Formation and Isolation of Leucocidin from *Pseudomonas aeruginosa*

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SUMMARY

A toxic substance, which destroyed leucocytes from man but was inactive against erythrocytes, was demonstrated in cultures of four out of 110 strains of *Pseudomonas aeruginosa* tested. The toxin, designated 'leucocidin', was cell-bound as a precursor toxin, exhibiting little or no toxicity. It was converted into toxin with maximum activity by various proteases including an endogenous elastase. The production of leucocidin was directly proportional to the number of bacteria and was not influenced by variations in media, iron concentration, pH or temperature. The best method for large-scale production of leucocidin was autolysis of washed bacteria.

INTRODUCTION

Sepsis due to *Pseudomonas aeruginosa* is often characterized by the absence of leucocytosis (Helm & Stille, 1972) or even by leucopenia (Schaffer & Oppenheimer, 1948; Foley *et al.*, 1969). Accordingly, local tissue neutropenia is a frequent symptom in necrotic lesions caused by *P. aeruginosa* (Fraenkel, 1912; Moncrief & Teplitz, 1964; Foley *et al.*, 1969). Neutrophils, if present, are found at the margin of the necrotic area and show pycnotic nuclei and disruption of the cytoplasm (Hubbard, King & Beamer, 1957; Teplitz, 1965). The reason for the absence of a polymorphonuclear leucocyte response had not yet been elucidated but may be due to toxic products of the bacterium (Teplitz, 1965). This is supported by the rapid disintegration of neutrophils after exposure to *P. aeruginosa in vitro* (Hubbard *et al.*, 1957).

Sensakovic & Bartell (1974) reported that the extracellular slime of *P. aeruginosa* caused leucopenia in mice and Liu (1974) detected a rapid reduction of circulating leucocytes after injection of exotoxin A into mice.

This paper reports the isolation from *P. aeruginosa* of another toxic substance which exerts a specific cytotoxic action on human leucocytes.

METHODS

Bacteria. One hundred and ten strains of *P. aeruginosa* were isolated from human and animal patients and from milk samples of cows. The strains were identified as *P. aeruginosa* according to Hugh (1970) and were pyocin typed by the 'fingerprinting' method of Farmer & Herman (1969). Strain 158, which was used for the production of leucocidin, had been isolated from milk. It was positive for the pyocin indicator strains 2, 3, 5, 6, 8, 13, 14, (16), 9, 22, 26 and for pyocins A to G, I, K, M to Q, and T.

Culture media. The following media were used: nutrient broth, brain-heart infusion

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broth, tryptic soy broth (TSB) and tryptic soy agar (TSA), all from Difco; the complex medium (MO) of Morihara *et al.* (1965); and a minimal medium (Pulverer & Korth, 1961) containing 2 % (w/v) glucose as carbon source.

Cultivation techniques. *Pseudomonas aeruginosa* was inoculated from fresh TSA slants into 100 ml Erlenmeyer flasks containing 20 ml medium. The flasks were shaken at 140 rev./min at 31 °C for 12 h. Erlenmeyer flasks (1 l) containing 50 ml medium were inoculated with 0.5 ml of this culture and incubated for 22 h at 31 °C on a rotating shaker at 140 rev./min. The bacteria were then separated from the culture fluid by centrifugation (27300 g, 20 min) and washed once with phosphate buffered saline (PBS) pH 7.2 (Dulbecco & Vogt, 1954).

Formation of enzymes. The production of protease was determined on TSA containing 1 % (w/v) sodium caseinate (Difco). Elastase was assayed on elastin agar plates (Sbarra, Gilfillan & Bardawil, 1960). Lipase production was detected on tributyrin agar (Oxoid). Lecithinase activity was determined on TSA containing 5 % (v/v) egg yolk (Oxoid).

Determination of growth. Viable count determinations were made by the pour plate method in which dilutions of the culture were added to molten TSA at 50 °C.

Assay of toxins and enzymes. Leucocidic activity was assayed by the microscopic slide adhesion method (Gladstone & van Heyningen, 1957) with human granulocytes. The slides had a number of fields (0.6 mm in diameter each) which were prepared by fixing a piece of punched parafilm on the glass. Serial dilutions of the toxin in PBS were incubated with the cells at 37 °C for 60 min in a moist chamber. One MLeD (minimal leucocidic dose) was the highest dilution of toxin which destroyed all the leucocytes of a field (about 6000 to 8000 granulocytes). The haemolytic activity was determined as described by Bernheimer & Schwartz (1963) with sheep erythrocytes. Protease activity was assayed as described previously (Scharmann & Balke, 1974) with casein (Hammarsten) as substrate. Leucine arylamidase (LAA) activity was measured by the method of Martinek, Berger & Broida (1964), the unit of activity being defined as the amount of enzyme which liberated 1 μ mol β -naphthylamine from L-leucyl- β -naphthylamide per hour at 37 °C and pH 7.1. The enzyme was not a leucine aminopeptidase, as L-leucine amide was not split. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Distintegration of bacteria. Washed bacteria resuspended in PBS at 10^{11} cells/ml were used. Ultrasonic disruption was done at 20 kHz in a 125 W Branson sonifier S 125 (Branson Instruments Inc., Danbury, Connecticut, U.S.A.). Treatment periods of 15 s were alternated with intervals of 1 to 2 min to avoid heat denaturation of the toxin. In addition, the treated tube was immersed in a -10 °C bath during sonication. After 10 to 15 sonications the sample was centrifuged (31 000 g, 20 min), and the supernatant collected.

As an alternative to sonication, bacteria were lysed for up to 72 h at 4 °C with 0.6 % (w/v) sodium deoxycholate. In lysis experiments with mitomycin C (0.5 μ g ml⁻¹) or carbenicillin (500 μ g ml⁻¹) the antibiotics were added to the culture in the exponential phase of growth (12 h after inoculation, 8×10^9 cells/ml), incubation was continued for 4 h at 37 °C and the supernatant collected by centrifugation (31 000 g, 20 min).

Chemicals. Trypsin, chymotrypsin, elastase from pig pancreas, pronase, DNAase from bovine pancreas, egg-white lysozyme, cysteine, 2-mercaptoethanol, *N*-1-naphthylethylene-diamine, L-leucine amide and polyethylene glycol 20000, were obtained from Serva, Heidelberg, Germany. L-Leucyl- β -naphthylamide was obtained from Sigma. Ammonium sulphamate was purchased from Fluka, Buchs, Switzerland. Carbenicillin was a generous gift from Beecham Pharma, Mainz, Germany. All other chemicals were obtained from Merck.

Fig. 1. Production of leucocidin as a function of time. *Pseudomonas aeruginosa* strain 158 was shaken at 31 °C in tryptic soy broth supplemented with 5 % (w/v) glucose. ○, Viable count; □, leucocidin activity in the supernatant of autolysed bacteria (washed, 10-fold concentrated); ■, leucocidin activity in culture supernatant; △, leucine arylamidase activity in culture supernatant.

RESULTS

Evidence for leucocidin

Preliminary experiments with *P. aeruginosa* had shown that individual strains of this species contained a leucocidin substance that was not identical with haemolysin. The cytotoxic effects of haemolysin were characterized by lysis of both erythrocytes and leucocytes from man. On the other hand 'leucocidin', as the other toxin was designated, damaged human leucocytes of all types in a specific manner but was inactive against erythrocytes. The cytopathic effect of leucocidin was marked by rapid and massive swelling of the leucocytes without rupture of the cell membrane (Scharmann, Jacob & Porstendörfer, 1976).

In TSB medium with 5 % (w/v) glucose, 110 strains of *P. aeruginosa* were examined for leucocidin activity and the toxin was found in the cell-free supernatants of four strains after 60 to 70 h growth at 37 °C. Strain 158 showed the highest leucocidin activity. The ability to produce leucocidin was lost during cultivation of the *Pseudomonads* on synthetic media. No correlation was found between leucocidin and various enzymes produced by the *Pseudomonads* such as proteinase (elastase), lecithinase C and lipase, since not only the leucocidin-positive, but also most of the leucocidin-negative strains produced these enzymes.

Haemolysin, which was also released into the culture supernatant in low amounts and interfered with the leucocidin test, could be eliminated from the supernatant by selective adsorption to aluminium oxide coated with cholesterol (Kanoe, Blobel & Schaeg, 1969).

Production of leucocidin

When *P. aeruginosa*, strain 158, was cultivated in TSB and shaken at 31 °C, leucocidin appeared in the cell-free supernatant only during the autolytic phase of growth between 24 and 80 h (Fig. 1). In contrast, leucine arylamidase activity which was used as an intracellular marker (Matheson, 1963) was detected as early as 10 to 12 h after inoculation of the medium and reached a peak at about 17 h. Leucocidin was also obtained from washed, autolysed cells: the bacteria were harvested at different phases of growth, washed once with PBS and resuspended in PBS, either in the original volume of the culture or in one-tenth of it. The suspension of washed cells was shaken for 73 h at 37 °C and the supernatant of the

Fig. 2. Liberation of leucocidin by autolysis of *P. aeruginosa*. A 1 l Erlenmeyer flask, containing 10^{18} bacteria in 100 ml PBS was shaken at 37 °C. ○, Viable count; □, leucocidin activity and △, protein content of supernatant of autolysed bacteria.

autolysate collected by centrifugation (37 000 g, 40 min). Leucocidin activity thus liberated by autolysis was found to be correlated with the number of viable cells of the growth cycle (Fig. 1).

To determine the optimal conditions for leucocidin production, *P. aeruginosa* strain 158 was grown in various media (Table 1) at 31 °C and the bacteria harvested at 20 h, washed and autolysed. MO medium gave the highest yield. With all media the formation of leucocidin was directly proportional to the number of bacteria. Autolysates of cultures grown at 31 °C yielded the highest leucocidin activity, since *P. aeruginosa* 158 grew better at this temperature than at 25 or 37 °C. Media adjusted to different pH values failed to reveal any direct influence of pH on toxin formation, and the highest leucocidin activity was measured between pH 7.0 and 8.0 where the maximum growth was obtained. Addition of Fe^{2+} (0.1 to 100 $\mu\text{g ml}^{-1}$), an important factor for the production of various toxins (Dubos & Geiger, 1946; Pappenheimer & Johnson, 1936), had no effect on the formation of leucocidin. Cultivation of *P. aeruginosa* on cellophane-over-agar (TSA), the method for the production of haemolysin and lecithinase C from *P. aeruginosa* chosen by Liu (1964), yielded only about half the amount of leucocidin that was obtained from broth cultures with the equivalent number of bacteria.

Solubilization of leucocidin from P. aeruginosa by autolysis and ultrasonication

Various methods for liberating leucocidin from the bacteria were tried but the best was autolysis by shaking the washed cells in PBS for 56 to 106 h at 37 °C (Fig. 2). Sodium thioglycolate (0.1 to 0.7 %; Seki *et al.*, 1954), toluene (0.5 %; Brown *et al.*, 1970) or KCl (0.5 to 1.0 M) had no effect on leucocidin activity in the autolysis supernatants. Only a part of the total activity that was bound to the organisms could be obtained by autolysis. The supernatant of a TSB culture contained a leucocidin activity three times higher than the supernatant of an autolysate with a comparable number of cells. Probably the loss of toxin was due to surface denaturation during shaking the autolysate at 37 °C. No inhibitor for leucocidin could be detected in the autolysate nor was the toxin destroyed by the purified protease (elastase) (Scharmann & Balke, 1974) of the same strain. When the washed bacteria were disrupted by ultrasonication, leucocidin activity in the homogenate or in its supernatant obtained by centrifugation was very low. However, the toxicity increased when the sonicated cultures were kept at 37 °C for 6 h and it increased up to 90-fold when the homogenate was

Table 1. *Production of leucocidin by P. aeruginosa strain 158 in various media*

The conditions for cultivation are described in Methods. Cultures were shaken for 20 h at 31 °C. Leucocidic activity was measured in the supernatant of autolysed washed bacteria.

Medium	$10^{-8} \times$ Viable count (c.f.u./ml)	$10^{-2} \times$ Leucocidin (MLeD/ml)	Protein (mg/ml)	Specific activity (MLeD leucocidin/mg protein)
MM	9	8	0.87	920
NB	50	40	1.23	3252
BHI	100	60	5.81	1032
TSB	100	80	5.43	1473
TSB, 1 % (w/v) glucose	80	55	5.10	1078
TSB, 2 % (w/v) glucose	70	50	5.70	877
TSB, 5 % (w/v) glucose	130	90	5.20	1730
MO	160	100	8.45	1183

MM, minimal medium; NB, nutrient broth; BHI, brain heart infusion broth; TSB, tryptic soy broth; MO, Morihara's medium; c.f.u., colony-forming units; MLeD, minimal leucocidic dose.

incubated with trypsin ($100 \mu\text{g ml}^{-1}$) or purified protease from *P. aeruginosa* strain 158, for 1 h. Control experiments showed that the enhancement of leucocidic activity was not due to synergistic action of protease and leucocidin. EDTA (0.1 to 0.02 M), 2-mercaptoethanol (0.6 M) or cysteine (10 mM) did not affect the toxic activity of the sonicated suspension. These results suggested that leucocidin was bound to subcellular structures of the bacteria as a 'precursor' toxin with no, or only low, specific toxicity. The conversion of the precursor into the toxin required partial degradation by the protease and probably also by other autolytic enzymes of the *Pseudomonads*.

Gel filtration confirmed the above speculations. When ultrasonicated homogenate was applied to a Sephadex G-150 superfine column, leucocidin with low toxicity eluted shortly after the void volume, trailing about half the length of the elution diagram; this was detected after incubation of the fractions with trypsin (Fig. 3*a*). The tubes containing leucocidin were then pooled, concentrated by dialysis against polyethylene glycol 20000 and fractionated again under identical conditions. Leucocidin then appeared in the middle of the elution diagram as a sharp peak (Fig. 3*b*). When the supernatant of autolysed bacteria was applied to the same column the toxin eluted at a similar position (Fig. 3*c*).

The release of active toxin from sonicated suspensions by several proteases of different peptide bond specificities was measured. Trypsin, chymotrypsin, pancreatic elastase, papain, subtilisin BNP¹, subtilisin Carlsberg and purified *P. aeruginosa* elastase all liberated the same amount of leucocidic activity, but pronase destroyed the toxin.

Release of leucocidin from P. aeruginosa by other methods

As autolysis of the bacteria was accompanied by a substantial loss of leucocidin and ultrasonication proved to be unsuitable for toxin production on a large scale, other methods for detaching leucocidin from the bacteria were tried.

To avoid denaturation of the toxin at 37 °C, lysis of washed bacteria was induced by sodium deoxycholate (0.6 %) at 4 °C, but only low leucocidic activity was obtained. This may have been due to a reduced action of the protease at 4 °C, since leucocidic activity increased upon incubation at 37 °C and after treatment with trypsin. However, this method did not yield higher amounts of leucocidin than autolysis (Table 2). Sodium deoxycholate (1.6 %, w/v) at 37 °C caused lysis but also a dramatic increase in viscosity due to the release of DNA. Only small amounts of precursor toxin could be separated from this jelly-like

Fig. 3. Gel filtration of different leucocidin preparations on Sephadex G-150 superfine. The column (2.5×45 cm) was equilibrated and eluted with PBS containing 0.2 M-NaCl. Fractions (4 ml) were collected. The following samples were applied: (a) Supernatant fluid of sonicated homogenate (24 mg protein); leucocidin activity in the fractions was assayed after trypsinization. (b) Pooled and concentrated leucocidin-containing fractions from experiment a. (c) Supernatant fluid of autolysed bacteria. —, Protein (E_{280}); $\Delta \dots \Delta$, leucocidin activity.

mass. The lysate was therefore liquefied by the addition of DNAase ($100 \mu\text{g ml}^{-1}$) and the supernatant collected by centrifugation. After dialysis and incubation with trypsin, its leucocidin activity was only slightly higher than that of the autolysate obtained from a similar number of bacteria (Table 2). Lysis of the bacteria with mitomycin C or carbenicillin yielded the highest leucocidin activity (Table 2). By these methods, 1 MLeD leucocidin was obtained from 3×10^6 bacteria. However, these antibiotics were too expensive for toxin production on a large scale.

Addition of EDTA ($1 \mu\text{mol ml}^{-1}$) to *P. aeruginosa*, which had been washed in PBS and resuspended in 0.03 M-tris solution, gave maximal lysis after 3 h incubation at 37°C but only a very small amount of active toxin was liberated, since the protease is inhibited by EDTA (Balke & Scharmann, 1974). After the addition of trypsin or CaCl_2 (0.1 M), toxicity

Table 2. Release of leucocidin by various treatments

For all experiments (except lysis by antibiotics) 10^{11} washed bacteria/ml were used. For lysis by antibiotics the results were extrapolated to 10^{11} cells/ml. The supernatant fluids of the cell homogenates (except autolysate) were incubated with trypsin (100 μ g/ml) for 1 h at 37 °C and dialysed exhaustively against PBS.

Method of disintegration	$10^{-2} \times$ Leucocidic activity (MLeD/ml) in the supernatant fluids
Autolysis	80
Ultrasonication	170
Deoxycholate (0.6 %, 4 °C)	65
Deoxycholate (1.6 %, 37 °C)	90
Mitomycin	265
Carbenicillin	250
Tris-EDTA	120

was enhanced (up to 70-fold) in the supernatant of the lysate to a level which was about 50 % higher than that of autolysate. Although maximal lysis of *P. aeruginosa* strain 158 with tris (pH 9)-EDTA alone took about 3 h, added lysozyme (50 μ g ml $^{-1}$) caused immediate lysis (Heilmann, 1972). High-speed centrifugation (40 000 g, 60 min) of the jelly-like mass yielded only a small quantity of supernatant. It contained the precursor leucocidin in amounts which were only slightly higher than those obtained by autolysis of a comparable number of bacteria.

DISCUSSION

These experiments demonstrate the production of a hitherto unknown toxic substance by *P. aeruginosa*. The toxin damaged leucocytes without causing haemolysis and was therefore designated 'leucocidin' by analogy with the Panton-Valentine leucocidin of *Staphylococcus aureus*. Leucocidin of *P. aeruginosa* did not correspond with other known cytotoxic substances such as exotoxin (Pavlovskis & Gordon, 1972), haemolysin (Scharmann, Köster & Blobel, 1973) or the protease (elastase) (Pflüger, Scharmann & Blobel, 1975). Only four of 110 tested *P. aeruginosa* strains revealed leucocidic activity. However, the occurrence of leucocidin-positive strains might be higher *in vivo*, since the ability to produce toxin is lost on synthetic media.

Leucocidin was cell-bound in the form of a precursor toxin with no, or only low, toxic activity. It was converted into toxin with high toxicity by the action of the endogenous protease (elastase) of *P. aeruginosa* and by six other proteases with known specificity for peptide bonds. Urea, EDTA, 2-mercaptoethanol or alkaline pH did not release toxic activity, suggesting that the toxin was covalently bound by a peptide chain.

The late appearance of leucocidic activity in the culture supernatant, compared with the early appearance of the intracellular leucine arylamidase, suggests a special mode of toxin liberation. The four leucocidin-positive strains also produced high protease (elastase) activity. The occurrence of precursor toxins or 'protoxins' converted into toxins by proteolytic enzymes had previously been described only for Clostridia, for example with *Clostridium botulinum* type A, B, E and F (Lamanna & Sakaguchi, 1971) and with *Cl. perfringens* type D (Turner & Rodwell, 1943).

The best method for making leucocidin on a large scale proved to be autolysis. However, about two-thirds of the toxin initially present in the bacteria was probably lost by surface denaturation.

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