SHORT ARTICLES

INHIBITION OF *CORYNEBACTERIUM ULGERANS* TOXIN PRODUCTION BY TWEEN 80

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CORYNEBACTERIUM ULGERANS, a gram-positive bacillus closely related to *C. diphtheriae*, has been isolated from cases of acute sore throat, tonsillitis and skin lesions, often from people dealing with cattle (Jebb, 1948; Saxholm, 1951; Tomlinson, 1966). Jayne-Williams and Skerman (1966) and Higgs *et al.*, (1967) reported *C. ulgerans* as a cause of clinical mastitis in cattle. Subcutaneous injection of a suspension of an overnight culture of a toxigenic strain kills guinea-pigs within 72 h, with changes resembling the effect of diphtheria toxin, except that neutralisation by diphtheria antitoxin is variable (Fox and Frost, 1974; Souckova and Soucek, 1974; Wilson and Miles, 1975).

Petrie and McClean (1934) reported that *C. ulgerans* produced two toxins; one immunologically identical to diphtheria toxin and the other related to the toxin of *C. ovis*. A close relationship of *C. ulgerans* to *C. diphtheriae* is supported by its susceptibility to lysogenisation by the β phage of *C. diphtheriae* leading to the production of diphtheria toxin (Maximescu, 1968; Barksdale, 1970). Maximescu *et al.* (1968) found that *C. ulgerans* and *C. ovis* lysogenised by *C. diphtheriae* phages produced their own toxin and a toxin identical with that of *C. diphtheriae*.

The production or action of diphtheria toxin is inhibited by iron (Pappenheimer, 1947; Clarke, 1958), glutamine, ammonium salts, glycamine and prolamine (Kim and Groman, 1965*a* and *b*). The absorption of β phage to *C. diphtheriae* is inhibited by Tween 80 (Groman and Bobb, 1955). During studies on a toxigenic and bacteriocinogenic strain of *C. ulgerans* (no. 378) we noticed that growth in media containing 0.05–0.5% Tween 80 yielded non-toxic culture supernates. This report described our preliminary investigation of the phenomenon.

MATERIALS AND METHODS

Organism. Toxinogenic and bacteriocinogenic *C. ulgerans* strain 378 was from the collection of corynebacteria in the Diphtheria Reference Laboratory, Welsh National School of Medicine, Cardiff.

Viable counts. Culture dilutions were made in serum-substitute broth (see below) and 0.1-ml samples were spread on blood-agar plates. Colonies were counted after incubation for 24 h at 35°C.

Protein content of bacteria. Cell pellets from 20 ml of centrifuged broth cultures were washed twice in 0.85% (w/v) saline by centrifugation, and resuspended in 5 ml of saline. Five ml of cold trichloroacetic acid (10%, v/v) was added to this suspension, the precipitate was washed twice with cold trichloroacetic acid (5%), and the protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

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Toxin preparation. *C. ulcerans* toxin was prepared by inoculation of serum-substitute broth (SSB), consisting of Proteose Peptone (Difco) 2% (w/v), sodium chloride 0.24% (w/v), glycerol 0.1% (v/v) and Casamino Acids (Difco) 0.2% (w/v), with an overnight culture of *C. ulcerans* strain 378 and incubation at 35°C for 72 h. The cells were removed by centrifugation (3000 r.p.m. for 15 min.) and the filtered supernate was used as source of toxin.

Assay of toxin. Toxicity was assayed by subcutaneous injection of 0.1 ml of filtrate into 300-g guinea-pigs and noting the time of death. The minimal lethal dose (MLD) was calculated from the standard dose-response curve of Baseman *et al.* (1970).

Ammonium sulphate precipitation. Toxin (200 ml of filtrate) was precipitated with solid ammonium sulphate to 2.4M, with continuous stirring at room temperature. The preparation was left at 4°C overnight and the precipitate was collected by centrifugation at 18 000 r.p.m. for 15 min at 4°C. The pellet was dissolved in 5 ml of 50mM tris HCl buffer (pH 7.8), dialysed overnight against the same buffer at 4°C, centrifuged as before, and the supernate was filtered through a 0.45 µm membrane filter and used as the concentrated toxin preparation.

Radial immunodiffusion. Fifteen-ml of molten 1% (w/v) agarose in PBS (pH 7.3) containing 2 ml of SSB and diphtheria antitoxin (Lister Institute, Elstree, Herts, 30 units/ml), were layered on a 10 × 10-cm glass slide and allowed to set; 5 µl holes were punched at 15 mm intervals and filled with filtrates. After 2 days at room temperature the plate was washed for several hours in PBS and distilled water, pressed, dried, stained for protein with Coomassie Brilliant Blue (Gurr, from Hopkin & Williams, Chadwell Heath, Essex) and examined for precipitation rings.

The effect of Tween 80 (BDH) on toxin production was investigated by adding various concentrations of it to 20 ml of SSB in screw-capped containers which were inoculated with 0.1 ml of bacterial suspension (*c.* 10⁷ cells) and incubated for 72 h. Cultures were centrifuged and the toxicity of the supernates was determined.

The effect of Tween 80 on growth and protein synthesis. The organisms were grown in 10 ml of SSB with or without Tween 80 0.1% (v/v) and incubated at 35°C for 18, 48 and 72 h. The optical density and viable count of each culture was determined, the organisms were separated by centrifugation and the protein concentration of the pellet and the toxicity of the filtrate were determined.

RESULTS

Toxin production was partially inhibited by Tween 80 at 0.05% (v/v) and completely inhibited by Tween 80 at 0.08%. This was confirmed by animal experiments (table I) and by the radial-immunodiffusion method. In the radial-immunodiffusion tests toxin-antitoxin precipitation rings appeared around wells that had received filtrates of cultures without Tween 80. Filtrates from Tween-grown cultures and uninoculated media gave no precipitate.

To determine whether toxin inhibition was due to direct interaction of Tween 80 with toxin, two concentration of Tween 80, higher than those used for growth (0.2 and 0.4%) were prepared in SSB, mixed in equal volumes with toxic filtrate, incubated at 35°C for 18 h and tested for toxicity as before. However, there was no evidence of a direct inhibition of preformed toxin because all guinea-pigs died and precipitation rings appeared in the radial-immunodiffusion plate.

Growth and protein synthesis. Compared with plain SSB, medium with Tween 80 doubled the growth of *C. ulcerans* during the first 18 h and gave an almost sevenfold increase by 48 h (table II). This led to more rapid death at 72 h with increased protein synthesis.

Because oleic acid is a potentially nutrient component of the Tween 80 molecule (Stinson and Solotorovsky, 1971; Marshavina and Gazaryan 1975), this substance and other fatty acids (linoleic, palmitic and stearic) at concentrations of 5–30 µg/ml, were substituted for Tween 80 in SSB medium and the experiments repeated. All 16 guinea-pigs that received 0.1 ml of each

TABLE I

Inhibitory effect of different concentrations of Tween 80 on toxin production by Corynebacterium ulcerans

Percentage of Tween 80 (v/v) in culture medium	Number of guinea-pigs dead/number inoculated*
0.000	10/10
0.001	8/8
0.010	8/8
0.050	2/8
0.080	0/5
0.100	0/8
0.150	0/6
0.500	0/6

* Each guinea-pig received 0.1 ml of culture filtrate.

TABLE II

Effect of Tween 80 0.1% (v/v) on growth and toxin production by Corynebacterium ulcerans

Incubation time (h)	Culture medium	Growth			Toxicity* (approx. number of MLD/0.1 ml)
		Optical density (E ₆₅₀)	Viable count (c.f.u./ml)	Bacterial cell-protein (µg/ml)	
18	SSB	0.23	1.9×10^9	30	0
	SSB + Tween	0.65	3.8×10^9	89	0
48	SSB	0.50	8.5×10^9	44	2
	SSB + Tween	0.60	60×10^9	95	0
72	SSB	0.54	3.2×10^9	60	2
	SSB + Tween	0.68	2.1×10^9	100	0

c.f.u. = Colony-forming units; SSB = serum-substitute broth.

* Each guinea-pig received 0.1 ml of culture filtrate; 0 = all animals survived.

preparation, corresponding to 2 MLD in an unsupplemented culture died within 72 h. It was therefore apparent that these fatty acids in the free state did not inhibit toxin production.

DISCUSSION

The inhibitory effect of Tween 80 on the toxicity of *C. ulcerans* culture filtrates may be due to direct interaction with free toxin or induction of defective toxin production. Because toxic filtrates to which various concentrations of Tween 80 had been added, were still toxic, the possibility that Tween 80 destroys free toxin could be ruled out. The notion that culture filtrates might contain defective toxin was not supported by the immunodiffusion tests which showed the usual pattern of precipitate.

Tween 80 has previously been reported as a major source of carbon and energy, and mycobacteria hydrolyse it to yield oleic acid and sorbitol or its polyoxyethylene ethers. The fatty acid is then utilised for carbon and energy (Yamane, Minami and Yasui, 1954; Stinson and Solorovsky, 1971). Our results are consistent with the latter authors' findings in that Tween 80

increased bacterial growth and protein synthesis. Because the corynebacteria, mycobacteria and nocardia groups are closely related (Barksdale, 1970), perhaps the observed increase in *C. ulcerans* growth with Tween 80 may follow such a mechanism. We also showed that oleic acid, a component of the Tween 80 molecule, neither destroyed the toxin nor inhibited its synthesis. Tween 80 seems to influence the physiology of the bacterial cells and, by accelerating growth, to repress toxin synthesis. The mechanism of this effect is obscure but may involve dissolution or derangement of cell-wall lipids with consequential effects on toxin synthesis or release.

SUMMARY

Tween 80 stimulated growth of *Corynebacterium ulcerans* but suppressed toxin production. It had no direct effect on preformed toxin. Toxin production was not inhibited by oleic, linoleic, palmitic or stearic acids.

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