Purification and Characterization of Leucocidin from *Pseudomonas aeruginosa*

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(Received 6 June 1975; revised 19 September 1975)

SUMMARY

Leucocidin from *Pseudomonas aeruginosa* strain 158 was released from bacteria by autolysis and purified 19-fold by ammonium sulphate precipitation (20 % saturation) and combined 'tandem' gel filtration on Sephadex G-100 superfine and Bio Gel P-100. The product gave a single band (mol. wt. 27000) after polyacrylamide gel electrophoresis with sodium dodecyl sulphate (SDS). However, it was separated into two active peaks (pI 5.0 and 5.2) by isoelectric focusing, and into five bands by disc electrophoresis without SDS. All bands contained leucocidic activity of about the same specific activity and retained their homogeneity.

The purified toxin was thermolabile and was inactivated by pronase, but not by several other proteases. The ultraviolet light absorbancy was typical of proteins. Antibodies directed against leucocidin were detected by passive haemagglutination and by toxin-neutralization. These antibodies inhibited the cytotoxic action of leucocidin bound to granulocytes. The toxin damaged all tested leucocytes (granulocytes of various animal species and lymphocytes of humans) and a number of tissue cultures, but was ineffective against erythrocytes, thrombocytes and isolated granules from polymorphonuclear leucocytes. The intravenous lethal dose for mice was about 1 µg.

INTRODUCTION

The occurrence of a leucocidin in *Pseudomonas aeruginosa* strain 158 is reported in the preceding paper (Scharmann, 1976). It was active on human polymorphonuclear leucocytes (Scharmann, Jacob & Porstendörfer, 1976), but not on erythrocytes.

This paper deals with the purification and characterization of the leucocidin and its toxicity for mice, leucocytes and various cell cultures.

METHODS

Bacterial strain. *Pseudomonas aeruginosa*, strain 158, was used for the production of leucocidin (Scharmann, 1976).

Buffer. Phosphate buffered saline pH 7.2 (PBS) was prepared according to Dulbecco & Vogt (1954).

Preparation of autolysate. Autolysate was prepared as described (Scharmann, 1976). Bacteria were cultivated for 22 h, washed once with PBS, resuspended in PBS to one-tenth of the original culture volume and shaken for 56 h at 37 °C. The supernatant of the autolysate was collected by centrifugation (37000 g, 40 min).

Measurement of leucocidic activity. Leucocidin was usually estimated as described in the preceding paper (Scharmann, 1976). In some experiments ⁵¹Cr-labelled target cells (2 × 10⁷)

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were incubated with leucocidin for 60 min at 37°C and the supernatant of the suspension collected by centrifugation (12000 g, 15 s, Eppendorf centrifuge; Eppendorf Gerätebau Netheler & Hinz, Hamburg, Germany). The radioactivity of the supernatant was measured in an autogamma spectrometer (Packard Instrument Co. Inc. La Grange, Illinois, U.S.A.) and expressed as a percentage of the total activity released from the target cells by 0.2 % (v/v) Triton X-100.

Leucocytes. Polymorphonuclear leucocytes from humans, rabbits, guinea pigs and mice were obtained by the slide adhesion method (Gladstone & van Heyningen, 1957). Granulocytes from cattle, sheep and dogs were prepared according to the method of Behrens & Esch (1963). One vol. blood was mixed with 2 vol. water. Isotonic conditions were restored after 55 s by the addition of 0.5 vol. of a 4.5 % (w/v) NaCl solution and the granulocytes collected by centrifugation at 150 g for 10 min.

Human lymphocytes were isolated by incubation of venous blood with glass beads and subsequent density gradient centrifugation on a mixture of Ficoll (Pharmacia) and Isopaque (Nyegaard & Co., Oslo, Norway) (Seiler *et al.*, 1972).

Thrombocytes. Human platelets were prepared as described by Cox, Hardegree & Fornwald (1974). A platelet suspension (1 ml) in PBS with an E_{650} of about 0.6 was examined for susceptibility to lysis by incubation for 1 h at 37°C with 2500 MLeD leucocidin, where 1 MLeD (minimal leucocidic dose) was the highest dilution of toxin which destroyed all the leucocytes of a field (about 6000 to 8000 granulocytes).

Granules. For isolating granules from bovine polymorphonuclear leucocytes, the method of Hegner (1968) was used. The granules were suspended in PBS to give an E_{405} of 0.5 and incubated for 60 min at 37°C with either 2500 MLeD leucocidin or 10 MHD haemolysin, where 1 MHD (minimal haemolytic dose) was the amount of toxin required to cause 50 % lysis of a 1 % (v/v) sheep erythrocyte suspension. The granule-free supernatant was obtained by centrifugation (12000 g, 2 min) and assayed for cathepsin, β -glucuronidase and alkaline phosphatase.

Target cells. Bovine polymorphonuclear leucocytes (1×10^8) in PBS were incubated for 30 min at 37°C with 100 μ Ci Na⁵¹Cr (Behringwerke, Marburg, Germany). After incubation the cells were washed three times with PBS.

Tissue cultures. The following tissue cultures were used: primary cultures of calf testicular cells, and rabbit kidney cells as diploid cells; serially propagated cultures of epithelioid green monkey kidney cells, L-cells, and HeLa cells as heteroploid cells. All cells were grown in Eagle's basal medium supplemented with bovine foetal serum (2 to 10 %, v/v), penicillin (100 u. ml⁻¹) and streptomycin (100 μ g ml⁻¹). Each culture contained about 2×10^6 cells/ml. For examining the cytopathogenic effect of leucocidin, the culture medium was replaced by PBS and the tissue cultures incubated with 100 MLeD leucocidin/ml for 30 min at 37°C.

Purification of haemolysin. Haemolysin from *P. aeruginosa* strain 158 was partially purified as described by Berk (1964).

Assays. β -glucuronidase was determined after 16 h of incubation with phenolphthalein glucuronidate (Fishman, Springer & Brunetti, 1948). Cathepsin was assayed according to Anson (1936), using haemoglobin. Lactate dehydrogenase was estimated with the Boehringer kit (Boehringer Mannheim, Mannheim, Germany) according to the suggestions of the manufacturer. Alkaline phosphate was measured by the method of Bessey, Lowry & Brock (1946) with *p*-nitrophenylphosphate as the substrate. Haemolytic and proteolytic activity and protein concentration were measured as described in the preceding paper (Scharmann, 1976).

Isoelectric focusing. An LKB 8101 (110 ml) column (LKB produkter, Stockholm-Bromma,

Sweden) was used to focus about 5 mg protein in a density gradient (0 to 50 %) of sucrose or urea. The carrier ampholytes (LKB; 0.9 to 3.75 %) ranged from pH 3 to 10 and from pH 4 to 6. Focusing was conducted for 26 h at 4 °C, the final potential being 1100 V.

Polyacrylamide disc gel electrophoresis. The general procedures and buffers described by Davis (1964) were used. The concentration of acrylamide in the separating gel was 7.5 % (w/v) unless otherwise stated. Gels were stained in an amido black 10 B solution and destained with 10 % (v/v) acetic acid. The positions of the proteins in unstained gels were determined by scanning the gels at 280 nm in a Gilford model 240 recording spectrophotometer (Gilford Instruments, Oberlin, Ohio, U.S.A.) fitted with a linear transporter. For calculating the molecular weight of leucocidin, sodium dodecyl sulphate–polyacrylamide gel electrophoresis was performed by the method of Weber & Osborn (1969) with bromophenol blue as tracking dye. The reference proteins, with their molecular weights, included: pepsin (35000), chymotrypsin (25700), trypsin (23300), lactoglobulin (18200), and myoglobin (17800).

Preparation of antisera. Samples containing 500 µg toxin/ml, purified by gel filtration, were injected into rabbits (average weight 3 kg). Injections were given at 3 to 4 day intervals. Five subcutaneous (0.2 to 0.8 ml) and two intramuscular (each 0.6 ml) injections were given. One week after the last injection, blood was collected by cardiac puncture and the serum fraction stored at –30 °C until used.

Passive haemagglutination test. The procedure was as described previously (Sezen, Scharmann & Blobel, 1975).

Determination of lethality to mice. Groups of four mice each weighing about 20 g were injected intravenously with 0.1 ml leucocidin dilutions in PBS.

Materials. Trypsin, alpha chymotrypsin, pancreatic elastase, pronase, pepsin, chymotrypsinogen, lactoglobulin, myoglobin (horse), haemoglobin (cattle), *p*-nitrophenylphosphate, phenolphthalein glucuronidate, iodoacetamide, *p*-hydroxymercuribenzoate, cysteine, Eagle's basal medium and polyethylene glycol 20000 were obtained from Serva, Heidelberg, Germany. Papain, subtilisin BPN', subtilisin Carlsberg, sphingomyelin, and brain extracts I, III and V were obtained from Sigma. Casein (Hammarsten), cholesterol and urea of analytical grade were purchased from Merck. Sephadex G-100 superfine was obtained from Pharmacia. Bio Gel P-100 was a product of Bio Rad Laboratories, Richmond, California, U.S.A.

RESULTS

Purification of leucocidin

After several preliminary experiments using different methods of precipitation and chromatography, the purification scheme shown in Fig. 1 was found to give the best results. All steps were carried out at 4 °C.

Step 1: ammonium sulphate fractionation. The supernatant fluid from autolysed *P. aeruginosa* cultures (150 ml) was cooled to 4 °C and 37.5 ml of a saturated ammonium sulphate solution pH 7.2 was added dropwise with stirring. Stirring was continued overnight and the precipitate collected by centrifugation at 22000 g for 15 min. The precipitate was dissolved in 10 ml PBS containing 0.2 M-NaCl, pH 7.2, stirred overnight, and the soluble fraction obtained by centrifugation (22000 g, 15 min.). The remaining precipitate was extracted once again in the same way. When the fractionation was performed with saturation grades higher than 20 % ammonium sulphate, the insolubility of the resulting precipitate increased so that no higher activities of soluble leucocidin were obtained. Moreover, at 20 % saturation only a small part of the protease (elastase) originally in the

Fig. 1. Purification procedure for leucocidin from *P. aeruginosa*

supernatant of the autolysate was precipitated. The protease interfered with further purification steps, since it has a similar molecular weight and isoelectric point (Scharmann & Balke, 1974).

Step 2: gel filtration. The supernatant fluid from step 1 (10 ml) was applied to a column (2.5 × 100 cm) of Sephadex G-100 superfine previously equilibrated with PBS containing 0.2 M-NaCl, pH 7.2, and eluted with the same buffer. Shortly before the leucocidic activity was expected to appear in the eluate, the effluent was transferred to a second column (2.5 × 100 cm) of Bio Gel P-100 equilibrated with PBS containing 0.2 M-NaCl, pH 7.2. This transfer was made by connecting the draining tube of the Sephadex column to the adapter of the Bio Gel column. This 'tandem' gel filtration separated leucocidin and protease (Fig. 2).

All other attempts to separate the two activities, e.g. recycling of the eluate or the use of various types of Sephadex or Bio Gel alone, were unsuccessful.

Fig. 2. 'Tandem' gel filtration of leucocidin on Sephadex G-100 superfine and Bio Gel P-100. Ten ml of solution containing a total of 3×10^5 MLeD leucocidin (87 mg protein) was applied to the column. Fractions of 5.4 ml h^{-1} were collected. —, Protein (E_{280}); $\bigcirc \cdots \bigcirc$, leucocidic activity; $\triangle \cdots \triangle$, proteolytic activity.

Fig. 3. Isoelectric focusing of 2.5×10^5 MLeD leucocidin (5 mg protein), previously purified by combined gel filtration on Sephadex G-100 superfine and Bio Gel P-100. Focusing was performed in ampholine buffer in the range of pH 4 to 6. Fractions (4 ml) were examined for pH ($\bullet \cdots \bullet$), dialysed against PBS at 4°C and assayed for E_{280} (—) and leucocidic activity ($\bigcirc \cdots \bigcirc$).

Table 1. *Purification of leucocidin*

Stage	Volume (ml)	$10^{-4} \times$ Total leucocidin activity (MLEd)	Total protein (mg)	Specific activity (MLEd/mg protein)	Recovery (%)
Supernatant of autolysed cells	150	240	1170	2051	100.0
Dissolved ammonium sulphate precipitate					
1st solution	10	32	34	9412	13.3
2nd solution	10	25	31	8064	10.4
Pooled Sephadex G-100/ Bio Gel A-100 eluate	122	42.7	12.2	35000	17.8
Concentrated and dialysed Sephadex/Bio Gel pool	12	42	10.9	38461	17.5
Isoelectric focusing					
Peak I	6	1.05	4.4	2365	0.4
Peak II	3	2.4	5.7	4233	1.0

Step 3: isoelectric focusing. The leucocidin purified by gel filtration was subjected to isoelectric focusing on a sucrose density gradient and pH gradients of pH 3 to 10 and pH 4 to 6. In both cases, two bands of precipitation were obtained corresponding to two zones of 280 nm extinction. Both peaks contained leucocidic activity. The isoelectric points were pH 5.0 ± 0.05 (peak I) and pH 5.2 ± 0.02 (peak II) (Fig. 3). Identical results were obtained when the toxin was focused in a density gradient of urea. Isoelectric focusing proved not to be a suitable method for the purification of leucocidin, since only 8 % of the applied toxic activity was recovered. The poor recovery-rate was presumably due to precipitation and inactivation of the toxin at its isoelectric point. A summary of the purification data is presented in Table 1.

Polyacrylamide disc gel electrophoresis

The leucocidin obtained by gel filtration was resolved into five protein bands by polyacrylamide disc gel electrophoresis (Fig. 4a). Disc electrophoresis of the same preparation in the presence of sodium dodecyl sulphate (SDS) yielded only a single band (Fig. 4b,c). Disc electrophoresis (without SDS) of the two peaks obtained by isoelectric focusing revealed 3 to 4 bands for peak I and 2 bands for peak II (Fig. 5a). However, only 1 band was observed each time that the electrophoresis was carried out in SDS. Subsequently, leucocidin peaks from isoelectric focusing were once again subjected to disc electrophoresis (without SDS) and the position of the protein bands determined in the unstained gels by scanning at 280 nm. Coincidence of leucocidic activity with all bands was demonstrated by eluting the individual protein bands of the gel and assaying leucocidic activity and protein. The eluted samples all had a similar specific activity. In disc electrophoresis without SDS, the individual leucocidin bands showed the same relative electrophoretic mobility as the preparations (peaks I and II) from which they were eluted (Fig. 5b). However, in the presence of SDS no differences in the electrophoretic mobility were observed (Fig. 5c).

Physicochemical properties of the purified leucocidin

The purified leucocidin (10000 MLEd/ml) was treated with various proteases such as trypsin, alpha chymotrypsin, pancreatic elastase, papain, subtilisin BPN', subtilisin Carlsberg (each 0.5 mg/ml) and pronase (35 PUK-u.). The toxin was completely inactivated by pronase within 30 min at 37 °C, but was not influenced by the other proteases. The u.v. light extinc-

Fig. 4. Polyacrylamide disc gel electrophoresis of leucocidin, previously purified by combined gel filtration on Sephadex G-100 superfine and Bio Gel P-100. (a) 125 μ g leucocidin in 7.5 % gel; (b) 55 μ g leucocidin in 10 % gel with SDS; (c) 55 μ g leucocidin in 7.5 % gel with SDS.

Fig. 5. Polyacrylamide disc gel electrophoresis. (a) Disc electrophoresis of leucocidin peak II, obtained by isoelectric focusing; (b) disc electrophoresis of the single protein bands, cut out and eluted from the unstained gel a; (c) disc electrophoresis of the isolated protein bands in gel with SDS. A 7.5 % gel was used. The position of the tracking dye was marked by indian ink.

tion of the purified leucocidin was typical for proteins. The maximum extinction was at 281 nm and the minimum at 250 nm. The molecular weight of the leucocidin, estimated by polyacrylamide gel electrophoresis in the presence of SDS, was 27 500 (Fig. 6).

Stability

A solution of purified leucocidin (14 000 MLeD/ml) lost about 15 % of its activity when kept at 4 °C for 7 days and about 50 % of its activity when kept at 22 °C for the same time. At 56 °C the toxin was inactivated within 60 min and at 100 °C within 30 s. At -30 °C the leucocidin retained full activity for at least 3 months. No differences of activity were detected when the toxin was diluted in a variety of 0.05 M buffers ranging from pH 5.5 to 9 and kept for 3 days at 4 °C.

Effect of various reagents

A solution of leucocidin (10 000 MLeD/ml) was adjusted to contain 0.01 M-CaCl₂ or 0.01 M-MgCl₂ and kept for 30 min at 22 °C. Then it was first dialysed against 0.02 M-EDTA and subsequently against demineralized water. Leucocidin treated in this way had the same activity as the control when measured with the slide adhesion test.

In the following experiments ⁵¹Cr labelled bovine granulocytes were used for the estimation of leucocidic activity: no effect was exerted on leucocidin by cysteine (10⁻³ M), iodoacetamide (10⁻² M), and *p*-hydroxymercuribenzoate (10⁻⁴ M); various lipids, including cholesterol, sphingomyelin and Folch' brain extracts I, III and V (each 100 μ g, ultrasonically dispersed in PBS) failed to inhibit 100 Folch of leucocidin.

Immunological studies

Purified leucocidin stimulated in rabbits the production of antibodies, which could be demonstrated in a passive haemagglutination test or by neutralization of the leucocidic activity. In the passive haemagglutination test, antibody titres of 1:5120 were measured. Normal serum did not induce the agglutination of sensitized erythrocytes. The neutralization test proved to be far less sensitive than the passive haemagglutination test. For a 100 %

Fig. 6. Determination of the molecular weight of leucocidin by polyacrylamide gel electrophoresis in the presence of SDS. The marker proteins, with their molecular weights, were: 1, Myoglobin (17800); 2, lactoglobulin (18200); 3, trypsin (23300); 4, α -chymotrypsinogen (25700); 5, pepsin (35000).

neutralization of 100 MLeD of leucocidin a serum dilution not higher than 1:64 was necessary the toxin was also inhibited by undiluted normal serum.

Leucocidin antibodies inhibited the action of leucocidin bound to leucocytes, as shown by the following experiment: bovine granulocytes were mixed with toxin at 4°C, kept for 1 h at the same temperature and washed twice in ice-cold PBS. No cytotoxic affect occurred as long as the cells were cooled. However, raising the temperature to 37°C resulted in an immediate destruction of the cells. On the other hand, no destruction occurred when the washed cells were treated with leucocidin antibodies before increasing the temperature.

Cytotoxic effect on various cell species

Leucocidin damaged granulocytes from humans and from all tested animal species (cattle, sheep, dog, rabbit, guinea pig and mouse). The degree of sensitivity varied among the granulocytes of the different animal species. About 1 MLeD leucocidin was needed to destroy about 8000 human granulocytes. However, the same number of granulocytes from rabbits was damaged by 0.05, from mice and guinea pigs by 0.25, from cattle by 2 to 4, from sheep by 4, and from dogs by 8 MLeD. Lymphocytes from humans were twice as sensitive as granulocytes (Fig. 7). The toxin did not induce the release of cathepsin, β -glucuronidase or alkaline phosphatase from granules isolated from bovine granulocytes. On the other hand, these enzymes were released by the action of haemolysin from *P. aeruginosa* strain 158.

No morphologic alterations could be detected in erythrocytes of humans, cattle, sheep, dogs, rabbits, guinea pigs, mice and chickens, even after the addition of high concentrations of toxin (5000 MLeD/ml). The same seemed to be true for thrombocytes of humans. Upon the addition of leucocidin no morphological changes of the platelets were observed and no lactate dehydrogenase activity was detected in the suspension medium of the platelets.

Tissue cultures of rabbit kidney cells (Fig. 8), L-cells (Fig. 9), calf testicular cells, HeLa-

Fig. 7. Cytotoxic effect of leucocidin on lymphocytes from man. G, polymorphonuclear granulocyte.

Fig. 8. Rabbit kidney cells, (a) before inoculation, and (b) 30 min after inoculation with leucocidin.

Fig. 9. L-cells, (a) before inoculation, and (b) 30 min after inoculation with leucocidin.

cells and epithelioid green monkey kidney cells showed a cytopathogenic effect similar to that observed in leucocytes when treated with leucocidin: the cells enlarged and became spherical, but the membrane persisted.

Lethality to mice

Purified leucocidin was shown to be lethal to mice, death occurring within 10 to 20 min from 4 to 8 μ g quantities. The minimal lethal dose was about 1 μ g.

DISCUSSION

Purified leucocidin exhibited the same properties as most of the other cytolytic toxins of bacteria (Bernheimer, 1970). It was inactivated by pronase, thermolabile, not dialysable, and gave rise to neutralising antibodies. Some of these characteristics, as well as the u.v. absorption spectrum of the toxin indicate its protein nature. The inability of *p*-hydroxymercuribenzoate and iodoacetamide to inhibit the leucocidal activity suggest that the active moiety of the toxin molecule does not contain free sulphydryl groups.

The purification of leucocidin was complicated by the presence of a protease (elastase) with a similar isoelectric point (pH 5.7 and pH 6.45) and molecular weight (22 300) (Scharmann & Balke, 1974). They were separated by a combined 'tandem' gel filtration on Sephadex G-100 superfine and Bio Gel P 100. This gave a toxin preparation which was homogeneous in molecular weight as shown by sodium dodecyl sulphate gel electrophoresis. However, this preparation was heterogeneous in charge, since it was separated by isoelectric focusing into two components exhibiting leucocidal activity. Moreover, these components could be further separated into several bands with toxic activity by means of disc electrophoresis without SDS. The isolated bands retained their homogeneity and electrophoretic mobility. The differences in the charge of the purified leucocidin preparation may have resulted from the transformation of cell-bound precursor toxin into the free toxin (Scharmann, 1976) or may be caused during the bacterial autolysis. The supernatant of autolysed cells exhibited the typical 5-band pattern of the gel-filtrated leucocidin. Six & Harshman (1973) described two forms of staphylococcal α -toxin, also showing different electrophoretic mobility in disc gel electrophoresis but identical mobility in SDS gel. Heterogeneity in charge of crystalline insulin due to a deamidation reaction has been reported by Carpenter & Hayes (1963). The insulin derivatives had been prepared either by mild acid treatment or by the action of trypsin or carboxypeptidase A on insulin.

Leucocidin damaged the leucocytes of all tested animal species and elicited a similar cytopathogenic effect on the cells of various tissue cultures. The toxin did not damage erythrocytes or thrombocytes. The relatively broad spectrum of cells sensitive to leucocidin suggests that the component which is essential for the reaction with leucocidin may be a frequent constituent of mammalian cells. Isolated leucocyte granules were not destroyed by leucocidin but by haemolysin of the same *P. aeruginosa* strain. These findings correspond to those of Bernheimer & Schwartz (1964), who reported that lysosomes are damaged only by those substances that also induce lysis of erythrocytes.

This work was supported in part by the Deutsche Forschungsgemeinschaft.

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