the concurrent experiment with *E. coli*, the bactericidal activity of this serum was manifested by a greater than 2 log reduction in the viability count of *E. coli* following 2 hr incubation with 10% NHS whilst there was no reduction when the bacteria were incubated without NHS for 2 hr. This indicated that the *in vitro* serum bactericidal system used was efficient.

Serum bactericidal activity on *P. pseudomallei* was not influenced by slight increases in serum concentration, growth phases of the bacterial culture, or buffer systems in which the assays were performed. NHS up to 20% final concentration displayed no considerable bactericidal activity on *P. pseudomallei* even after 2 hr incubation. The viability count of the bacteria, however, was slightly reduced by

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Table 1. Viability count of *P. pseudomallei* incubated with 10% normal serum

Strains tested	Colony-types	Sources	Time	Serum incubated cfu/ml ($\times 10^6$)	PBS incubated cfu/ml ($\times 10^6$)
P. pseudomallei UKMKL	Smooth	Patient in Kuala Lumpur, Malaysia	0	2.10	2.10
			30	1.76	3.75
			60	2.82	3.10
			120	3.12	7.56
P. pseudomallei 13 smooth	Smooth	Soiled water in Krabi, Thailand	0	3.76	7.23
			30	3.46	9.23
			60	9.50	1.23
			120	9.86	2.79
P. pseudomallei 25 Donut	Donut	Soil in Ubol, Thailand	0	7.53	7.40
			30	8.60	11.80
			60	10.03	14.80
			120	30.6	34.70
P. pseudomallei 1188 HP-R	Rough	Soiled water in Prachuab, Thailand	0	13.10	12.50
			30	12.70	14.50
			60	31.50	12.40
			120	33.20	23.00
P. pseudomallei USAMRU str. 4	Smooth	Soiled water in Pangna, Thailand	0	17.90	13.90
			30	23.00	15.50
			60	13.20	11.20
			120	25.50	16.70

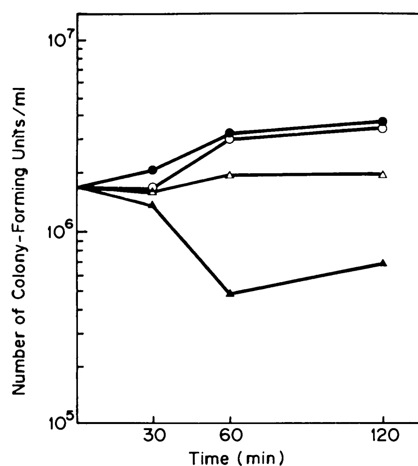

Fig. 2. The effect of different concentrations of normal human serum on viability of *P. psuedomallei*: ○, no serum; ●, 10% serum; △, 20% serum; ▲, 30% serum. Mean of 4 replicates.

a half log reduction after 1 hr incubation with 30% NHS (Fig. 2).

P. pseudomallei was resistant to 10% NHS when incubated for 2 hr whether the bacteria was taken from early log phase, late log phase, or stationary phase. The

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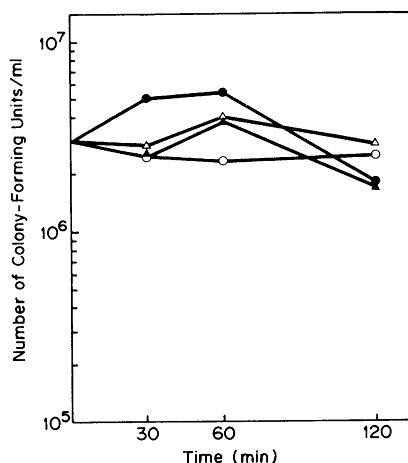

Fig. 3. The effect of 10% NHS on *P. pseudomallei* at different growth phases: ○, 8 hr culture (early log phase); ●, 18 hr culture (late log phase); △, 24 hr culture (stationary phase); ▲, 18 hr culture with no serum. Mean of 3 replicates.

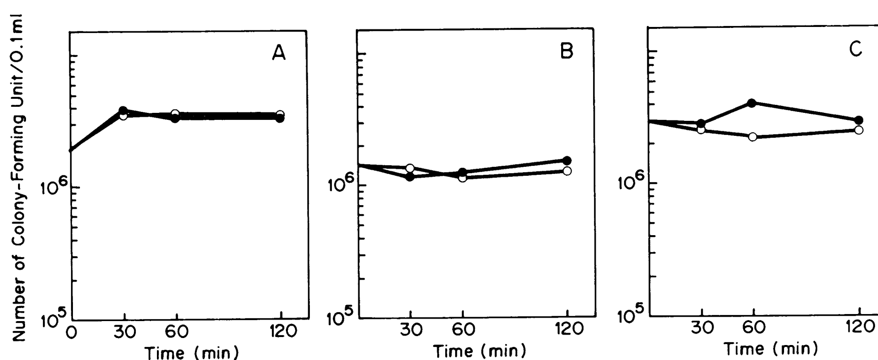

Fig. 4. Influence of different buffers on serum bactericidal assay for *P. pseudomallei* using 10% NHS. The bacteria were incubated with (●) or without (○) NHS for 0, 30, 60, or 120 min in PBS, pH 7.2 (A), GVBS (B), or 0.02 M Tris, pH 7.2 (C) before viability of the bacteria was determined. Mean of 3 replicates.

viability count of the bacteria at different growth phases was the same as that of the control (Fig. 3).

The viability count of *P. pseudomallei* in different buffer systems was similar irrespective of whether the assays were performed in PBS (pH 7.2), 0.02 M Tris (pH 7.2), and gelatin veronal buffered saline (GVBS) (Fig. 4).

DISCUSSION

It is evident from the data that *P. pseudomallei* is resistant to the normal serum

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bactericidal mechanism. The universality of serum resistance property of *P. pseudomallei* is indicated by our test results employing strains isolated from patients as well as from water and soil habitats. Serum resistance also appears to be exhibited by test organisms obtained from smooth, rough, and donut colony-types. Reduction in CFU of more than 2 logs after 24 hr of incubation with 10% NHS was observed when *E. coli* were used as test organisms. Similar treatments of *P. pseudomallei* failed to show any effects of NHS on the viability of this organism. Serum bactericidal activity on *P. pseudomallei* was only apparent at the highest serum concentration (i.e., 30%) tested. Even so, the reduction in CFU was only in the order of one-half log viability of this organism.

Earlier investigators have established that serum susceptibility of bacteria is influenced by the growth conditions employed for preparation of the inoculum (16). *E. coli* for instance are more readily killed by serum when in stationary phase (3, 13). However, in this study, inoculum preparation of *P. pseudomallei* obtained during early logarithmic or stationary phases exhibited similar degree of resistance to serum bactericidal killing.

The method for preparation of cell inoculum used in the serum bactericidal system is believed to also determine the result of the assay (4, 17). For instance, centrifugation of bacteria at 0–4 C or extensive washing may result in an overestimation of the serum susceptibility test. Taking cognizance of these possible limiting factors, our serum bactericidal assay was performed under conditions approaching optimum. Each bacterial inoculum was centrifuged once to pellet the organism and resuspended in buffer without washing. This serum bactericidal assay is also influenced by the buffer types used (1, 20). Employing three different buffers, our results showed that *P. pseudomallei* affirmed its serum-resistant property in all the buffers tested. Clearly the ineffectiveness of normal human serum in the killing of *P. pseudomallei* was not due to non-specific inhibition afforded by the buffer systems.

Serum resistance of bacteria has been suggested as an important determinant in providing a significant survival advantage to the pathogen when present in the blood system (12). Previous investigators have reported the isolation of higher proportions of serum-resistant bacterial strains from blood cultures of bacteremic patients than from other sources such as urine or stool cultures (5, 19, 23). Thus, it can be conjectured that for successful invasion of the host blood stream, bacterial pathogens with serum-resistant properties possess a survival advantage over sensitive strains. Resistance to bactericidal action of serum has been associated with the ability of *Neisseria gonorrhoeae* to cause disseminated gonococcal infection in persons with a functionally intact complement pathway (7, 14). *P. pseudomallei* infection is known to produce fatal septicemic melioidosis where the organisms invade the blood stream and multiple abscesses develop in various organs, most commonly in the lungs, liver, and spleen (10, 18, 21). It is most probable that the ability of *P. pseudomallei* to resist elimination from the host and cause systemic diseases in human is partly due to its serum-resistant property.

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We gratefully acknowledge the kind cooperation of the Laboratory Staff, Microbiology Department, Faculty of Life Science, Universiti Kebangsaan Malaysia. We thank Mrs. Noraini Abdul Hamid for typing the manuscript.

REFERENCES

1) Clas, F., and Loss, M. 1980. Killing of the S and R forms of *Salmonella minnesota* via the classical pathway of complement activation in guinea-pig and human serum. Immunology **40**: 547–556.
2) Collins, F.M. 1967. Serum mediated killing of three group D salmonellas. J. Gen. Microbiol. **46**: 247–253.
3) Davis, S.D., and Wedgewood, R.J. 1965. Kinetics of the bactericidal action of normal serum on gram-negative bacteria. J. Immunol. **95**: 75–79.
4) De Matteo, C.S., Hammer, M.C., Baltach, A.L., Smith, R.P., Sutphen, N.T., and Michelsen, P.B. 1981. Susceptibility of *Pseudomonas aeruginosa* to serum bactericidal activity. A comparison of three methods with clinical correlations. J. Lab. Clin. Med. **98**: 511–518.
5) Fierer, J., Finley, F., and Braude, A.L. 1972. A plaque assay on agar for detection of gram-negative bacilli sensitive to complement. J. Immunol. **109**: 1156–1158.
6) Johnston, R.B., Jr., and Stround, R.M. 1977. Complement and host defense against infection. J. Pediatrics **90**: 169–179.
7) Lee, T.J., Utsinger, P.D., Snyderman, R., Yount, W.J., and Sparling, P.F. 1978. Familial deficiency of the seventh component of complement associated with recurrent bacterimic infections due to Neisseria. J. Infect. Dis. **138**: 359–368.
8) Mackie, J.T., and Finkelstein, M.H. 1931. Natural bactericidal antibodies: observations on the bactericidal mechanism of normal serum. J. Hyg. **31**: 35–55.
9) Mackie, J.T., and Finkelstein, M.H. 1932. The bactericidins of normal serum: their characters, occurrence in various animals and the susceptibility of different bacteria to their action. J. Hyg. **32**: 1–24.
10) Magee, H.R., Mitchell, R.M., Fitzwater, J.J., Christie, D.G.S., and Rao, A. 1967. Melioidosis. Med. J. Aust. 1180–1183.
11) Olling, S. 1977. Sensitivity of gram-negative bacilli to the serum bactericidal activity: a marker of the host-parasite relationship in acute and persisting infections. Scand. J. Infect. Dis. (Suppl.) **10**: 1–40.
12) Roantree, R.J., and Rantz, L.A. 1960. A study of the relationship of the normal bactericidal activity of human serum of bacterial infection. J. Clin. Invest. **39**: 72–81.
13) Rowley, D., and Wardlaw, A.C. 1958. Lysis of gram-negative bacteria by serum. J. Gen. Microbiol. **18**: 529–533.
14) Schoolnik, G.K., Buchanan, T.M., and Holmes, K.K. 1976. Gonococci causing disseminated infection are resistant to the bactericidal action of normal human serum. J. Clin. Invest. **58**: 1163–1173.
15) Stanton, A.T., and Fletcher, W. 1932. Melioidosis. Studies Inst. Med. Research Kuala Lumpur, No. 21.
16) Taylor, P.W. 1978. The effect of the growth environment on the serum sensitivity of some urinary *Escherichia coli* strains. FEMS Microbiol. Lett. **3**: 119–122.
17) Taylor, P.W. 1983. Bactericidal and bacteriolytic activity of serum against gram-negative bacteria. Microbiol. Rev. **47**: 47–83.
18) Thin, R.N.F., Brown, M., Stewart, J.B., and Garret, C.J. 1970. Melioidosis: a report of ten cases. Quart, J. Med. **39**: 115–118.
19) Vosti, K.L., and Randall, E. 1970. Sensitivity of serologically classified strains of *Escherichia coli* of human origin to the serum bactericidal systems. Am. J. Med. Sci. **259**: 114.
20) Wardlaw, A.C. 1962. The complement-dependent bacteriolytic activity of normal human serum. I. The effect of pH and ionic and the role of lysozyme. J. Exp. Med. **115**: 1231–1248.
21) Whitmore, A. 1913. Melioidosis of Rangoon. J. Hyg. **13**: 1–7.

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22) Wright, S.D., and Levine, R.P. 1981. How complement kills *E. coli* 1. Location of the lethal lesion. J. Immunol. **127**: 1146–1151.
23) Young, L.S., and Armstrong, D. 1972. Human immunity of *Pseudomonas aeruginosa* 1. *In vitro* interaction of bacteria, polymorphonuclear leukocytes and serum factors. J. Infect. Dis. **126**: 257–276.

(Received for publication, August 20, 1987)

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