TREATMENT OF *PSEUDOMONAS AERUGINOSA* INFECTIONS WITH PYOCINES

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PYOCINES are bacteriocines produced by strains of *Pseudomonas aeruginosa* and inhibitory to other strains of the same species. They may be classified in two main groups on the basis of their molecular weight. The high molecular-weight group (mol. wt. 10^6 – 10^7) is composed of two types of particle: the contractile and the filamentous, both visible in the electron microscope and apparently similar to certain defective bacteriophages. The structure and activity of these pyocines have been described in detail by several workers (Kageyama, 1964; Higerd, Baechler and Berk, 1967; Takeya *et al.*, 1969; Govan, 1974*a* and *b*). The other major group of pyocines consists of substances of lower molecular weight (c. 10^5), which are not sedimentable by ultracentrifugation and have not been resolved in the electron microscope, but are apparently simple proteins resembling some colicines (Ito, Kageyama and Egami, 1970; Ohkawa, Kageyama and Egami, 1973). These pyocines are known as S type or small.

P. aeruginosa poses considerable problems in hospitals because it readily attacks debilitated patients and, because of its resistance to many antibiotics, treatment of infections is difficult. The specific inhibitory activity of pyocines is well documented and recently the therapeutic use of these agents has been suggested.

Bird and Griebel (1969) injected an infective dose of *P. aeruginosa* into chick embryos, followed immediately by a suitable pyocine preparation, and found that a single dose of pyocine increased the survival of infected embryos from 3% to 46%. Similarly, Merrikin and Terry (1972) recorded an improvement in survival of mice infected with *P. aeruginosa* and treated intravenously with pyocine. However, it is difficult to assess the future of pyocine therapy from these reports, because neither group of workers purified the pyocine preparations, nor did they define clearly the type of pyocine used.

The aim of this investigation was to examine the therapeutic use of a representative of each of the three types of pyocine in infections caused by a single sensitive strain of *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains. *P. aeruginosa* strain P14 was kindly supplied by Dr R. J. Jones, Birmingham Accident Hospital, and was used throughout as the pyocine-sensitive indicator

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and infecting strain. This strain has the advantage of belonging to pyocine-type 16 (Gillies and Govan, 1966) and has a distinctive inhibition pattern. The other strains of *P. aeruginosa* were from the collection in the Bacteriology Department, Edinburgh University Medical School.

Selection of pyocinogenic strains. One-hundred and seventy strains of *P. aeruginosa* were examined for the production of pyocines inhibitory to indicator-strain P14. The methods used are described in detail by Williams (1974), but briefly, pyocine production was detected by inoculating the producer strain into Tryptone Soya Agar (Oxoid CM131) with or without trypsin. After overnight incubation at 32°C, the growth of *P. aeruginosa* was killed with chloroform and a soft-agar overlay containing indicator-strain P14 was applied.

Production of high molecular-weight pyocines resulted in a small inhibition zone around a point inoculum, which was unaffected by the presence of trypsin. Further tests were required to differentiate between contractile and filamentous pyocines. Small pyocines gave wider inhibition zones, but these were not formed in the presence of trypsin.

Strains 1577, 5882 and H108 were selected as representatives of pyocinogenic strains that produced respectively contractile, filamentous and small pyocines. Each strain appeared to produce only one type of pyocine inhibitory to strain P14. The pyocines were designated by the same number as their producer strains.

Preparation of purified pyocine. A standard procedure was devised for the purification of the three pyocines to be used in in-vivo studies. Five-ml volumes of an overnight nutrient broth (Oxoid Nutrient Broth no. 2, CM67) culture of the pyocine producer strain were used to inoculate three 200-ml volumes of sodium glutamate broth (Kageyama and Egami, 1962) in 2-litre flasks. The cultures were incubated at 32°C in an orbital incubator at 100 r.p.m. in the dark. After 4 h each culture was induced by the addition of a solution of Mitomycin C (Kyowa Hakko Kogyo Co. Ltd) to give a final concentration of 1.5 µg per ml, and incubation was continued for a further 4 h.

Extracellular slime was removed from the cultures by the addition of 24 ml of a filtrate obtained by mixing molar solutions of manganous chloride and sodium hydroxide in the ratio 2 : 1 and filtering off the precipitate (Brown, 1973). The fluffy slime precipitate was removed by centrifugation.

Ammonium sulphate was added to the pyocine-containing supernate to give 75% saturation and held at 4°C for 24 h before centrifugation at 2100 *g* for 60 min. in the cold. The supernate was discarded and the deposit resuspended in 25 ml of buffer (0.02M Tris HCl containing 0.02M MgCl₂, pH 7.6) and dialysed against two changes of the same buffer for 72 h. The dialysed pyocine was ultracentrifuged at 80 000 *g* for 90 min. under refrigeration in a Spinco Angle 40 rotor. The supernate was discarded and the deposit resuspended in 12 ml of buffer. (With pyocine H108, the supernate was retained and the deposit discarded.)

Six ml of the pyocine preparation was applied to a DEAE-Sephadex A-25 (Pharmacia) ion-exchange column, allowed to become adsorbed, washed with buffer and eluted with a sodium-chloride gradient in buffer (0–1.0M NaCl). The protein content of the samples was estimated spectrophotometrically, and samples with high readings were assayed against strain P14 for pyocine activity. Samples containing the peak of pyocine activity were dialysed against buffer for 5 h and then ultracentrifuged as described above. The deposit was resuspended in a small volume (c. 10 ml) of buffer and titrated against strain P14.

The small pyocine H108 was not adsorbed on to the DEAE-Sephadex resin but was collected in the preliminary buffer wash. It was not then ultracentrifuged but was used without further treatment in experiments *in vivo*.

A critical-dilution assay was used throughout for the estimation of the pyocine activity of samples. Doubling dilutions of the pyocine preparation were spotted on a lawn of strain P14 and incubated overnight at 37°C. The titre of pyocine activity, expressed in units per ml, was read as 50 times the highest dilution causing complete lysis of the indicator lawn.

The activity of pyocines in vivo. Female CFE mice, in three groups of five, were given injections of 0.3-ml volumes of one of the pyocine preparations intravenously, intraperitoneally or subcutaneously. The activities of the preparations of pyocines 1577, 5882 and

H108 were 8.0×10^5 , 4.0×10^4 and 3.2×10^3 units per ml respectively. Control animals received sterile sodium-glutamate broth by the same routes. One mouse from each group was killed 0.5, 1, 3, 5 and 24 h after inoculation and bled from the posterior vena cava. The blood was allowed to clot and the serum removed and assayed for inhibitory activity on lawns of strain P14.

The effect of pyocines on P. aeruginosa infections initiated by intraperitoneal administration of the bacteria. The infective dose was prepared by seeding 100 ml of nutrient broth 20% (v/v) in physiological salt solution (NB₂₀S) with a single colony of strain P14 and incubating under agitation at 37°C for 16 h. A 20-ml volume of this culture was centrifuged at 1850 g for 30 min., and the deposit resuspended in 50 ml of NB₂₀S. Viable counts of cell suspensions prepared by this method were in the range $1.0-3.0 \times 10^8$ bacteria per ml and 0.5-ml volumes injected intraperitoneally into mice gave 100% mortality.

The pyocines 1577 and 5882 were purified as described and had activities of 1.6×10^6 and 5.0×10^4 units per ml respectively. CBA mice were caged in groups of six and given intraperitoneal injections on day 1 of 0.5-ml volumes of pyocine and bacteria as shown in table I. The mice were observed daily for 14 days and the spleen of animals that died during the experiment was examined for *P. aeruginosa* by homogenising in 2 ml of saline, and seeding the homogenate on blood agar and cetrimide agar. Isolates of *P. aeruginosa* were pyocine-typed by the method of Gillies and Govan (1966). At the end of the experiment the surviving mice were killed and the spleen of each was treated as described.

The effect of pyocine H108 on *P. aeruginosa in vivo* was studied in three groups of four CFE mice. The mice in group 1 were given 0.5 ml of the suspension of strain P14 followed by 0.5 ml of buffer; group 2 received 0.5 ml of the suspension followed by 0.5 ml of NB₂₀S, and group 3 received 0.5 ml of the suspension followed by 0.5 ml of pyocine H108 (activity 3.2×10^3 units per ml). All injections were intraperitoneal.

The effect of topically-applied pyocines on P. aeruginosa infections of burns. The methods used for burning and infecting mice were based on those described by Jones, Jackson and Lowbury (1966). Male hairless mice were used throughout. Each mouse was kept in an individual cage that had been sterilised before the start of the experiment. Bedding was provided in the form of sterile paper towels which were changed daily. The mice received food and water *ad lib*.

TABLE I
Schedule for intraperitoneal injections on groups of six mice

Group no.	Time of injection (hours from start)		
	0	3	6
1	C, P*	...	...
2	C	P	...
3	C	...	P
4	C	B	...
5	P	C	...
6	P	...	C
7	P	B	...
8	NB ₂₀ S	NB ₂₀ S	...

C = *P. aeruginosa* strain P14 cells suspended in nutrient broth 20% (v/v) in physiological salt solution (NB₂₀S).

P = Pyocine 1577 or 5882 suspended in buffer (0.02M Tris HCl + 0.02M MgCl₂, pH 7.6).

B = Buffer (see footnote above).

NB₂₀S = See footnote above.

... = No injection.

* = Mice in group 1 were given strain P14 into one side of the peritoneal cavity and then pyocine into the other side.

The mice were anaesthetised by intraperitoneal injection of Nembutal solution (1 part of concentrated Nembutal to 9 parts of physiological salt solution; 0.1 ml per g body weight). A burn was made on the back of each mouse by applying for 10 s a 19-mm sq. brass block weighing 119 g heated in boiling water.

The infective dose of *P. aeruginosa* strain P14 was prepared by seeding the organism over the entire surface of six plates of Columbia Agar (Oxoid) and incubating these at 37°C overnight. The growth was scraped off the plates with a bacteriological loop into 10 ml of NB₂₀S and centrifuged at 1850 *g* for 30 min. The supernatant was discarded and the cells were resuspended in 10 ml of NB₂₀S. Three h after burning, 0.1 ml of the bacterial suspension was delivered by pipette on to each burn and spread over it with a bacteriological loop. Uninfected control animals received NB₂₀S in the same manner.

A purified preparation of pyocine 5882 was used in treatment. Three groups of four mice were burned, and two of these groups were infected with *P. aeruginosa* strain P14 on the first day. The burns were treated with pyocine three times daily, at 2-h intervals, on the 2nd and 3rd days of the experiment. Treatment was effected by rubbing a sterile cotton-wool swab, soaked in pyocine, over the burned area. A fresh swab was used for each treatment of each mouse. One group of infected mice was treated with pyocine 5882 and the other infected group and the uninfected controls were treated with buffer.

The mice were observed daily for 14 days. The bacterial flora of the burns was monitored by swabbing before and after infection and treatment. The swabs were cultured on MacConkey's agar, and isolates of *P. aeruginosa* were typed to confirm that they were of pyocine-type 16.

Small petri dishes (diameter 48 mm) were filled to the brim with nutrient agar. One h after the last pyocine treatment on day 2, two impression cultures were made of the burn of each mouse; one plate was incubated at 37°C overnight to give an indication of the bacterial flora on the burn surface. The second impression culture of each burn was used to demonstrate the presence of inhibitory activity. The plate was exposed to chloroform vapour for 5 min., the chloroform was allowed to disperse, and the plate was then flooded with a 4-h

TABLE II

The inhibition of P. aeruginosa strain P14 by mouse serum collected at given times after injection into mice of pyocine 1577, 5882 or H108 by various routes

Route of injection	Time after injection at which mouse was killed	Pyocine activity of serum (units per ml) after injection of pyocine		
		1577	5882	H108
Intravenous	0.5	50 000	25 000	100-200
	1	25 000-50 000	25 000	100-200
	3	25 000	12 800-25 000	100
	5	12 800-25 000	3200	50
	24	800	100-200	0
Intraperitoneal	0.5	50*	3200-6400	0
	1	12 800-25 000	6400-12 800	0
	3	25 000	12 800-25 000	50
	5	12 800	12 800	100
	24	1600	800	0
Subcutaneous	0.5	0	0	0
	1	0	0	0
	3	50	50	0
	5	50	100	0
	24	0	200	0

* Insufficient serum for dilutions.

nutrient broth culture of strain P14. Excess culture was removed, the plates were allowed to dry, and were then incubated overnight at 37°C.

The experiment was repeated and the number of mice in each group was increased to ten. Treatment with pyocine 5882 was given four times daily, at 2-h intervals, on the 2nd and 3rd days of the experiment.

The effect of topical or systemic administration of pyocine on P. aeruginosa infections of burns. Twenty-four mice were arranged in groups of four (although each mouse was caged individually). The mice were burned and infected as described previously and pyocine 1577, administered either topically or intravenously, was used for treatment. Topical treatment was effected by rubbing a sterile cotton-wool swab soaked in pyocine over the burned area three times daily on the 2nd, 3rd and 4th days of the experiment. Systemic pyocine treatment was given by intravenous injection of 0.3-ml volumes of pyocine into the tail vein once daily on the 2nd, 3rd and 4th days of the experiment. The mice were weighed daily for 14 days and the bacterial flora of the burns was monitored by frequent swabbing.

RESULTS

The activity of pyocines in vivo

Sera from the control mice that had been given injections of sodium glutamate broth did not show any inhibitory activity against *P. aeruginosa* strain P14, whereas sera from mice that had been given injections of pyocine inhibited the growth of strain P14; the inhibitory activities are shown in table II.

Infections initiated by intraperitoneal administration of P. aeruginosa

The effect of pyocines 1577 and 5882. The pyocine preparations alone had no adverse effects on the mice, neither did the suspending agent for the cells (NB₂₀S). These results confirmed that two injections alone were harmless.

The infective dose of *P. aeruginosa* strain P14 caused 100% mortality among the control mice (group 4, table I). Mice that received bacteria before the injection of pyocine died, whereas animals that received pyocine before or along with the organisms had a considerably lower mortality (table III).

TABLE III

Survival of mice infected with P. aeruginosa strain P14 and treated intraperitoneally with pyocine 1577 or pyocine 5882

Time of injection of organisms (hours from start)	Time of injection of pyocine (hours from start)	Survivors per group of 6 mice after treatment with pyocine	
		1577	5882
0	0	5	5
0	3	0	0
0	6	0	0
3	0	5	6
6	0	4	4
0	NI	0	0
NI	0	6	6

NI = no injection.

P. aeruginosa of pyocine-type 16 (i.e., the same type as the infecting strain P14) was found in spleen homogenates of all the mice that died during the experiment. No organisms were isolated from the spleen of animals that survived to the end of the experiment.

The effect of pyocine H108. All the mice given strain P14 died within 8–24 h of injection, regardless of whether or not they had also received pyocine H108. Mice that were given pyocine alone survived.

The effect of pyocine 5882 applied topically to mouse burns infected with P. aeruginosa

The mortality of burned mice in the first experiment is shown in table IV. The mice that were treated with pyocine showed better survival than those treated with buffer, but the burn remained colonised with *P. aeruginosa* even after treatment. The impression plates showed that *P. aeruginosa* was isolated from infected burns 1 h after pyocine treatment and that the burn surface did not show any inhibitory activity.

When the experiment was repeated on larger numbers of mice, treatment with pyocine 5882 was less effective in reducing mortality (table V). Cultures of swabs taken before and after pyocine treatment of infected burns did not reveal any obvious reduction in the number of *P. aeruginosa* on the burn. At the end of the experiment, 60% of mice whose burn was infected with *P. aeruginosa* strain P14 and treated with pyocine 5882 were dead; by comparison, 70% of infected but untreated mice had died. The mean time to death of the treated mice was 6.3 days compared with 7.3 days for the untreated group. *P. aeruginosa* was not isolated from the uninfected burned mice and these remained healthy throughout the experiment.

The effect of pyocine 1577 administered topically or systemically to mice with burns infected with P. aeruginosa.

Twenty-four h after infection with *P. aeruginosa* strain P14, the burns showed some growth of this organism on culture and by 48 h all the infected burns yielded a heavy growth of *P. aeruginosa* pyocine-type 16. Despite topical

TABLE IV
Mortality in groups of four burned mice infected with P. aeruginosa strain P14 and treated topically with pyocine 5882

Group no.	Infected with strain P14	Treatment	Number of mice that	
			died	survived
1	Yes	Buffer	3	1
2	Yes	Pyocine 5882	1	3
3	No	Buffer	0	4

DISCUSSION

Highly active preparations of the small pyocine H108 were difficult to obtain, and the preparation tested did not appear to exert any protective effect in mice when injected simultaneously with *P. aeruginosa* P14.

TABLE V
*Survival of burned mice infected with P. aeruginosa strain P14
and treated topically with pyocine 5882*

[illegible]

Topical therapy was thought to be a more likely use for pyocines and, in the first burns experiment described, treatment with purified pyocine 5882 appeared to improve the chances of survival of burned mice infected with strain P14. However, this result may have been fortuitous. Agar impressions of the burns taken after pyocine treatment showed only a slight reduction in the number of *P. aeruginosa* on infected burns treated with pyocine 5882. A second impression, processed to demonstrate inhibitory activity on the burn surface, showed that this was absent 1 h after pyocine treatment.

The experiment was repeated with larger groups of mice, and the results showed that the regime of topical pyocine therapy did not significantly improve the survival rate of burned mice infected with *P. aeruginosa* strain P14. Higher doses of pyocine might have given better results, but increased concentrations of purified pyocine were difficult to obtain in large volume.

In the experiment in which pyocine was given by two routes, the topical treatment was aimed at reducing the surface infection with *P. aeruginosa*, while intravenous therapy was designed to counteract septicaemic spread of the bacteria. However, the results suggested that the infective dose of *P. aeruginosa* was too small and the mortality of the infected but untreated mice was very low. In view of the lack of clear positive controls it was difficult to draw any conclusions about the efficacy of either topical or systemic pyocine therapy.

Although these studies are limited, it is concluded that the outlook for pyocine therapy is not favourable, and alternative agents for the treatment of *P. aeruginosa* infections should be sought. The results given may suggest a prophylactic role for pyocines, but their high degree of strain specificity would make this unlikely to succeed.

SUMMARY

The interactions of a contractile, a filamentous and a small pyocine with a sensitive strain of *Pseudomonas aeruginosa* (no. P14) were examined *in vivo*. The purification procedure used yielded high-activity pyocine preparations that were not toxic to mice. The inhibitory activity of such preparations, when injected into mice by various routes, was retained for up to 24 h. However, high molecular-weight pyocines given intraperitoneally in the presence of a lethal dose of strain P14 administered by the same route did not prevent the fatal outcome of infection unless they are given before or together with the bacteria. The small pyocine had no protective effect.

In burned mice infected with strain P14, topical application of a filamentous pyocine failed to improve the chances of survival.

The results suggest that there is little future for pyocine therapy.

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