PURIFICATION OF A PROTEIN TOXIN FROM *CORYNEBACTERIUM ULGERANS*

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PLATE XL

CORYNEBACTERIUM ULGERANS, which is usually associated with mastitis in cattle, has been reported as a cause of sore throat and tonsillitis in man (Jebb, 1948; Saxholm, 1951; Tomlinson, 1966). Subcutaneous injection of this organism into guinea-pigs produces features, such as haemorrhage and oedema at the site of injection and haemorrhagic adrenals, which are characteristic of diphtheria toxin. Diphtheria antitoxin neutralises the lethal effect of toxin from some strains of *C. ulcerans* (Fox and Frost, 1974) but not from others (Souckova and Soucek, 1974). In this study the purification and preliminary characterisation of a toxin from *C. ulcerans* strain 378 is reported.

MATERIALS AND METHODS

Bacteria. Bacteriocinogenic and toxinogenic *C. ulcerans* strain 378 (Diphtheria Reference Laboratory, Welsh National School of Medicine, Cardiff) was used for the production of toxin.

Culture medium. Soft serum-substitute agar (SSA) contained Proteose Peptone (Difco) 20 g, Casamino Acids (Difco) 3 g, NaCl 4.8 g, glycerol 1 ml, agar 6 g, distilled water 1.2 litres. It was sterilised by autoclaving at 120°C for 20 min. and dispensed into petri dishes.

Chemicals. Sudan Black B, Oil Red O, SDS Gel and all enzymes and proteins were from Sigma Chemical Co. Ltd, Poole, Dorset; Sephadex G-200 and Blue Dextran 2000 from Pharmacia Fine Chemicals, Hounslow, Middlesex; DEAE-cellulose DE-52 from Whatman, Maidstone, Kent and Ultrogel AcA22, AcA 54 from LKB Produkter AB, Sweden. Acrylamide N,N,N',N'-tetramethylethylenediamine and methylenebisacrylamide were from Koch-Light Laboratories, Poole, Dorset; Bromphenol blue and Coomassie Brilliant Blue from Hopkin Williams, Romford, Essex and 2-mercaptoethanol from British Drug Houses, Poole, Dorset.

Crude toxin extract. Crude toxin preparations were obtained from cultures of *C. ulcerans* strain 378 grown at 35°C for 24 h in SSA medium. The media from 30 plates were pooled and frozen at -15°C overnight then thawed at room temperature to release the toxin. After centrifugation of the slurry at 2500 r.p.m. for 60 min., the supernate was filtered through a 0.45 µm filter, ammonium sulphate was added up to 1.6M and the whole was allowed to stand at 4°C overnight. The mixture was centrifuged at 18 000 r.p.m. for 20 min. at 4°C and the supernate brought to 3.2M with additional ammonium sulphate and allowed to stand at 4°C overnight. The precipitate was collected by centrifugation, dissolved in 10 ml of 50mM tris HCl buffer (pH 7.8) and dialysed against the same buffer at 4°C for 10 h.

Assay of toxin and protein. Various amounts of filtrate preparations (0.05, 0.1, 0.2, 0.3, 0.4, 0.5 ml) were injected subcutaneously into guinea-pigs weighing about 300 g and the time of death was noted. The toxicity in terms of minimal lethal dose (MLD) was then calculated from a standard dose-response curve (Baseman *et al.*, 1970). Specific activity of toxin was expressed as MLD per mg of protein; the protein was measured by the method of Lowry *et al.*, (1951) with bovine serum albumin as standard.

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Polyacrylamide-gel electrophoresis. SDS-gel electrophoresis was done by the method of Weber and Osborn (1969). The gels were stained for protein with Coomassie brilliant blue (Weber and Osborn, 1969), for lipid with oil red O or Sudan black B (Sargent and George, 1975) and for carbohydrate by the periodic acid Schiff reaction (Crowle, 1961). In each experiment the following proteins were run in parallel to serve as molecular-weight markers: bovine serum albumin (mol. wt 66 000), ovalbumin (43 000), pepsin (35 000) and cytochrome C (12 400).

Gelfiltration. A column (2.5 × 40 cm) of Ultrogel AcA54 was equilibrated at room temperature with 50mM tris HCl buffer (pH 7.8) containing 0.02% NaN₃ at a flow rate of 24 ml/h. Samples were applied in a 2-ml volume, 4-ml fractions were collected and the eluate was monitored at 280 and 420 nm. Fractions were also tested for toxicity by injection of 0.2 ml subcutaneously into guinea-pigs. The elution volume of the marker proteins, bovine serum albumin, ovalbumin and cytochrome C and of the toxin were determined and, from the standard curve of elution volume/void volume (V_e/V_o) plotted against molecular weight, the molecular weight of *C. ulcerans* toxin was estimated. At the start of every run, Blue Dextran was added to estimate the V_o .

RESULTS

Purification of *C. ulcerans* toxin. Five ml of ammonium sulphate-precipitated toxin was applied to an Ultrogel AcA22 column. The toxin was in the second peak (fig. 1). Pooled toxin fractions 33–51 were concentrated by lyophilisation and dissolved in 5 ml of distilled water. After dialysis the toxin was further purified by ion-exchange chromatography on DEAE cellulose. Toxin was eluted with the bulk of the absorbed protein at a molarity of 0.08–0.37 in a single peak (fig. 2). Active fractions 3–10 were pooled, concentrated by lyophilisation and dissolved in 2.5 ml of distilled water. On gel filtration through Ultrogel AcA54, the toxin was eluted in a single peak of protein (fig. 3). After lyophilisation, fractions 20–30 yielded 1 mg

FIG. 1.—Purification of *C. ulcerans* toxin by chromatography on Ultrogel AcA22. Fractions (3.5 ml) were collected and examined for absorbance at 280 nm (●—●) and toxicity (+).

FIG. 2.—Elution and activity profile of *C. ulcerans* toxin (50 MLD) on a DEAE-cellulose column. The toxin was applied in 50mm tris HCl (pH 7.8) containing 0.2% sodium azide to a 1 × 20-cm column of DEAE-cellulose equilibrated in the same buffer. After washing until no further protein was eluted, a linear gradient to 1M NaCl in the tris buffer was applied. ●—●=Absorbance at 280 nm, □—□=number of MLD, —=molarity of NaCl.

FIG. 3.—Further purification of *C. ulcerans* toxin (35–40 MLD) by chromatography on Ultrogel AcA54. Fractions (4 ml) were collected and tested for absorbance at 280 nm (●—●) and toxicity (+). The elution position of the marker proteins, bovine serum albumin (BSA), ovalbumin (OVA) and cytochrome C (Cyt C) are shown for reference.

protein/ml with specific activity of 33 MLD/mg of protein. This was 66-fold higher than that found in the original preparation (0.5 MLD/mg) and the yield was 16%.

Assessment of purity and molecular weight. Polyacrylamide-gel electrophoresis at pH 7.0 in the presence or absence of 0.1% SDS-gel and 2-mercaptoethanol was used as the criterion of homogeneity. Purified *C. ulcerans* toxin appeared as a single band staining for protein (fig. 4) but not for lipid or carbohydrate. Comparison with marker proteins of known molecular weight indicated a molecular weight of 13 000 (fig. 5). On gel filtration in a calibrated column of Ultrogel AcA54 the molecular weight was 15 000 (fig. 6).

FIG. 5.—Determination of the molecular weight of *C. ulcerans* toxin by electrophoresis in SDS-polyacrylamide gel (10%). BSA = bovine serum albumin; Ov = ovalbumin; Pep = pepsin; Cyt C = cytochrome C; ■ = toxin.

DISCUSSION

C. ulcerans toxin purified by a four-step procedure yielded a protein with specific activity of 33 guinea-pig MLD/mg protein which was homogeneous as judged by SDS-polyacrylamide gel-electrophoresis and had an apparent molecular weight of 13 000. Because pre-treatment with 2-mercaptoethanol did not reduce the molecular weight, it appears that the toxin protein is composed of a single polypeptide chain.

These data show that *C. ulcerans* toxin, with lower toxicity and molecular weight, is distinct from diphtheria toxin (Collier and Kandel, 1971; Gill and Dinius, 1971). However, both toxins cause similar pathological changes, and toxins from some strains of *C. ulcerans*, including strain 378, are neutralised by diphtheria antitoxin. In gel diffusion, the antigenic cross reactivity

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FIG. 4.—SDS-polyacrylamide-gel (10%) electrophoresis of 0.03 mg of purified *C. ulcerans* toxin.

FIG. 6.—Determination of the molecular weight of *C. ulcerans* toxin in a calibrated column of Ultrogel AcA54. Symbols as in fig. 5.

between *C. ulcerans* strain 378 toxin and diphtheria antitoxin suggests that the two entities are related (Abreham and Zamiri, unpublished).

Souckova and Soucek (1974) separated two toxin proteins from *C. ulcerans* strain ATCC9015 by ammonium sulphate precipitation and ion-exchange chromatography on Sephadex CM-C50. One had the ability to produce haemorrhagic necrosis with oedema and pyogenic reaction in rabbits. This toxin was identical to sphingomyelinase D with a molecular weight of about $17\,000 \pm 1000$. The second protein was shown to be dermonecrotic and lethal for experimental animals but an estimate of its molecular weight was not made. These toxins, unlike *C. ulcerans* strain 378 toxin, could not be neutralised by diphtheria antitoxin, although in immunodiffusion they gave a line of identity with diphtheria toxin.

In this study, *C. ulcerans* strain 378 toxin has been much more highly purified (66-fold and homogenous on SDS-polyacrylamide-gel electrophoresis) and only one toxin protein was detected. This toxin was dermonecrotic as well as lethal to guinea-pigs and rabbits. The relationship of *C. ulcerans* strain 378 toxin to sphingomyelinase D remains to be clarified.

SUMMARY

Toxin from *Corynebacterium ulcerans* strain 378 was purified 66-fold by ammonium sulphate fractionation, dialysis, gel filtration on Ultrogel AcA22, ion-exchange chromatography on DEAE-cellulose and gel filtration on AcA54. On polyacrylamide-gel electrophoresis the purified material was homogeneous, staining for protein but not carbohydrate or lipid. The molecular weight of *C. ulcerans* toxin was 13 000 as determined by SDS-polyacrylamide-gel electrophoresis and 15 000 by gel filtration on AcA54.

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