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Growth, toxigenicity and virulence of *Pseudomonas aeruginosa*

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More than 80 years has elapsed since the first recognition of *Pseudomonas aeruginosa* as a human pathogen (Gessard, 1882), and its importance has increased considerably in the last decade because of its antibiotic resistance. The species as a whole has been known to be an opportunist which invades the host tissue and establishes infections most readily when the host is already weakened by other causes, such as burn, malignancy or diabetes mellitus. The factor which determines the virulence of the organisms, however, is very little understood. In a previous study (Liu, Abe & Bates, 1961) various products of this species were separated into five fractions and their roles in the pathogenesis were elucidated. This work was done with two virulent strains of this species and the question as to what determines the virulence of *Ps. aeruginosa* was left unanswered.

The conventional methods for the study of the virulence of bacteria usually express the mortality of animals injected with the organisms. The results are presented numerically, either as the number of animals that died or as the numbers of bacteria that were required to kill 50% of the animals, i.e. the LD 50. These methods, however, are grossly inadequate in the study of the virulence of *Ps. aeruginosa* for several reasons. First, the organisms usually produce localized infections, such as wounds of skin, otitis media and urinary tract infections, and sepsis that results in the death of the host is rare. Death from sepsis of experimental animals, therefore, does not really express the true mode of action of *Ps. aeruginosa*. Secondly, the virulence of this species is usually very low compared with that of other pathogens, such as *Salmonella* or *Pasteurella* and, therefore, the relatively large number of organisms required to cause death may result in the toxic death of the host, without showing the true virulence of the the organisms, which includes invasiveness as well as toxigenicity. Thirdly, the resistance of mature man and animals to the infections of *Ps. aeruginosa* appears to be quite high owing to the presence of antibodies probably acquired as the results of latent infections; therefore, the data of mortality of animals infected with this organism are influenced by the resistance of the hosts as well as the virulence of the organisms. It was decided, therefore, to study the virulence of *Ps. aeruginosa* by the development of skin lesions in the same individual animal.

In order to establish infection in a host it is obvious that the organisms must be able to grow in the tissue of the host. A study in this direction has already been made by Colebrook, Lowbury & Hurst (1960) who found that some strains of *Ps.*

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aeruginosa were able to grow in fresh human serum but others were not able to do so. This study, however, left unanswered the question of what determines the virulence, because it was not possible to show that the ability of this organism to grow in human serum was related to virulence of man. As pointed out previously from this laboratory (Liu *et al.* 1961), the cells of *Ps. aeruginosa* are relatively non-toxic, and, therefore, even if the organism can grow freely in the host tissue the growth of this type of non-toxic cells alone is unlikely to explain the pathological picture produced by this infection. On the other hand, we have found that many strains of *Ps. aeruginosa* that were able to produce considerable amounts of extracellular toxic substances, such as haemolysin, lecithinase and protease, were quite non-virulent when injected intracutaneously into rabbits, i.e. they failed to produce the lesions which were produced when the toxins formed *in vitro* were injected. In order to evaluate the relative importance of toxigenicity and ability to grow in the host tissue in the manifestation of virulence, a large number of strains of *Ps. aeruginosa* were tested for their ability to grow in fresh serum of rabbits. The virulence of these organisms was also tested in the same rabbits from which the sera were obtained. The results were then compared with the ability of the organisms to produce various extracellular toxic substances *in vitro*. The present communication describes one of these experiments which appears to throw some light on the factors that determine the virulence of *Ps. aeruginosa*.

MATERIALS AND METHODS

Four groups of *Ps. aeruginosa* were selected for this study. The first group, represented by strains P-A-7 and P-A-214, was the type which grew readily in fresh rabbit serum and produced large amounts of haemolysin, lecithinase and protease, which will be referred to collectively as the extracellular toxins (ET). The second group, represented by the strain P-A-4, was the type which produced considerable amounts of ET but failed to grow in the fresh rabbit serum. The third group, represented by the strain 1419, was the type which grew readily in fresh serum but failed to produce appreciable amounts of ET. The fourth group, represented by the strains 117 and 1184, was that which failed to grow well in fresh serum and also failed to produce appreciable amounts of ET. All the 6 strains described here were isolated from human sources.

The blood of the rabbits was obtained from the marginal vein of the ear and the serum was Seitz-filtered. The growth of the organisms in the sera as well as in the trypticase soy broth (Baltimore Biological Laboratory) was estimated by inoculating a sufficient number of cells to make the initial concentration to about 10^5 per ml., taking samples of the cultures (37° C.) at intervals, making tenfold dilutions in saline, and then plating 0·1 ml. of each dilution in duplicate on trypticase soy agar. The average of the colony counts of the two plates was taken to express the rate of growth. The virulence of the organism was observed by the development of a lesion in the back of the rabbit after intracutaneous injection of 0·1 ml. of an 18 hr. broth culture. The production of the ET *in vitro* and their titrations were carried out as described previously (Liu *et al.* 1961).

RESULTS

In Text-fig. 1 are shown the growth curves in trypticase soy broth of the 6 strains of *Ps. aeruginosa*. The strains P-A-7, P-A-214, P-A-4 and 1419 did not show significant differences in their rates of growth in this medium. The strains 117 and 1184 were definitely slower than the other 4 strains in their rates of growth. The titres of haemolysin and extracellular enzymes produced by these organisms are listed

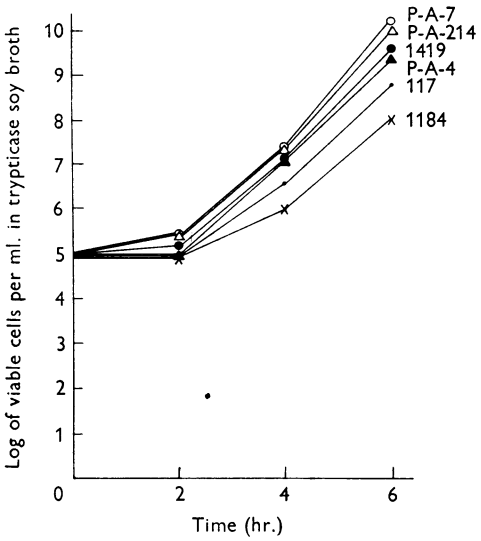


Fig. 1

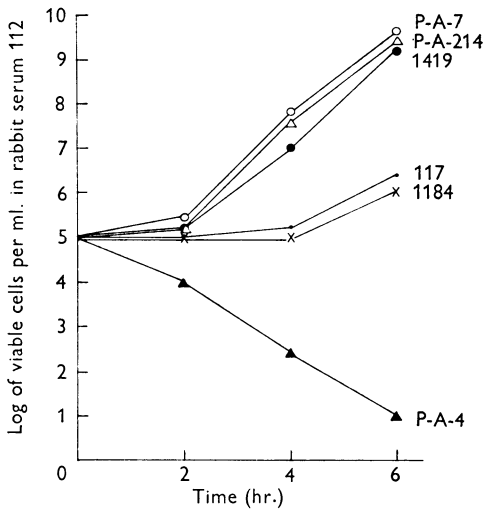


Fig. 2

Text-fig. 1. Growth curves of 6 selected strains of *Ps. aeruginosa* in trypticase soy broth.

Text-fig. 2. Growth curves of the same 6 strains of *Ps. aeruginosa* in the fresh serum of rabbit no. 112. Strains P-A-7, P-A-214 and 1419 grew well in this serum while 117 and 1184 hardly grew at all. Strain P-A-4 appeared to have been killed by this serum.

Table 1. *Toxigenicities* in vitro *of six selected strains of* Pseudomonas aeruginosa

Strain	Haemolysin	Lecithinase	Protease
117	8	0	8
1184	< 4	0	4
1419	< 4	0	8
P-A-4	32	16	64
P-A-7	64	32	128
P-A-214	32	32	128

Reciprocals of the highest dilutions of the crude preparations showing a complete reaction are listed as the titres.

in Table 1. Strains P-A-4, P-A-7 and P-A-214 produced considerable amounts of these extracellular toxic substances, but strains 117, 1184 and 1419 were very poor producers. The growth curves of these 6 strains in the serum of rabbit no. 112 are shown in Text-fig. 2. As will be seen in this figure, the strains P-A-7, P-A-214

and 1419 grew readily while the other 3 strains did not. One-tenth ml. of 18 hr. broth cultures of these 6 strains of *Ps. aeruginosa* were then inoculated intradermally into rabbit no. 112 from which the serum was obtained. In Pl. 1 are shown the results 48 hr. after the inoculation. Strains P-A-7 and P-A-214 produced large areas of induration and redness of skin; considerable necrosis was noted also in the centre of the lesions. These findings were expected because both of these strains grew well in the serum of this rabbit and they produced large amounts of ET. Strains 117 and 1184 failed to show any lesion. These findings were also expected because neither of these strains grew well in the serum of this rabbit and they did not produce ET in significant amounts. The most interesting part of this experiment, however, was the behaviour of the strains P-A-4 and 1419. The strain 1419 grew well in this serum but failed to produce significant amounts of ET. The strain P-A-4, on the contrary, produced considerable amounts of ET but failed to grow in this serum. It was hoped that the behaviour of these two strains, representing two groups of *Ps. aeruginosa*, would provide an answer to the question whether the toxigenicity or the ability to grow in the sera of animals would constitute the determinant factor of the virulence of *Ps. aeruginosa*. As shown in Pl. 1 neither strain P-A-4 nor 1419 was able to produce a significant lesion in the skin of rabbit no. 112. It appears, therefore, that neither the ability to grow in the sera of animals, nor the ability to produce various extracellular toxic substances alone is sufficient to make a strain of *Ps. aeruginosa* virulent to animals. In other words, a strain of *Ps. aeruginosa* must be able both to grow in the serum of an animal and to produce various types of extracellular toxins in order to be virulent to that animal. Although all the rabbits used in this study were supposed to be normal animals that had never been used in any experiment, considerable differences in pathological pictures were observed from one rabbit to another using the same group of *Ps. aeruginosa*. The differences were usually found among the group of *Ps. aeruginosa* which were able to produce large amounts of ET, in which the virulence of the organisms appeared to be a function of their ability to grow in the sera of animals. Those strains which failed to produce appreciable amounts of ET were usually nonvirulent regardless of their ability to grow in the sera of animals, and, therefore, the results were consistent from one experiment to another. The ability of a serum sample to inhibit the growth of a strain of *Ps. aeruginosa* appeared to depend largely on the presence of a type-specific antibody, because the inhibitory effect of the serum could be absorbed out by cells of the susceptible strains of *Ps. aeruginosa*, but not by those of the strains which grew readily in the same serum. These findings were not surprising because of the ubiquitous nature of *Ps. aeruginosa*. Many of the so-called normal animals one purchases from animal dealers were probably exposed to some strains of *Ps. aeruginosa* at some time in their life span. The presence of *Ps. aeruginosa* antibodies in human sera has been described by many workers (Gaines & Landy, 1955; Whitby, Michael, Woods & Landy, 1961). These specific antibodies which exist in the sera of animals and man have most likely been produced by exposure of the individuals to latent infections. In order to circumvent this possibility we have tested many samples of human placental serum for their ability to inhibit the growth of *Ps. aeruginosa*. Text-figs.

3 and 4 show the growth curves of the 6 strains of *Ps. aeruginosa* with two samples of placental sera. As will be seen in these figures, sample A permitted the growth of strains P-A-7, 1419, 1184 and 117 but inhibited the growth of P-A-4 and P-A-214. Sample B, on the other hand, permitted the growth of P-A-214 but inhibited 117.

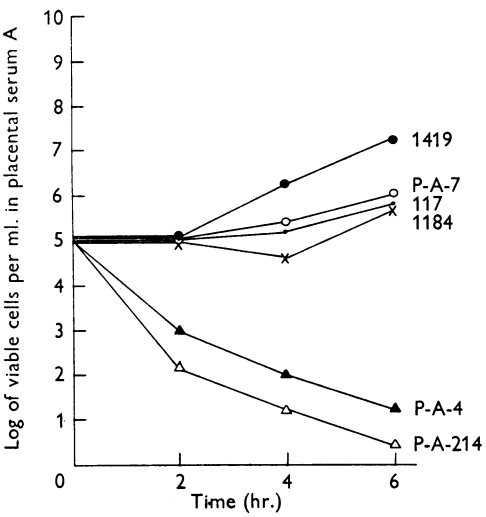


Fig. 3

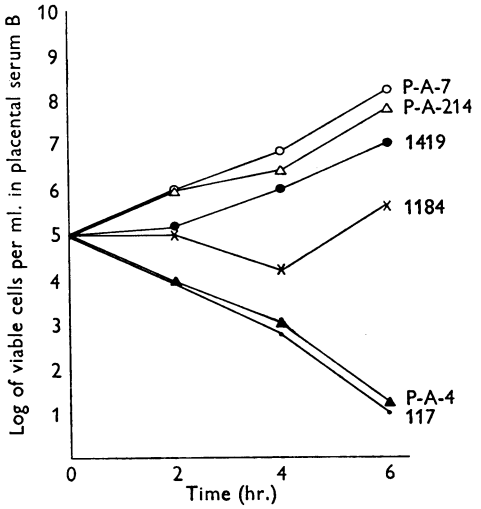


Fig. 4

Text-fig. 3. Growth curves of the 6 strains of *Ps. aeruginosa* in the placental serum A.

Text-fig. 4. Growth curves of the 6 strains of *Ps. aeruginosa* in the placental serum B.

DISCUSSION

The results of this study appear to indicate that the virulence of *Pseudomonas aeruginosa* depends on both the toxigenicity and the ability to grow in the serum of animals. Since the serum of many animals contains antibodies to various serological types of *Ps. aeruginosa*, a given strain of this species can show virulence to a particular animal only when its serum does not contain sufficient antibody to inhibit the growth of the organism, and then only if the organism is capable of producing various types of extracellular toxic substances. The fact that many samples of placental serum of man, and possibly those of various animals, contain antibodies in different degrees to different serological types of *Ps. aeruginosa* makes it very difficult, if not impossible, to describe the virulence of this species in general terms. Earlier observations on the antibody transfer in the human from mother to infant were concerned with diphtheria (Fischl & von Wunschheim, 1895) and tetanus (Polano, 1904). The subject of placental permeability to antibody has been reviewed by Doerr (1941). A reference to the virulence of *Ps. aeruginosa*, therefore, can be made with certainty only with regard to one particular strain of this species against one particular individual animal. This finding will explain the fact that some strains of *Ps. aeruginosa* isolated from severe cases of the infections, such as sepsis, do not

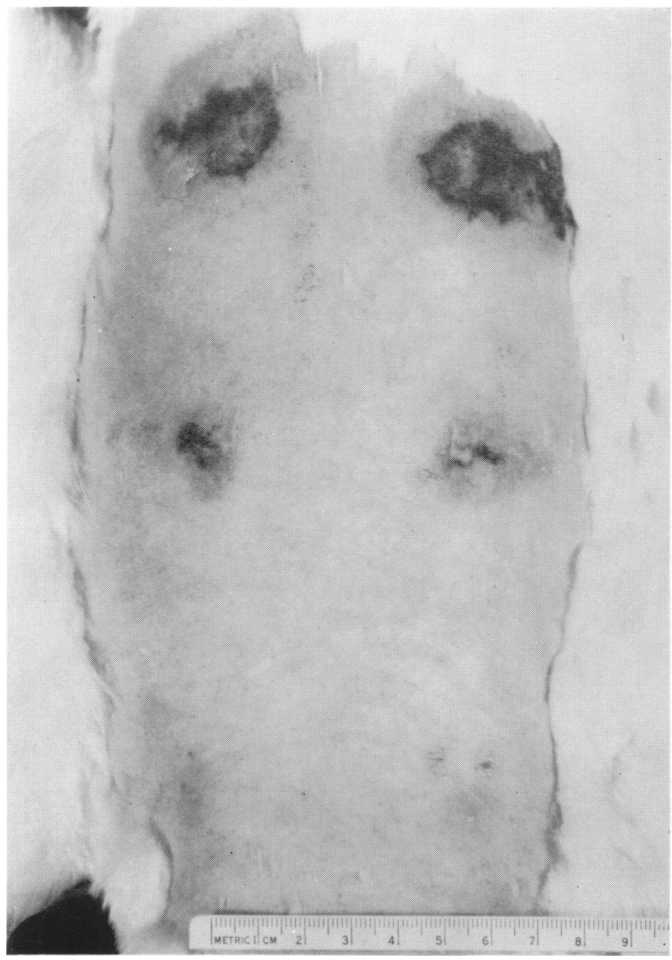
necessarily show more virulence in laboratory animals than those strains isolated from mild and trivial infections.

The relative resistance of man to infections with *Ps. aeruginosa* is probably due to both passive immunity acquired through the placenta and active immunity acquired through exposure to latent infections. Gaines & Landy (1955) have reported that antibody titres in man to the lipopolysaccharide of *Ps. aeruginosa* increased with age, and by 8–15 years practically all of those tested showed antibodies to this substance to some degree. It is interesting to note, however, that these antibodies were detected with the haemagglutination procedures. The same authors also mentioned that they were not able to demonstrate the presence of agglutinating antibody to bacterial cells in those sera containing haemagglutinating antibodies. Attempts to demonstrate incomplete antibody of the Coombs or blocking type also failed. Actually the failure to agglutinate the cells of *Ps. aeruginosa* by the sera containing antibody to the lipopolysaccharide can be explained by the fact that the cells of these organisms are usually covered by slime which could not be removed even by repeated washing with acid (Liu *et al.* 1961). Most of the bacterial suspensions used in the agglutination tests were prepared by exposure of these cells to formalin, phenol, alcohol, or heating which certainly will not remove these slimes on the surface of the cells of *Ps. aeruginosa*. It has been pointed out in a previous communication from this laboratory, (Liu *et al.* 1961) that it is the antibody to the slime, not the antibody to the lipopolysaccharide, that is responsible for both agglutination and protection. The slimes of *Ps. aeruginosa* have been shown to be a complex of large molecules containing carbohydrate, ribonucleic acid, deoxyribonucleic acid, and possibly others (Eagon, 1962). It did not appear to be as good an antigen as the lipopolysaccharide of the organism and, therefore, in the previous study mentioned above (Liu *et al.* 1961) an extensive use of Freund's adjuvant was made to produce an antibody to these substances. The poor antigenicity of slimes explains the fact that many sera of man or animals containing antibodies to the lipopolysaccharide of *Ps. aeruginosa* do not contain antibodies to the slimes.

Recently, Landy, Michael & Whitby (1962) described a method for measuring small amounts of antibody to Gram-negative bacilli which uses the bactericidal effect of the serum. These workers emphasized the fact that with this technique they were able to detect smaller amounts of antibody to enteric bacilli than by other serological tests. In the case of *Ps. aeruginosa*, however, such a technique would detect antibody to the slimes instead of those to the lipopolysaccharide. By testing the ability of *Ps. aeruginosa* to grow in undiluted sera of animals, we were actually performing quite a similar test to those described by Landy *et al.* (1962).

SUMMARY

The virulence of *Pseudomonas aeruginosa* appears to depend on both its ability to grow in the serum of animals and its ability to produce various types of extracellular toxins. No strain of *Ps. aeruginosa* lacking either of these qualifications was ever found to be virulent to animals.


METRIC 1 CM 2 3 4 5 6 7 8 9

The ability of various sera of animals to inhibit growth of *Ps. aeruginosa* appears to depend largely on their content of specific antibodies to each serological type of the surface antigens (the slime layer) and, therefore, susceptibility of animals to the infections of *Ps. aeruginosa*, even within one species, varies considerably from one individual to another.

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EXPLANATION OF PLATE 1

Lesions produced by 0·1 ml. of broth cultures of the 6 strains of *Ps. aeruginosa* in the skin of the rabbit no. 112. On the top, from left to right, are those produced by P-A-7 and P-A-214, both of which grew well in the serum of this rabbit and also produced considerable amounts of the extracellular toxins. In the middle, left side, is the lesion produced by P-A-4 which produced considerable amounts of the extracellular toxin *in vitro* but failed to grow in the serum of this rabbit. In the middle, right side, is the lesion produced by 1419 which grew well in this serum but failed to produce much extracellular toxins *in vitro*. On the bottom, from left to right, are those produced by 117 and 1184, neither of which grew well in this serum or produced much extracellular toxin. The last two strains hardly produced any change in the skin.