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Toxic activity against alveolar macrophages of products of *Pseudomonas aeruginosa* isolated from respiratory and non-respiratory sites

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SUMMARY

The toxic effect of certain products of *Pseudomonas aeruginosa* on guinea-pig alveolar macrophages has been studied in an attempt to account for the apparent infrequency with which certain strains of this species are associated with respiratory infection. Tests were carried out on strains derived from the respiratory tract, strains from infection at other sites, and strains from the inanimate hospital environment which were believed not to have been responsible for infection ('environmental' strains).

Haemolysin, pigments, enzyme-containing fractions, slime and cell-wall fraction all exhibited toxic activity against macrophages in an *in vitro* system, although for any given strain of *Ps. aeruginosa* the haemolysin was by far the most potent factor. The activity of this factor against macrophages was directly proportional to its haemolytic activity against human erythrocytes. The haemolysin fractions of environmental strains, which have previously been found to have little activity on erythrocytes, were also less active against macrophages than haemolysin preparations from 'infective' strains.

It is therefore postulated that the ability of a strain of *Ps. aeruginosa* to initiate respiratory infection may be related to the degree of haemolysin production. The activity of other fractions against macrophages is more variable, but they may contribute in different ways to the development of infection once entry into the lung has been achieved.

INTRODUCTION

Pseudomonas aeruginosa is a well-recognized cause of infections in the respiratory tract. Broadly, such infections are most often seen in two groups of patients: those suffering from cystic fibrosis, in whom the infecting strains are usually of a characteristic 'mucoid' type (Iacocca, Sibinga & Barbero, 1963; Doggett, 1969), and those receiving intensive therapy, including positive-pressure ventilation. Strains responsible for the latter type of infection are usually non-mucoid (Phillips, 1967; Martin, 1973; Al-Dujaili & Harris, 1975); it is unknown whether these non-mucoid strains possess other specific factors which confer on them the ability to colonize the respiratory tract, or whether in fact all non-mucoid strains are capable of doing so. However, *Ps. aeruginosa* infection is not an inevitable con-

sequence of intensive therapy (Harris & Gray, 1974) and there is some evidence to suggest that certain strains may exist in the environment of patients without causing clinical infection (Al-Dujaili & Harris, 1975).

The production of infection by *Ps. aeruginosa* is considered to be associated with its ability to produce a range of toxins including a haemolysin, various fractions possessing enzymic activity, slime and certain 'lethal' toxins (Liu, Abe & Bates, 1961; Morihara, 1963, 1964; Diener, Carrick & Berk, 1973; Liu, 1973; Liu, Yoshii & Hsieh, 1973). Wretlind, Héden, Sjöberg & Wadström (1973) found that all strains of *Ps. aeruginosa* isolated from infective lesions produced some of these toxins. However, it was shown (Al-Dujaili & Harris, 1975) that the degree of toxin production *in vitro* was much lower in strains isolated from the inanimate hospital environment and unassociated with infection during a 14-month period of observation, than in strains isolated from infective lesions. This difference was particularly noticeable in the case of the haemolysin. It was thought that the apparent inability of the environmental strains to produce sufficiently large amounts of the various toxins (especially the haemolysin) might result in a failure to overcome the defence mechanisms of the respiratory tract and consequently account for the infrequency with which these strains were encountered as causes of infection.

The likelihood of a bacterium successfully initiating pulmonary infection is dependent on, among other things, the organism's ability to withstand the bactericidal activity of the alveolar macrophages (Laurenzi, Berman, First & Kass, 1964; Green & Kass, 1964). The present study was undertaken in an attempt to discover whether the toxic products of *Ps. aeruginosa* are injurious to alveolar macrophages, and whether any difference exists in this respect between the products of 'infective' and 'environmental' strains.

MATERIALS AND METHODS

Strains of Ps. aeruginosa

Twenty-seven strains of *Ps. aeruginosa* were selected for study from among those isolated by Al-Dujaili & Harris (1975). They comprised 19 strains isolated from the respiratory tract, 1 from a wound, 1 from a urinary infection and 6 which had been isolated from sites in the inanimate environment of patients. None of the environmental strains was derived from contaminated ventilation equipment. Pyocine typing and sero-typing were carried out as described previously (Al-Dujaili & Harris, 1974). For 18 strains, the activity of Liu's fractions II and III was tested against appropriate substrates by the methods of Liu, Abe & Bates, (1961); for the remaining 9 strains, only the activity of fraction II (the haemolysin) was assessed. The relevant characteristics of the 27 strains are shown in Table 1.

Harvesting of alveolar macrophages

Guinea-pig alveolar macrophages were obtained by a method similar to that used in rats by Brain & Frank, (1968). Young adult male guinea-pigs weighing

Table 1. *Source, identification and degree of toxin production of the* 27 *strains of* Ps. aeruginosa *studied*

Strain no.	Pyocine; serotype Combination	Source	Infections associated with this combination	Reciprocal of titres			
				Fraction II	Fraction III		
				Haemolysin	Protease	Lecithinase	Lipase
32	1(*c*) ; 8	Wound	7	16	16	8	< 2
1	3 ; 6	Urine	8	16	32	4	2
24	3 ; 6	Respiratory	8	32	32	16	16
23	3 ; 6	Respiratory	8	32	16	4	2
61	1(*c*) ; 8	Respiratory	7	16	8	2	2
150	1(*a*) ; 5	Respiratory	2	16	8	16	< 2
158	10 ; 11	Respiratory	5	32	4	16	< 2
64	6 ; NA	Respiratory	1	16	4	2	4
58	1(*a*) ; 7	Respiratory	2	8	8	4	4
157	UC ; 6	Respiratory	6	8	8	8	2
54	2 ; PA	Respiratory	1	4	16	4	2
97	1(*a*) ; NA	Respiratory	1	4	4	4	< 2
67	22 ; 3	Respiratory	1	4	8	4	4
179	10 ; 11	Respiratory	5	8	—	—	—
191	14 ; 3	Respiratory	1	8	—	—	—
197	3 ; 6	Respiratory	8	16	—	—	—
210	1(*c*) ; 5	Respiratory	4	8	—	—	—
219	1(*a*) ; 9	Respiratory	2	32	—	—	—
222	1(*f*) ; 6	Respiratory	2	16	—	—	—
229	9 ; 3	Respiratory	3	16	—	—	—
242	3 ; 2	Respiratory	2	32	—	—	—
17	10 ; 13	Environment	1	2	16	16	8
26	1(*g*) ; 6	Environment	0	4	4	2	2
15	UC ; NA	Environment	0	2	32	8	2
171	34 ; 3	Environment	0	2	8	8	2
70	10 ; 11	Environment	5	2	16	< 2	4
69	1(*a*) ; 7	Environment	3	4	—	—	—

NA, Non-agglutinable; PA, polyagglutinable; UC, unclassifiable pyocine inhibition pattern.

approximately 300 g. were killed by injecting 0·8 ml. 'Nembutal' intraperitoneally. Immediately after death the chest cavity was opened, and the upper part of the trachea dissected free and ligated just below the larynx. A needle was inserted into the trachea between the cartilages, and the lungs were flushed with physiological saline. This was instilled over a period of 1 min., allowed to remain in the lungs for a further minute, and withdrawn during a third minute. The procedure was repeated 10 times on each animal, employing 5 ml. saline for the first washing then increasing the volume for subsequent washings to 10 or 12 ml. The washings were pooled and centrifuged at 1500 rev./min. for 10 min. After discarding the supernatant, the cell button was resuspended in a suitable volume of Hanks' balanced salt solution to give a concentration of 20–30 cells per high-power field (normally 3–6 ml. for each harvest). Experiments on any given batch of macrophages were completed on the day of harvest.

Preparation and dilution of extracellular toxins of Ps. aeruginosa

Pyocyanin, other pigments, haemolysin, enzymes, slime and cell-wall fractions were prepared from 24 hr. cultures on tryptone glucose extract agar (Difco) according to the methods of Liu *et al.* (1961). Fractions were stored in the form of 'standard preparations' (see Al-Dujaili & Harris, 1975).

Technique

Preliminary tests on undiluted 'standard preparations' of the various toxins showed that all had definite toxic activity against guinea-pig alveolar macrophages. Dilution revealed differences in the activity of the toxins. In order to allow tests with different toxins to be carried out over a consistent time scale, and to facilitate graphical comparison, different toxins were diluted to a variable extent with Hanks' solution before use in the tests to be described. The empirical final concentrations of the various toxins used in the tests were as follows:

Fraction Ia	(pyocyanin)	33·3 %	Fraction III	(enzymes)	5 %
Fraction Ib	(other pigments)	25 %	Fraction IV	(slime)	10 %
Fraction II	(haemolysin)	2 %	Fraction V	(cell walls)	5 %

An appropriate amount (0·5 ml.) of the toxin under study was added to 0·5 ml. of a suspension of macrophages in a sterile plastic universal container, which was then incubated in a 37° C. water bath. Samples of 0·02 ml. were removed at approximately 20 min. intervals with a Pasteur pipette and placed on microscope slides. One 2 mm. loopful of 1 % Trypan blue solution (George T. Gurr Ltd) was added and, after placing a coverslip on the slide, the preparation was examined under a 6 mm. objective. Blue-staining of the macrophage nuclei was taken as an indication of non-viability. The percentage of viable macrophages was calculated for each sample.

A positive control was essential, since macrophages derived from different animals varied in their inherent viability. For this reason, a standard toxin was used as control for all experiments. In order to allow comparison between experiments performed on different batches of macrophages, results were finally

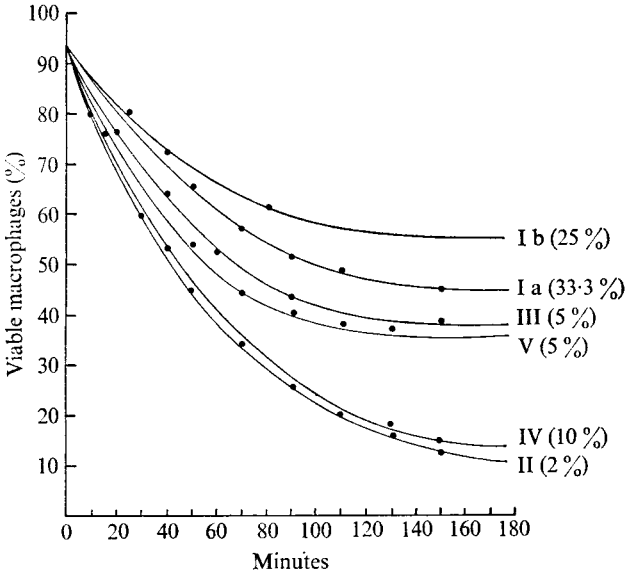

Fig. 1. Lethal effect of fractions Ia–V from a strain of *Ps. aeruginosa* on guinea-pig alveolar macrophages.

expressed as the difference between the percentage of macrophages killed by the toxin under test and the percentage of the same batch of macrophages killed by the standard toxin under identical conditions.

RESULTS

Although all the fractions studied displayed toxic activity against macrophages in the concentrations used, the degree of activity varied considerably between different toxins. A series of results obtained with the toxins of one strain of *Ps. aeruginosa* (No. 24) is shown in Fig. 1, which illustrates the far greater killing power of the haemolysin (at a concentration of 2 %) as compared with that of the pigments (at concentrations of 25 % or 33·3 %). The relative activities of the toxins shown in this graph were typical of the results obtained with 9 of the 18 strains in which the effect of all 6 toxins was studied (numbers 23, 24, 32, 58, 61, 64, 150, 157 and 158). A further strain (No. 1) produced almost identical results, except that fraction III was marginally less active than fractions Ia and Ib.

These 10 strains were all derived from clinical infections, and 8 of them produced haemolytic titres of 16 or 32 when fraction II was tested against human erythrocytes; the remaining 2 strains produced titres of 8.

The results obtained with strains 15, 17, 26, 54, 67, 70, 97 and 171 produced a different picture when represented graphically. In strains 15, 17, 26, 70 and 171 there was a closer approximation of the various curves; also, in strains 17, 26, 70 and 171 the haemolysin (at the concentration used) was not the most toxic fraction, its activity being exceeded by fraction IV in the first three strains and by fraction Ib in the last. All these strains had been isolated from environmental sources (see Table 1) and only one (no. 17) might possibly have been a cause of in-

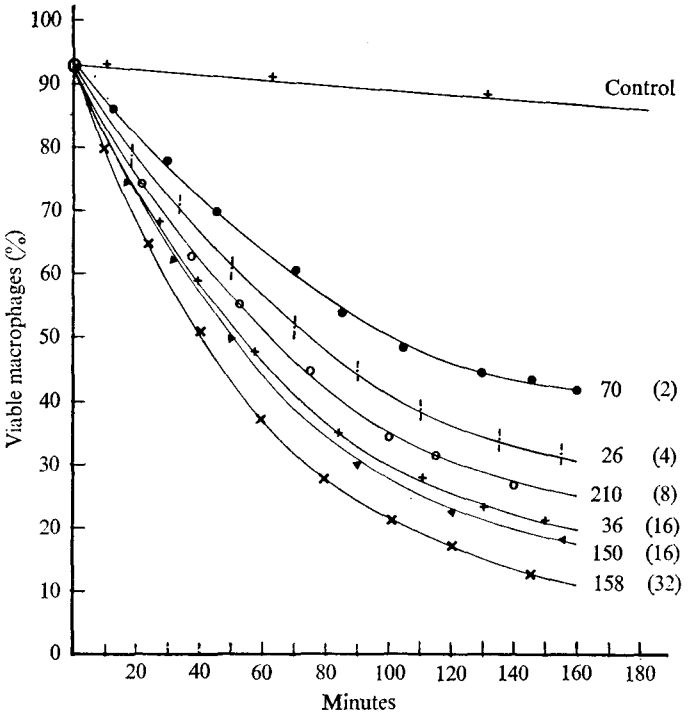

Fig. 2. Lethal effect of a 2% concentration of fraction II (haemolysin) from strains 26, 36, 70, 150, 158 and 210 on guinea-pig alveolar macrophages. (Figures in parentheses represent the haemolytic titre of the fraction for human erythrocytes.)

fection in the same ward. They were all characterized by low haemolytic titres when tested against human erythrocytes (see Table 1). The remaining 3 strains (nos. 54, 67 and 97) were unusual in that although all 3 were isolated from respiratory infections, the relative activity of their toxins against macrophages closely resembled that of the group of environmental strains. Reference to Table 1 shows that this resemblance was also reflected in the haemolytic activity of fraction II. Strains 54 and 67 were mucoid strains derived from patients suffering from cystic fibrosis; strain 97 was isolated from the respiratory tract of a patient receiving intensive care, and was of typical colonial morphology.

Fig. 2 shows a comparison between the effects on macrophages of the same fraction derived from different strains of *Ps. aeruginosa*, demonstrating that great differences in activity existed between 2% concentrations of fraction II extracted from the various strains. The curves also show that the activity of this toxin against alveolar macrophages is directly proportional to the haemolytic titre obtained when the fraction is tested against human erythrocytes.

Fig. 3, in which the percentage of viable macrophages remaining after 2 hr. incubation is shown in relation to the haemolytic activity of the strains, shows that this direct relationship holds good over the whole range of strains tested. This figure also illustrates the distinction between the activities of infective and environmental strains, with the strains from cystic fibrosis patients (54 and 67) showing only marginally greater activity than those derived from the environment.

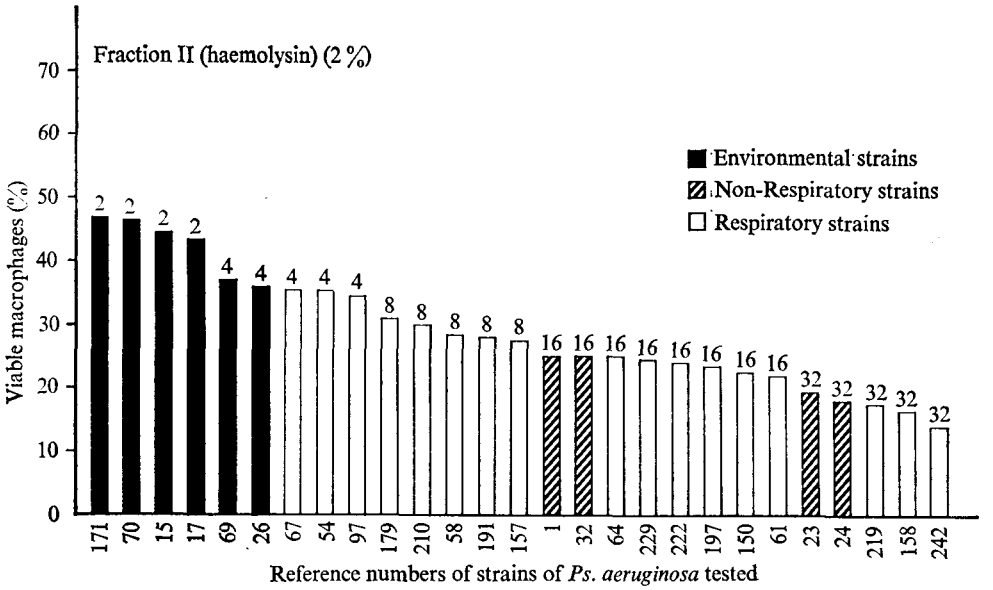

Fig. 3. Lethal effect of fraction II from 27 strains of *Ps. aeruginosa* on guinea-pig alveolar macrophages, showing the survival rate after 2 hr. incubation with the toxin. (Figures at the head of the columns represent the haemolytic titre of the fraction for human erythrocytes.)

When the results obtained with other toxic fractions were plotted graphically, it was again possible to demonstrate differences in the activity of the same fractions derived from different strains. In the case of fraction III, there was again a distinction between the activity on macrophages of fractions derived from infective and environmental strains, the former being more toxic than the latter. However, it was not possible to correlate the results precisely with the results of the tests of enzymatic action shown in Table 1. For example, 5 % concentrations of fraction III derived from strains 15, 17 and 70 (which had produced titres of 16 or 32 in tests of protease activity) were not significantly more active against macrophages than the same fraction of strain 26 (which produced a protease titre of 4).

The slime fraction of strain 26 was considerably more toxic to macrophages than the slime of other environmental strains, and in fact more toxic than some of the slime fractions from infective strains. However, in this respect it was exceptional.

Whereas, for the fractions considered so far, there appeared to be an association between the source of the strain and the toxicity of the particular fraction for macrophages, this association was not found in the case of fractions Ia, Ib, or V, the pigments and cell walls of the various strains being more or less toxic than others irrespective of the site from which the strain had originally been isolated.

DISCUSSION

Respiratory infection caused by *Ps. aeruginosa* is virtually always dependent on some degree of host debility. Consequently, an investigation such as the present study, which concentrates exclusively on properties of the bacterium, explores only one aspect of the pathogenesis of these infections. However, the results obtained tend to confirm previous impressions (Al-Dujaili & Harris, 1975) that factors produced by the species contribute to the impairment of pulmonary defence mechanisms, and that strains of *Ps. aeruginosa* may vary in their ability to produce infection.

Previous workers have employed various approaches in the study of *Ps. aeruginosa* toxins and their role in pathogenesis. Some have employed animal models (Liu, Abe & Bates, 1961; Klyhn & Gorrill, 1967; Carney & Jones, 1968; Kobayashi, 1971) whilst others have utilized tissue-culture systems (Nelson & Berk, 1960; Armstrong, Stewart-Tull & Roberts, 1971; Kamps & Ludovici, 1974). The results obtained, and the opinions on their significance, have been conflicting. However, none of these studies was directly concerned with the effect of *Ps. aeruginosa* products on the pulmonary defence system, and it cannot be assumed that results obtained with such models will necessarily be relevant to the pathogenicity of *Ps. aeruginosa* for the respiratory tract. The importance of using an appropriate model is emphasized by the finding of Shimizu *et al.* (1974) that the relative virulence of two strains of *Ps. aeruginosa* as measured by intraperitoneal inoculation of mice was reversed when the strains were administered intranasally to mink. Consequently the finding in the present work that products of *Ps. aeruginosa* exert a direct toxic effect on alveolar macrophages is of considerable interest, particularly so in the case of the haemolysin, since it was with this fraction that a distinction could most clearly be drawn between those strains which were known to have caused infection and those which were apparently not so associated. The toxicity of the fraction to macrophages was directly proportional to its haemolytic titre.

Although fraction III from environmental strains was generally less active against macrophages than the same fraction derived from infective strains, this finding could not be correlated with the results of tests of enzymatic activity, since some of the environmental strains were active producers of protease and lecithinase.

These findings strongly suggest that the haemolysin of *Ps. aeruginosa* may play a critical part in facilitating the entry of the species into the lung through its lethal powers against alveolar macrophages. However, it is uncertain how closely the empirical concentration used in the test corresponds to the amount of haemolysin produced *in vivo*. Although the enzymes contained in fraction III have been considered to play a major role in the pathogenesis of *Ps. aeruginosa* pneumonia (Liu, 1974), it seems unlikely from the present results that they contribute significantly to the destruction of the alveolar macrophage; it is more probable that they are responsible for damage to the lung tissue once access has been gained.

The toxic effects produced by the cell-fraction are interesting, since Liu *et al.* (1961) found that even a 50% concentration of this fraction was non-lethal to

mice on intraperitoneal injection and non-toxic to Hela cells. No record has been found of previous work on the toxicity of cell-fractions to alveolar macrophages, and it is conceivable that there might exist a cell-bound toxin which is lethal to these particular cells. One such toxin might be the intracellular heat-labile substance described by Liu in 1973. The toxic effect of the cell-fraction was not especially marked in the 'infective' as opposed to the 'environmental' strains, and in this respect it differed from the effects of the diffusible fractions (haemolysin, enzymes, pigments and slime).

It would appear from this investigation that environmental strains producing small quantities of toxin (and especially those producing little haemolysin) are ill-equipped to gain access to the respiratory tract of patients.

However, the results do not exclude the possibility that continued active production of diffusible toxins is dependent on frequent passage between susceptible hosts, and that existence in the inanimate environment under unfavourable nutritional conditions may result in diminished production of these factors. Forsberg & Bullen (1972) showed that the virulence for mice of a strain of *Ps. aeruginosa* isolated from a clinical infection was increased on serial passage. Further work will be required to determine whether the environmental strains described above can exhibit increased powers of toxin production after passage through a susceptible host.

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