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The exotoxins of *Corynebacterium ulcerans*

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SUMMARY

The exotoxins produced by ten strains of *C. ulcerans* (two human, six bovine and two equine) have been studied. On the criteria of toxin–antitoxin neutralisation and immunoprecipitation tests using highly specific diphtheria and *C. ovis* antitoxins with crude toxic filtrates, $(NH_4)_2SO_4$ concentrates, and partially purified chromatographic preparations of these, together with the presence or absence of inhibition of the action of staphylococcal beta-haemolysin, and the reaction produced when injected intradermally into rabbits, two toxins could be identified, namely diphtheria toxin and *C. ovis* toxin. There was no evidence for the production of a third toxin specific for *C. ulcerans*. Five strains produced both diphtheria and *C. ovis* toxins. In four diphtheria toxin predominated, but in the fifth *C. ovis* toxin predominated. Two strains produced only diphtheria toxin and two only *C. ovis* toxin, though there was good but not complete evidence that a third strain (Revell) also fell into this latter group. Considerable variation occurred in the concentration of each toxin and, where both were present, in the proportion of each.

INTRODUCTION

The name *Corynebacterium ulcerans* was first given by Gilbert & Stewart (1927) to corynebacteria isolated from human throat lesions in New York State, U.S.A. In the virulence test as used for *C. diphtheriae*, these organisms gave rise to a marked local reaction with ulcer formation, both in normal guinea-pigs and in those previously injected with diphtheria antitoxin. Filtrates of liquid cultures were toxic, and this toxicity was neutralized by antiserum prepared in a horse. It was observed that, before immunization, the serum of this horse contained less than 0·002 of a unit of diphtheria toxin per ml, whereas after immunization over 18 months it had risen to approximately 30 units per ml. These observations suggested that these strains of *C. ulcerans* produced two exotoxins, the predominant one being diphtheria toxin.

Barratt (1933) at the Lister Institute, London, reported observations on a group of so-called 'aberrant' corynebacteria, isolated from lesions of the human nasopharynx, which appeared to be similar to Gilbert & Stewart's *C. ulcerans*. The cultural characters and pathogenic properties of these organisms in guinea-pigs

were compared with those of *C. diphtheriae* and *C. ovis* (*pseudotuberculosis*) and it was observed that whenever the characters of the aberrant strains differed from those of *C. diphtheriae*, they approached those of *C. ovis*. Petrie & McClean (1934) at the Elstree Laboratories of the Lister Institute studied the toxins produced by five of Barratt's organisms and the toxin-antitoxin relationships of these and of *C. diphtheriae* and *C. ovis*. They reported that, first, they found no relationship between the exotoxins of *C. ovis* and *C. diphtheriae*; secondly that one of the aberrant strains produced two toxins: the predominant one was immunologically identical with diphtheria toxin; the second was not neutralised by diphtheria antitoxin but 'had an affinity' with *C. ovis* toxin.

Although a considerable number of reports on organisms of the *C. ulcerans* type have been published, the validity of a specific taxonomic status has not been generally accepted (Skerman, McGowan & Sneath, 1980) and the identity of the second exotoxin produced has not been fully established. It is evident from published papers and our own observations on 125 strains isolated from man, cattle, buffaloes, horses and a monkey (to be published elsewhere) that organisms that have been included under the name *C. ulcerans* show considerable variation in morphology and biochemical reactions; as regards their toxigenic properties, whilst the majority of human strains described produce predominantly diphtheria toxin with smaller amounts of a second toxin having an affinity with *C. ovis* toxin, some strains produce the latter type of toxin only, and others are non-toxigenic. Furthermore, some organisms that have been described as *C. ovis*, isolated from suppurative lesions in horses, cattle and buffaloes, differ in a number of their properties from typical *C. ovis* isolated from characteristic lesions of caseous lymphadenitis of sheep.

The presence of two distinct toxins in liquid cultures of many strains of *C. ulcerans* raised the question whether such cultures might be a mixture of two different bacteria, each producing its own characteristic toxin. Our observations on repeated subcultures of single colonies picked from plates sown with single cell suspensions of 8 human strains have failed to support such a possibility.

There is now good evidence that the predominant toxin produced by many strains of *C. ulcerans* is diphtheria toxin, but a number of factors have contributed to the uncertainty of the nature of the second exotoxin produced by most strains. First, no detailed knowledge of the characteristics of the exotoxin of *C. ovis* (which it was thought by Petrie & McClean to resemble), became available until the papers of Souček & Součkova (1971, 1974), Carne & Onon (1978) and Onon (1979). These have shown that *C. ovis* toxin is a basic glycoprotein, MW 14500 ± 1000, which is a phospholipase D; it attacks sphingomyelin to produce ceramide phosphate and choline; when inoculated intradermally or subcutaneously in guinea-pigs, rabbits or sheep it causes marked leakage of plasma from blood and lymph vessels, producing local oedema; it does not produce congestion of the adrenal cortex of the guinea-pig, which is characteristic of diphtheria toxin. It thus differs clearly from diphtheria toxin which is an acidic protein, MW 62000–63000, which inhibits protein synthesis by inactivating elongation factor 2, an enzyme necessary for peptide synthesis, by catalysing its adenine-5-diphosphate ribosylation using nicotinamide adenine dinucleotide as substrate (Collier, 1975).

We have also shown that diphtheria toxin and *C. ovis* toxin are antigenically completely distinct, their antitoxins being quite specific, there being no reciprocal cross-neutralization or precipitation by their respective antitoxins. These differing properties are contrasted in Table 7.

Another source of confusion in toxin–antitoxin neutralization experiments with toxic filtrates of *C. ulcerans* resulted from the fact, unknown to some earlier investigators, that normal horses, prior to experimental immunization with toxic filtrates, frequently have present in their serum variable but significant amounts of antitoxins which specifically neutralize diphtheria toxin and/or *C. ovis* toxin. Petrie & McClean (1934), and Stănică *et al.* (1968) drew attention to this; the former noted as an example that the serum of one normal horse before immunization with *C. ovis* toxin neutralized 1000 guinea-pig minimal reacting doses (m.r.d.) of diptheria toxin and approximately 200 guinea-pig m.r.d. of *C. ovis* toxin. After a course of immunization with crude toxic filtrate of *C. ovis*, 1 ml of serum still neutralized 1000 guinea-pig m.r.d. of diphtheria toxin, but now neutralized 500 guinea-pig m.r.d. of *C. ovis* toxin. The origin of these two types of 'normal' antitoxins in healthy horses is of considerable interest. Although the production of diphtheria antitoxin may result from occasional unrecognized or inapparent infection of horses with true *C. diphtheriae*, *C. ovis* has never been found in indigenous sheep, cattle or horses in Great Britain. However, Maximescu *et al.* (1974) have reported the isolation of 35 strains of *C. ulcerans* from the nasopharyngeal flora of normal horses in Romania, where 50% of those examined were found to be carriers. Although *C. ulcerans* infections of the throat in man and of the mammary glands in cows are not common in the U.K., no report of the occurrence of this organism in horses has yet been published. It seems more likely that unrecognized infection by *C. ulcerans* could be the source of such 'normal' diphtheria and *C. ovis* antitoxins in horses.

Součkova & Souček (1974) reported the concentration and partial separation of two toxins present in culture filtrates of *C. ulcerans* ATCC 9015 isolated from a human throat. The organism was grown for 3–5 days in Todd–Hewitt broth and the toxins were precipitated by methanol or ammonium sulphate and separated by ion-exchange chromatography on a Sephadex CM 50 column. A toxin resembling diphtheria toxin was eluted from the column by 0·08 M-NaCl; this was lethal for guinea-pigs with characteristic congestion of the adrenal glands, and in immunodiffusion–precipitation tests gave fusion of identity with diphtheria toxin. Guinea-pigs, however, were not protected by 6000 units of diphtheria antitoxin administered previously. The second toxin (resembling that of *C. ovis*) was eluted from Sephadex CM 50 by 0·15 M-NaCl. It showed sphingomyelinase D activity and reaction to intradermal injection of rabbits characteristic of *C. ovis*. It was noted, however, that separation of the two was not complete, as the diphtheria toxin eluates contained traces of the second toxin.

Abrehem & Zamiri (1980) have recently described a toxin produced by *C. ulcerans* strain 378 of the Diphtheria Reference Laboratory, Welsh National School of Medicine, Cardiff. It was purified 66-fold by ammonium sulphate fractionation, dialysis, gel filtration on Ultragel AcA22, ion-exchange chromatography on DEAE

cellulose. Toxin was eluted at a molarity of 0·08–0·37 in a single peak. On gel filtration on Ultragel AcA54, toxin was eluted in a single peak. This purified toxin had a molecular weight of 13000–15000 and a lethal action in the guinea-pig of 33 M.L.D./mg by subcutaneous injection. The authors state that the toxin of this strain of *C. ulcerans*, of lower toxicity and molecular weight, is distinct from diphtheria toxin, but that it produced similar changes in the guinea-pig and is neutralized by diphtheria antitoxin; the authors also state that in gel diffusion–precipitation tests the antigenic cross-reactivity between *C. ulcerans* strain 378 toxin and diphtheria antitoxin suggests that the two entities are related, but they give no details. These observations are difficult to interpret. It is possible that the purified toxin was in fact *C. ovis* toxin. The estimated molecular weight (13000–15000) corresponds fairly closely with that of *C. ovis* toxin (14000 ± 1000 (Onon, 1979)). The pathological changes produced in guinea-pigs by subcutaneous inoculation of lethal doses of *C. ovis* and diphtheria toxins are not readily distinguished when very high doses are employed, but with moderate doses which cause death after 48 h or longer, diphtheria toxin produces characteristic congestion and haemorrhage in the adrenal cortex. *C. ovis* toxin in contrast produces no significant naked-eye change in the adrenal glands, but a marked gelatinous subcutaneous oedema extending from the site of inoculation over a large area of the abdomen and thorax. The authors do not mention the presence or absence of these lesions in their guinea-pigs.

Our paper is primarily concerned with the nature of the non-diphtheria toxin of *C. ulcerans* and presents evidence that this is identical with the distinctive specific exotoxin of *C. ovis*. This conclusion is based on experimental observations on the exotoxins produced by two of Petrie & McClean's (1934) human strains, six bovine and two equine strains, and comprising toxin–antitoxin neutralization and immunodiffusion–precipitation tests using highly specific diphtheria and *C. ovis* antitoxins with both crude toxic filtrates and concentrated toxins partially purified and separated by chromatography. We have found no evidence for the production of a specific exotoxin by *C. ulcerans*, distinct from diphtheria toxin and *C. ovis* toxin.

MATERIALS AND METHODS

General plan of investigation

This comprises five successive stages as follows.

(1) Batches of crude toxic Millepore filtrates of 20 h cultures in a special liquid medium were prepared from a series of strains of *C. ulcerans* and the m.r.d. on intradermal injection (i.d.) in rabbits was determined. For comparison, a series of artificial mixtures of purified diphtheria toxin and *C. ovis* toxin in varying proportions was similarly injected. Because of the distinctive differences between the reactions to these two toxins, special attention was paid to the time of first appearance and characters of the reactions.

(2) Neutralization tests were then carried out to determine the effect of diphtheria antitoxin and *C. ovis* antitoxin separately and mixtures of both these

antitoxins on fixed amounts of known toxicity of crude filtrates, again using intradermal injection in rabbits to measure free toxin.

(3) Attempts were made to separate the two different toxins present in crude toxic filtrates by chromatography. The separated fractions were freeze-dried and their m.r.ds determined by i.d. injection in rabbits. Neutralization tests were then carried out with these fractons using antitoxic sera prepared with crystalline diphtheria toxin and purified *C. ovis* toxin (1DE).

(4) Chromatographic fractions were tested for inhibition of staphylococcal beta-haemolysin, a characteristic property of *C. ovis* toxin.

(5) Immunodiffusion–precipitation tests using (*a*) Elek plates with streak cultures and wells with diphtheria antitoxin and *C. ovis* antitoxin, and (*b*) radial double diffusion from wells punched in agarose gels, using antitoxic sera as in (*a*) and chromatographic fractions of *C. ulcerans* toxic filtrates.

Bacterial strains. Toxin production by the following 10 strains of *C. ulcerans* and two strains of *C. ovis* has been studied.

From human throat. Strains 'Mair' and 'Revell' (Petrie & McClean, 1934).

From bovine mastitis. JW 32, JW 34, JW 36, JW 39 from Dr D. J. Jayne-Williams, National Institute for Research in Dairying, Shinfield, U.K. and strain FCN 1 from Dr F. C. Nelson, Ontario Veterinary College, Guelph, Canada.

From bovine ulcerative lymphangitis: Strain Bov K 519 from Dr F. G. Davies Veterinary Research Laboratory, Kabete, Kenya.

From nasopharynx of horse. Strain 298G, 510C from Dr Paula Maximescu, Cantacuzino Institute, Bucarest, Romania (Maximescu, 1968).

C. ovis (pseudotuberculosis). Ov $137C_2$, Ov 133 – both isolated from cases of caseous lymphadenitis in Australian sheep and extensively studied since 1930.

The ten strains of *C. ulcerans* conformed to the properties usually attributed to this organism, namely, their morphology tends to be coccoid or short bacillary, they ferment starch and trehalose, split urea and do not reduce nitrate to nitrite.

Stock cultures were maintained freeze-dried; subcultures on tryptic digest of ox heart or Medium I (see below).

Preparation of toxins

C. ovis *toxin.* Toxigenicity of strains varies considerably, so highly toxigenic strains should be selected (e.g. Ov $137C_2$, Ov 133 listed above). Although *C. ovis* grows readily in many types of broth in common use (including those employed for production of diphtheria toxin), special media are required for good yields of toxin. We have used mainly 'Medium I', a brain–heart infusion with added lactalbumin hydrolysate and yeast extract (details in Onon, 1979). Best yields are obtained by providing liberal aeration either by shaking or by vortex aeration with a magnetic stirrer during incubation at 37 °C. Toxin production under such conditions reaches a peak at approximately 20 h, and toxicity subsequently declines rather rapidly so that by 48 h the yield of active toxin may have fallen almost to zero. For sterilization by filtration, membrane filters (e.g. Millepore a.p.d. 22 μm) should be used as toxin is retained by siliceous filters (e.g. Chamberland,

Berkfeld, Seitz). The m.r.d. of filtrates determined by intradermal inoculation in the rabbit is of the order of $0{\cdot}2 \times 10^{-3}$ to 10^{-4} ml.

C. ulcerans *toxins*. We have used the same method of production as for *C. ovis*. In view of the fact that production of *C. ovis* toxin tends to be poor in some of the media commonly employed for production of diphtheria toxin, a number of strains of *C. ulcerans* were grown on a variety of different culture media, but none was found to give significantly better yields of either of the two types of toxin, nor did the proportions of each type of toxin vary significantly.

Diphtheria toxin. Three samples of toxin were employed, namely:

J.G. 664: a dry crystalline toxin (Wellcome Research Laboratories, Beckenham, Kent), m.r.d. for rabbit $0{\cdot}2 \times 10^{-5}$ mg.

RX 6679: dry toxin of high specific toxicity (Wellcome), m.r.d. for rabbit $0{\cdot}2 \times 10^{-5}$ mg.

PW8 (Alb 103): liquid toxic filtrate (Lister Institute, Elstree), m.r.d. for rabbit 0·0002 ml.

Measurement of toxins by intradermal injection

For comparative studies on the toxins of *C. diphtheriae*, *C. ovis* and *C. ulcerans* the rabbit is the experimental animal of choice for the following reasons: the reactions to intradermal injection of diphtheria toxin and *C. ovis* toxin are not only more clearly distinguishable than in the guinea-pig, but because of the looseness of the dermal tissue in the rabbit the process of injection of 0·2 ml doses appear to be virtually painless and up to 30 inoculatons can be accommodated on the back of a full-grown animal. Also the slower rate of hair growth in the rabbit facilitates reading reactions that take several days to reach full development. We have never found 'normal' diphtheria or *C. ovis* antitoxins in the sera of non-immunized animals. To determine the m.r.d., 0·2 ml of appropriate dilutions of toxin in phosphate-buffered saline (PBS) containing 0·4 molar $MgCl_2$ were injected into the closely clipped skin and reactions read at 24, 48 and 72 h.

The m.r.d. of diphtheria toxin. Defined as a circular area approximately 1·0 cm in diameter, evenly pink all over, raised slightly above the general surface of the skin *with a well-defined margin*: it *usually does not appear before* 24 *h or even later* and attains its maximum development at 72 h or more. There is no significant oedema of the tissues surrounding the margin. With higher doses (e.g. 10 m.r.d.) the reaction again does not usually appear before 24 h, the diameter of the area of congestion is increased to 25 mm or more, with a faint pink colour, coarsely mottled with white over the whole area; by 72 h a wider pink rim is seen just within the margin, and pale whitish central area. After 100 m.r.d. or more, central areas of haemorhage and necrosis develop.

The m.r.d. of C. ovis *toxin*. A circular, pink, low dome-shaped swollen area approximately 1·0 cm in diameter *develops within* 24 *h*; the *margin is ill-defined* and there is moderate congestion but no necrosis. By 48 h there is little change, but the reaction is clearly subsiding by 72 h. After 10 m.r.d., within 24 h there is an area of *oedematous swelling* approximately 3 cm in diameter with ill-defined borders and a central area of necrosis approximately 8 mm diameter with irregular outline.

The surrounding oedema fluid tends to gravitate downwards. After 100 m.r.d. or more, the oedematous pink swelling may reach 5 cm or more in diameter with ill-defined margins; there is well-marked congestion and a central area of haemorrhagic necrosis.

Titration of C. ovis *toxin by inhibition of staphylococcal beta-haemolysin*

Inhibition of the action of staphylococcal beta-haemolysin on sheep erythrocytes, first described by Lovell & Zaki (1966) and subsequently shown by Onon (1979) to be a useful *in vitro* method of measurement of *C. ovis* toxin, was employed as described by Onon (1979) as an additional test for the presence of *C. ovis* toxin in crude filtrates and chromatographic fractions prepared from them.

Concentration and purification of toxins

C. ovis *toxin.* The method was as described by Onon (1979), consisting of precipitation by 35–65 % $(NH_4)_2SO_4$, dialysis, ion-exchange chromatography on a 9 cm × 2 cm column of Sephadex CM-50 buffered with 0·1 M sodium acetate, pH 6·1. After the unbound material had been eluted, a linear NaCl gradient was applied. The active fraction designated 'CM 3' was force-dialysed and freeze-dried. Fraction CM 3 was then applied to a column of DEAE-cellulose exchanger buffered with 0·05 M Tris/HCl, pH 8·0. A linear salt gradient was applied after all the unbound material had emerged from the column. The active fraction (1 DE) was force-dialysed and freeze-dried. This effected a 438-fold purification, the final product having an m.r.d. of 0·0003 mg on intradermal inoculation in the rabbit.

C. ulcerans *toxins.* Toxic filtrates were concentrated with $(NH_4)_2SO_4$ as with *C. ovis* toxin. The 35–65 % fraction was dialysed against 0·1 M sodium acetate, pH 6·1, and then applied to a column of Sephadex CM-50. A flow rate of 30 ml/h was maintained. After the unbound material had been eluted, a linear salt gradient (500 ml of buffer and 500 ml of buffer containing 2 M-NaCl) was applied. Tubes of eluate were divided into 3 groups designated CM 1 (the first to emerge from the column), CM 2 (the second peak) and CM 3 (the final peak). The three groups were then dialysed and freeze-dried. Each of these fractions was then tested for toxicity and the ability of *C. ovis* and *C. diphtheriae* antitoxins to neutralize this toxicity determined.

Separation of the two types of toxin in C. ulcerans *filtrates.* Gill & Dinius (1971) demonstrated that diphtheria toxin could be concentrated from toxic filtrates of *C. diphtheriae* by precipitation wth $(NH_4)_2SO_4$ and dialysis against 0·01 M phosphate at pH 6·8 or 7·0. Then when applied to a column of DEAE-cellulose equilibrated and flushed with the same buffer, toxin could be eluted by 0·05 M phosphate. T. W. Patrick (unpublished observation in this laboratory) found that if an artificial mixture of *C. ovis* and *C. diphtheriae* exotoxins is bound to a column of DEAE-cellulose, the diphtheria toxin could be eluted by increasing the phosphate concentration to 0·1 M and thus separated from the *C. ovis* toxin which starts to be eluted at a concentration of 0·15 M, but complete elution was only achieved when the concentration was raised to 0·5 M.

In view of these findings the following modification of our first method above was used for certain filtrates of *C. ulcerans.* The 35–65 % $(NH_4)_2SO_4$ fraction after

dialysis was first applied to a Sephadex CM-50 column and then eluates CM 1 and CM 3 from this were put onto separate DEAE-cellulose columns. Then 0·1 M phosphate was applied to the first column yielding eluate (CM 1–DEAE–0·1 M). This was followed by 0·5 M phosphate yielding eluate (CM 1–DEAE–0·5 M). CM 3 was applied to the second DEAE column equilibrated with 0·5 M Tris/HCl, pH 8·0. This yielded fraction CM 3–DEAE. The three eluates were dialysed, freeze-dried and tested for toxicity by intradermal inoculation of rabbits and subsequently the ability of *C. ovis* and *C. diphtheriae* antitoxins to neutralize the toxicity was determined.

Antitoxins

C. ovis *antitoxin*. Antitoxins have been produced in the horse, sheep, turkey, guinea-pig and rabbit using crude toxic filtrates. All animals were first tested for freedom from 'normal' *C. ovis* antitoxin. Initial doses were of formol-toxoid prepared by addition of 0·2 % formalin to toxic filtrates prepared from our standard stock strains of *C. ovis* Ov $137C_2$ and Ov 133 having an m.r.d. of 0·0002–0.00002 ml, and holding at 37 °C or room temperature until non-toxic. Conversion to toxoid is slow (several weeks) and if the concentration of formalin is increased, antigenicity of the toxoid is markedly lowered. An initial dose of toxoid combined with Freund's incomplete adjuvant was followed a month later by weekly subcutaneous or intramuscular doses of toxoid and later of active toxin. *C. ovis* toxin and toxoid are rather poor antigens, and long courses of subcutaneous or intramuscular injections are required to produce satisfactory levels of antitoxin. The following are examples of levels of antitoxins attained.

Horse 'Doll': 1·0 ml antitoxin neutralized; 200 M.L.D. crude toxic filtrate
Sheep A236: 1·0 ml antitoxin neutralized; 4000 m.r.d. crude toxic filtrate
Turkey 937: 1·0 ml antitoxin neutralized; 2000 m.r.d. crude toxic filtrate
Rabbit P75: 1·0 ml antitoxin neutralized; 2400 m.r.d. crude toxic filtrate
Guinea-pig: a group of 17 which had received 12 doses of crude toxoid over a period of 4 months showed no reaction to intracutaneous injection of 100 m.r.d. of crude toxic filtrate. The actual levels of antitoxin in their sera were not determined.

These antitoxic sera prepared from crude toxic filtrate were used in a series of early neutralization experiments but were subsequently replaced by antitoxins prepared in rabbits using purified *C. ovis* toxin as follows.

C. ovis *antitoxin prepared in rabbits using purified* C. ovis *toxin* (*1DE*). Purified toxin (see 1 DE above), m.r.d. 0·001–0·0001 mg; initial doses of formol-toxoid + Freund's incomplete adjuvant intramuscularly, followed by toxoid and active toxin as above; 38 intramuscular doses over 20 months. Titres attained – 1·0 ml neutralized 4000–10000 m.r.d. of 1 DE. These antitoxins gave good precipitin lines in immunodiffusion tests with purified *C. ovis* toxin Ov 133-1 DR. Some samples were concentrated fivefold by means of the Minicon B 15 (Amicon Corp., Lexington, Mass. U.S.A.).

Diphtheria antitoxins

It was important to ensure that specific diphtheria antitoxic sera, especially those prepared in horses, were free from 'normal' antitoxin against *C. ovis* toxin. The three following diphtheria antitoxins were obtained through the courtesy of Dr M. Sterne and Mr P. A. Knight from Burroughs Wellcome Research Laboratories, Beckenham, Kent and all were found free from such 'normal' antitoxins.

Ex 4627, prepared in a horse immunized exclusively with crystalline diphtheria toxin. This contained 560 units of antitoxin/ml, and in immunodiffusion–precipitation tests, undiluted antitoxin gave good precipitin lines with diphtheria toxin JG 664 (2 mg/ml) and at progressive dilutions in these proportions. This antitoxin was used for our main experimental observations.

For some of the preliminary experiments two other diphtheria antitoxins were used, namely:

DP 2593–2, prepared in a horse: contained 3450 units/ml.

RX 6164/63, prepared in a sheep: contained 3000 units/ml.

Toxin-antitoxin neutralization tests with C. ulcerans *toxins*

In initial tests crude toxic filtrates of known m.r.d. were mixed with:

(*a*) *Diphtheria* antitoxin prepared with crude filtrates in horse and sheep and of known neutralizing capacity.

(*b*) *C. ovis* antitoxin prepared in horse, sheep and turkey and of known neutralizing capacity.

(*c*) mixtures of *C. diphtheriae* and *C. ovis* antitoxins as in (*a*) and (*b*).

Mixtures were held at 37 °C for 1–2 h and then 0·2 ml doses were inoculated intradermally into rabbits and reactions recorded at 24, 48 and 72 h.

In subsequent tests purified fractions CM 1 and CM 3 of toxic filtrates were mixed with diphtheria antitoxin (Ex 4627) prepared from crystalline diphtheria toxin, and *C. ovis* antitoxin prepared in rabbits immunized with purified *C. ovis* toxin (1 DE).

Immunodiffusion–precipitation tests

(*a*) *Elek plates.* Medium I agar poured in 9 cm diameter plastic Petri dishes. Three wells for sera 5 mm diameter punched on both sides 10 mm distant from central streak of culture 1·0 cm wide across the diameter of plate. Incubated at 37 °C for 48 h, then held at room temperature.

(*b*) *Radial double diffusion* from circular wells 5 mm diameter, centres 1·0 cm apart, punched in 1 % agarose gel on 25 cm^2 photographic glass plates; held in Petri dishes with moist filter paper at room temperature.

RESULTS

The reaction to intradermal injection of C. ulcerans *toxins*

The reaction to intradermal injection in rabbits of crude toxic filtrates varied according to the presence and concentration of a single toxin (either diphtheria

toxin or *C. ovis*), or both of these. When both toxins were present, the character of the reaction varied according to the proportion and concentration of each toxin. Thus, for example, the reaction to 1 m.r.d. of crude toxin filtrate of strain Mair, in which diphtheria toxin predominated, appeared within 24 h (i.e. earlier than with pure diphtheria toxin), but in other respects it conformed closely to the reaction to 1 m.r.d. of pure diphtheria toxin, the reaction having a sharply defined margin, the skin slightly thickened, and it persisted with little change over 4 days or longer. In contrast, crude toxic filtrate of strain Bov K519 in which *C. ovis* toxin predominated produced an early reaction, well developed by 24 h, showing congestion, an ill-defined margin and well-marked oedema, and the reaction had begun to subside after 48 h, thus conforming closely to the reaction produced by pure *C. ovis* toxin.

Intradermal injection in rabbits of experimental mixtures of diphtheria toxin and *C. ovis* toxin in various proportions produced reactions which closely resembled those of *C. ulcerans* crude toxins described above.

Chromatographic fractions CM1 and CM3, which it was anticipated should contain mainly diphtheria toxin and *C. ovis* toxin respectively, were prepared from crude toxic filtrates of 3 strains of *C. ulcerans* (Mair, JW 32, JW 34) and the m.r.d. of each was determined by intradermal injection in rabbits with the following results.

Strain	CM1 m.r.d. (mg)	CM3 m.r.d. (mg)
Mair	0·0008	0·008
JW32	0·1	0·1
JW34	0·02	0·02

The reactions to the CM3 fractions were indistinguishable from those produced by *C. ovis* toxin; those produced by CM1 fractions, although closely resembling the reaction to diphtheria toxin, appeared earlier (at 24 h), their margins were less sharply defined and they were similar to reactions produced by experimental mixtures of diphtheria toxin with smaller amount of *C. ovis* toxin.

It was found that the CM2 fractions contained greater amounts of the two mixed toxins and were therefore not examined so extensively as fractions CM1 and CM3.

Measurement of C. ovis *toxin by its inhibition of staphylococcal beta-haemolysin*

This test, although not as sensitive as intradermal injection of rabbits, had proved to be a valuable *in vitro* method for determining the presence and approximate concentration of *C. ovis* toxin during processes of concentration and purification in crude filtrates. Table 1 gives the results of tests by this method of the 35–65% $(NH_4)_2SO_4$ concentrate, and CM1 and CM3 fractions prepared from it by chromatography on Sephadex CM-50 of five strains of *C. ulcerans*.

It will be seen that of the two chromatographic fractions, the greatest concentration of the (*C. ovis* toxin) inhibitor was present in the CM3 fractions but that CM1 also contained a small amount, indicating that separation of the two toxins was not complete. This corresponded with the findings in toxin–antitoxin neutralization tests (see below Tables 3 and 5).

Table 1. *Staphylococcal beta-haemolysin inhibition tests on* 35%–65% $(NH_4)_2SO_4$ *fractions and Sephadex CM-50 fractions CM* 1 *and CM* 3 *on five strains of* C. ulcerans

	Fractions (1 mg/ml)		
Strain	35–65% $(NH_4)_2SO_4$ Ring diameter* (cm)	CM 1	CM 3
Mair	1·5	0·6	1·3
Revell	2·3	1·1	2·1
JW 32	1·65	0·75	1·85
JW 34	2·05	0·9	1·85
298G	1·6	1·0	1·85

* Diameter of circle of inhibition around central well of 0·5 ml.

Table 2. *Toxin–antitoxin tests with crude toxic filtrates*

(0·2 ml of mixture contains 50 m.r.d. in every case.)

Strain	Toxin (ml)	Diphtheria antitoxin (ml)	*C. ovis* antitoxin (ml)	PBS (ml)		Reaction
Mair	0·5 (= 250 m.r.d.)	0·25[a]	—	0·25	±	early, *C. ovis* type
	0·5	—	0·25[c]	0·25	+ + +	*C. diphtheriae* type
JW 32	0·5 (= 250 m.r.d.)	0·25[a]	—	0·25	±	early, *C. ovis* type
	0·5	—	0·25c	0·25	+ +	*C. diphtheriae* type
JW 34	0·5 (= 250 m.r.d.)	0·25[a]	—	0·25	±	*C. ovis* type
	0·5	—	0·25[d]	0·25	+ +	*C. diphtheriae* type
JW 36	0·2 (= 100 m.r.d.)	0·2[a]	—	—	+	*C. ovis* type
	0·2	—	0·2[d]	—	+ + +	*C. diphtheriae* type
JW 39	0·2 (= 100 m.r.d.)	0·2[a]	—	—	−	
	0·2	—	0·2[d]	—	+ + +	*C. diphtheriae* type
FCN 1	1·0 (= 500 m.r.d.)	1·0[b]	—	0·5	+ + +	*C. ovis* type
	1·0	—	0·25[d]	0·75	−	
298G	0·5 (= 250 m.r.d.)	0·5[b]	—	—	+ +	*C. ovis* type
	0·5	—	0·5[e]	—	−	
510C	0·5 (= 250 m.r.d.)	0·25[b]	—	0·25	−	
	0·5	—	0·25[e]	0·25	+	*C. diphtheriae* type
	0·5	0·25[b]	0·25[e]	—	−	

[a] Diphtheria antitoxin Ex 4627; 1·0 ml neutralizes 120000 m.r.d. of diphtheria toxin JG 664.
[b] Diphtheria antitoxin sheep RX 6164/63: contains 3000 units of antitoxin/ml.
[c] *C. ovis* antitoxin rabbit 194; 1·0 ml neutralizes 4000 m.r.d. of purified *C. ovis* toxin (1 DE).
[d] *C. ovis* antitoxin sheep A 236; 1·0 ml neutralizes 4000 m.r.d. of purified *C. ovis* toxin (1 DE).
[e] *C. ovis* antitoxin turkey 973; 1·0 ml neutralizes 2000 m.r.d. of *C. ovis* crude toxic filtrate.
PBS = phosphate-buffered saline.

Toxin–antitoxin neutralization tests with crude toxic filtrates

First, we demonstrated that the three diphtheria antitoxins employed had no neutralizing action on *C. ovis* toxin when the latter was in the form either of crude toxic filtrate or purified 1 DE; similarly, that *C. ovis* antitoxins prepared by immunization of horse, sheep, turkey and rabbits failed to neutralize diphtheria toxin. The majority of neutralization tests with *C. ulcerans* toxins were carried out with diphtheria antitoxin Ex 4627 prepared by immunization of a horse with crystalline diphtheria toxin, and *C. ovis* antitoxin prepared in rabbits with purified *C. ovis* toxin 1 DE. In reciprocal neutralization tests with these it was found that 10 m.r.d. of either toxin was not neutralized by an amount of the opposite antitoxin capable of neutralizing 1000 m.r.d. of its own specific toxin.

Table 2 sets out the results of toxin–antitoxin neutralization tests with crude toxic filtrates prepared from eight strains of *C. ulcerans* (one from an ulcerated human throat, five from cases of bovine mastitis and two from the nasopharynx of normal horses). From 100 to 500 m.r.d. were mixed with a series of diphtheria antitoxins and *C. ovis* antitoxins, all of which had been tested for freedom from 'normal' diphtheria and *C. ovis* antitoxins. It will be seen that four filtrates (Mair, JW 32, JW 34, JW 36) showed evidence of the presence of two toxins neutralized respectively by diphtheria and *C. ovis* antitoxins. The remaining four appeared to contain only one toxin; in two instances (JW 39 and 510C) this was neutralized by diphtheria antitoxin and in the remaining two (FCN 1 and 298G) the single toxin was neutralized by *C. ovis* antitoxin.

Toxin–antitoxin neutralization tests with chromatographically separated fractions of C. ulcerans *toxic filtrates, and antitoxins prepared with crystalline diphtheria toxin and purified* C. ovis *toxin*

With the preceding evidence of the presence of both diphtheria toxin and *C. ovis* toxin in toxic filtrates of strains Mair JW 32, JW 34 (Table 2), attempts were then made to separate these two toxins by the methods described above involving differential elution from columns of Sephadex CM-50. The eluates were freeze-dried, their toxicity measured by intradermal injection in rabbits, and neutralization experiments carried out with diphtheria antitoxin Ex 4627 prepared in a horse immunized with crystalline diphtheria toxin, and *C. ovis* antitoxin prepared in rabbits immunized with purified *C. ovis* toxin (1 DE). Table 3 gives the results of neutralization tests on the first (CM1) and last (CM3) eluate peaks in which diptheria toxin and *C. ovis* toxin respectively would be anticipated to be present.

It is evident that by the methods employed complete separation of the two toxins was not obtained. The CM1 fractions obtained by elution by 0·1 M buffer contained only diphtheria toxin, but the CM3 fractions obtained by elution by 0·5 M buffer apparently contained a mixture of predominantly *C. ovis* toxin mixed with a smaller amount of diphtheria toxin.

Bov K-519 is a strain of special interest. It was isolated from an outbreak of bovine lymphangitis and lymphadenitis in Kenya and was considered to be *C. ovis*. Crude toxic filtrate in Medium I had an m.r.d. of 0·002 ml, the reaction being

Table 3. *Toxin–antitoxin neutralization tests with chromatographic fractions CM 1 and CM 3 and antitoxins prepared with crystalline diphtheria toxin and purified* C. ovis *toxin*

(0·2 ml of mixture contains 50 m.r.d. of toxin in every case.)

Strain	Toxic *fraction* (*ml*)	Diphtheria *antitoxin* (*ml*)	*C. ovis antitoxin* (*ml*)		*Reaction*
Mair	CM 1, 250 m.r.d.				
	in 0·5 ml	0·5	—	−	
	in 0·5 ml	—	0·5	+	*C. diphtheriae* type
	CM 3, 250 m.r.d.	0·5	—	+ + +	*C. ovis* type
	in 0·5 ml	—	0·5	+ +	*C. diphtheriae* type
JW 32	CM 1, 250 m.r.d.	0·5	—	−	
	in 0·5 ml	—	0·5	+ + +	*C. diphtheriae* type
	CM 3, 250 m.r.d.	0·5	—	+ + + +	*C. ovis* type
	in 0·5 ml	—	0·5	+ + +	*C. diphtheriae* type
JM 34	Cm 1, 250 m.r.d.	0·5	—	−	
	in 0·5 ml	—	0·5	+ + +	*C. diphtheriae* type

CM 3 (not done; insufficient for test)

Diphtheria antitoxin Ex 4627; 1·0 ml neutralizes 120000 m.r.d. of diphtheria toxin JG 664

C. ovis antitoxin; 1·0 ml neutralizes 4000 m.r.d. of *C. ovis* toxin (1 DE)

	CM 1 m.r.d. mg	CM 3 m.r.d. mg
Mair toxin	0·0008	0·0008
JW 32 toxin	0·1	0·1
JW 34 toxin	0·02	(insufficient for testing)

Table 4. *Toxin–antitoxin neutralization tests of purified fraction CM 3–DEAE of the toxin of Bov K* 519

Strain	Toxin fraction CM 3–DEAE (ml)	Diphtheria antitoxin (ml)	*C. ovis* antitoxin (ml)	0·2 ml of mixture contains (m.r.d.)		Reaction
Bov K 519	0·6 (= 500 m.r.d.)	0·4	—	100	+ + + +	*C. ovis* type
	0·6	—	0·4	100	+ +	*C. diphtheriae* type
	0·6	0·2	0·2	100	−	
	Toxin control	—	—	15	+ + +	mixed type

Diphtheria antitoxin Ex 4627: 0·2 ml neutralized 24000 m.r.d. of diphtheria toxin JG 664.
C. ovis antitoxin rabbit 194: 0·2 ml neutralized 800 m.r.d. of purified *C. ovis* toxin (1 DE).

essentially of the *C. ovis* type. After concentration with $(NH_4)_2SO_4$ it was put on a Sephadex CM-50 column and, after all the unbound material had emerged from the column, a linear salt gradient was applied. The last peak (Bov K 519–CM 3) was then applied to a column of DEAE-cellulose exchanger buffered with 0·5 M Tris/HCl pH 8·0 and maintained with a flow of 30 ml/h, and the active fraction (1 DE) was force-dialysed and freeze-dried. The resulting dried fraction had an

Table 5. *Toxin–antitoxin neutralization tests of Bov K519–CM1–DEAE eluates with* 0·1 M *and* 0·5 M *buffer and diphtheria and* C. ovis *antitoxins*

(0·2 ml of mixture contains 50 m.r.d. in every case)

	Freeze-dried toxic fraction in PBS (ml)	Diphtheria antitoxin (ml)	*C. ovis* antitoxin (ml)	Reaction	
0·1 M eluate	0·5 (= 250 m.r.d.)	0·5	—	++++	*C. ovis* type
	0·5	—	0·5	+	Mixed type
	0·5	0·25	0·25	−	
0·5 M eluate	0·5	0·5	—	++++	*C. ovis* type
	0·5	—	0·5	+	Mixed type
	0·5	0·25	0·25	−	

Diphtheria antitoxin Ex4627: 0·25 ml neutralizes 3000 m.r.d. diphtheria toxin JG664.
C. ovis antitoxin rabbit 194: 0·25 ml neutralizes 1000 m.r.d. *C. ovis* toxin (2DE).
0·1 M eluate (freeze–dried): 1 m.r.d. = 0·04 mg.
0·5 M eluate (freeze-dried): 1 m.r.d. = 0·004 mg.

m.r.d. of 0·002 mg and produced a characteristic *C. ovis*-type reaction on intradermal injection in the rabbit. Neutralization tests of this fraction were then carried out and the results are set out in Table 4. These indicate that this organism produces two toxins; the major one is neutralized by *C. ovis* antitoxin and the minor one by diphtheria antitoxin, and the identification of these two toxins is confirmed by the results of immunodiffusion–precipitation tests (see below).

An attempt was then made to separate the two toxins by the following modification of the procedure employed in the preceding experiments. The 35–65 % $(NH_4)_2SO_4$ fraction after dialysis was first applied to a Sephadex CM-50 column and the eluates Bov K519–CM1 and Bov K519–CM3 prepared as described above. It was anticipated that diphtheria toxin would be found in the former and *C. ovis* toxin in the latter. Bov K519–CM1 was then applied to a column of DEAE-cellulose equilibrated with 0·1 M phosphate, pH 6·3, and the unbound material which came through (Bov K519–CM1 + DEAE 0·1 M) was expected to contain the diphtheria toxin. The buffer was then changed to 0·5 M phosphate and in the material which came through (Bov K519–CM1–DEAE–0·5 M) it was anticipated that any *C. ovis* toxin should be found. The two eluates were dialysed, freeze-dried and tested for toxicity by i.d. inoculation into rabbits with the following results.

Bov K519–CM1–DEAE – 0·1 M m.r.d. = 0·04 mg
Bov K519–CM1–DEAE – 0·5 M m.r.d. = 0·004 mg

Toxin–antitoxin neutralization tests with diphtheria and *C. ovis* antitoxins were then carried out with the results shown in Table 5. It will be seen that, as in the preceding experiment (Table 4) the attempt to obtain complete separation of the two toxins was unsuccessful.

Table 6. *Immunodiffusion–precipitation tests in Elek plates*

Culture streak	Sera in wells: Diphtheria antitoxin Ex 4627 undiluted	Sera in wells: *C. ovis* antitoxin rabbit 194 undiluted
C. ulcerens		
Mair	++	+
JW32	++	+
JW34	++	+
Bov K519	+	++
C. ovis		
Ov 133	−	+++

Immunodiffusion–precipitation tests

(a) *Elek plates.* The results of tests with four strains of *C. ulcerans* and one of *C. ovis* are set out in Table 6.

Diphtheria toxin–antitoxin precipitate lines were just visible after 24 h, but by 7 days appeared as sharply defined narrow lines. *C. ovis* toxin–antitoxin precipitates were perceptible by 48 h, but were broader and more hazy than those of diphtheria toxin–antitoxin precipitates. Precipitates were most marked with *C. ovis* Ov 133 (control) and formed a semicircle with their convex side towards the culture streak and located several millimetres from the edge of the well. With the *C. ulcerans* cultures, precipitates with *C. ovis* antitoxin formed a distinct hazy halo which was not so dense and extended further around the wells. The addition of 2% polyethylene glycol to the culture medium did not sharpen the precipitin lines, but they were much better defined, with sharper margins when the *C. ovis* antitoxin serum was concentrated fivefold.

Whereas the precipitates produced by strains Mair, JW 32 and JW 34 with diphtheria antitoxin were more clearly marked than with *C. ovis* antitoxin, the reverse was the case with Bov K-519, which produced well-defined lines of precipitate with diphtheria antitoxin, but with *C. ovis* antitoxin two parallel semicircles of precipitate formed, separated by a gap of 1–2 mm.

(*b*) *Radial double diffusion precipitation in agarose gels.* As is well known, good precipitation lines are produced by diphtheria toxin and antitoxin. We have found that good precipitation also occurs between purified *C. ovis* toxin (1 DE) and antisera prepared in rabbits immunized with this antigen, but long courses of immunization are required to produce good titres. Similar tests using chromatographically separated fractions CM 1 and CM 3 of *C. ulcerans* toxins (Mair, JW 32, JW 34), although toxic and neutralizable, failed to give clear precipitin lines with either diphtheria or *C. ovis* antitoxins.

DISCUSSION

All of the 10 strains of *C. ulcerans* examined were toxigenic. On the criteria of toxin–antitoxin neutralization and immunoprecipitation tests using highly specific

diphtheria and *C. ovis* antitoxins with crude filtrates, ammonium sulphate concentrates and partially purified chromatographic preparations of these, and the reactions produced on intradermal injection in rabbits, two toxins could be identified, namely diphtheria toxin and *C. ovis*. toxin. There was no evidence for the production of a third toxin specific for *C. ulcerans*. As indicated above, the identification of *C. ovis* toxin in toxic filtrates of many strains of *C. ulcerans* has had to await the determination of the detailed characteristic properties of *C. ovis* toxin recently provided by Souček, Michaelec & Součkova (1971), Souček & Součkova (1974), Carne & Onon (1978) and Onon (1979). The problem has also been complicated by the fact that while the majority of strains of *C. ulcerans* produce both diphtheria toxin and *C. ovis* toxin, the proportions of each toxin can vary, and some strains produce only one or the other of the two. The significant differences between the properties of diphtheria toxin and *C. ovis* toxin are contrasted in Table 7.

The evidence that the second toxin (other than diphtheria toxin) produced by most strains of *C. ulcerans* is *C. ovis* toxin is as follows.

(1) It is neutralized by *C. ovis* antitoxin prepared in rabbits from purified *C. ovis* (1 DE), Strain Ov 133, but is unaffected by diphtheria antitoxin (Table 2).

(2) It can be almost completely separated by chromatography from diphtheria toxin produced simultaneously by the same bacterium, and the purified preparations are also neutralized by *C. ovis* antitoxin (Tables 3, 4, 5).

(3) It inhibits the action of *Staphylococcus aureus* beta-haemolysin on sheep red cells (Table 1).

(4) Specific immunodiffusion–precipitation is demonstrable in Elek plates using *C. ovis* antitoxin.

Five strains (Mair, JW 32, JW 34, JW 36 and Bov K519) produced both diphtheria and *C. ovis* toxins. In four of these diphtheria toxin predominated in their filtrates (Mair, JW 32, JW 34, JW 36), but in the fifth (Bov K519) *C. ovis* toxin predominated. Of the remaining five strains two (JW 39, 510C) produced only diphtheria toxin, and two (FCN1, 298G) produced only *C. ovis* toxin. There was good but incomplete evidence that the final strain (Revell) fell into this latter group, its crude toxin being neutralized by *C. ovis* antitoxin but not by diphtheria antitoxin; it also inhibited staphylococcal beta-haemolysin, but owing to a mishap in refrigeration the chromatographic fractions prepared from it were lost, with the result that the full series of experimental tests could not be completed. Variation in the concentration of each toxin and, where both were present, in the proportion of each, was reflected in the character of the local reaction to intradermal injection in the rabbit.

Factors which appear to have led to confusion in results obtained by some earlier workers include variation in toxigenic properties of the strains studied; the importance for toxin production of culture media and methods of cultivation; the difficulty in obtaining high-titred specific *C. ovis* antitoxin; the presence of variable amounts of 'normal' diphtheria and *C. ovis* antitoxins in the serum of horses used for antitoxin production, and the use of whole cultures for the preparation of antisera, which therefore contained antibacterial as well as antitoxic immune

Table 7. *Contrasting properties of diphtheria toxin and* C. ovis *toxin*

Property	Diphtheria toxin	*C. ovis* toxin
Chemical nature	Acid protein	Basic protein
Molecular weight	62000–63000	14000 ± 1000
Biochemical properties	An enzyme inhibiting protein synthesis by inactivating elongation factor 2	Phospholipase D sphingomyelinase and permeability factor
Action on staphylococcal beta-haemolysis	Does not inhibit	Inhibits
Chromatography on Sephadex CM-50	Mainly in first peak of eluate (CM1) with linear salt gradient	Mainly in last peak of eluate (CM3) with linear salt gradient
Chromatography on DEAE-cellulose	Mainly in eluate with 0·1 M phosphate	Mainly in eluate with 0·5 M phosphate
Pathogenic action		
Intradermal inoculation of 1 m.r.d. in rabbit	Circular pink area, approximately 1·0 cm diam., slightly raised but not 'domed'; sharply defined margin, no significant oedema; *usually does not appear before* 48 *h* attains maximum at 72 h or later	Circular pink, swollen area (dome-shaped) approximately 1·0 cm diam; *develops within* 24 *h*; margin ill defined; subsides after 48 h
With larger doses intradermally	Diameter of area of congestion increases; may be central area of necrosis and haemorrhage	Extensive *oedematous* swelling 3–5 cm diam., ill-defined margins; central area of necrosis and with haemorrhage
Subcutaneous lethal doses	*Acute congestion*, often with *haemorrhage, in adrenal glands* Paralysis	No significant changes in adrenal glands. *Extensive subcutaneous, transparent gelatinous, oedema* at site of inoculation, often spreading over large area of abdomen and thorax; subserous and submucous haemorrhages, especially along alimentary canal No paralysis
With diphtheria antitoxin	Neutralization and immunodiffusion–precipitation	No neutralization No immunodiffusion–precipitation
With *C. ovis* antitoxin	No neutralization or immunodiffusion–precipitation	Neutralization and immunodiffusion–precipitation

Diphtheria antitoxin. An appropriate dose will not only protect a guinea-pig against diphtheria toxin, but also against the establishment of an infection following injection of live *C. diphtheriae.*

C. ovis *antitoxin.* While neutralizing *C. ovis* toxin, it does not protect a guinea-pig against the establishment of a pyogenic infection which will follow injection of live *C. ovis.*

globulins. Some investigators also appear to have been unaware that, whereas in infection by *C. diphtheriae* toxin is the main cause of injury to the host, and experimental animals can be completely protected against injection of live *C. diphtheriae* by antitoxin, with *C. ovis* the major pathogenic role is played by its pyogenic property, to which different species of experimental animals show a range of degrees of susceptibility. The guinea-pig is the most susceptible and can be infected experimentally with doses of less than 10 bacilli. These give rise to a suppurative reaction with progressive spread by lymph and blood leading to abscess formation in various internal organs and death within 4–8 weeks. Large doses of live *C. ovis* may cause death in a few days from acute intoxication. Furthermore, protection against the pyogenic action of *C. ovis* is not conferred by even large doses of purely antitoxic serum or by active immunization with toxoid, and non-toxigenic strains are still capable of setting up infection in guinea-pigs, but progressive extension of lesions is much reduced in the absence of toxin production.

While most strains of *C. ulcerans* produce some *C. ovis* toxin and give rise to some degree of local suppuration, with ulcer formation at the site of intradermal injection of modest doses of live organisms in guinea-pigs, we have found that if the guinea-pigs do not die of acute intoxication (usually from the diphtheria toxin produced by most strains), the local suppurative and ulcerative lesion resolves without extension of the pyogenic process.

These variations in the properties of '*C. ulcerans*' raise again questions not only of the relationships between *C. diphtheriae*, *C. ulcerans* and *C. ovis* (Barksdale, 1970; Silea, Pollice & Barksdale, 1980) but the appropriate nomenclature of such organisms as Bov K519. The latter is of special interest in that it shares certain properties with all three species. It resembles *C. ovis* in producing predominantly *C. ovis* toxin and gives rise to a progressive pyogenic infection in experimental guinea-pigs; it resembles *C. diphtheriae* in producing a small amount of diphtheria toxin, and it resembles *C. ulcerans* (as at present defined) in its biochemical properties of fermenting starch and trehalose, splitting urea, and failing to reduce nitrates. Organisms of similar type isolated from African cattle have been regarded by some as strains of *C. ovis* (Purchase, 1944). However, it is noteworthy that although caseous lymphadenitis due to *C. ovis* is widespread in sheep in Australia, there is no record of the occurrence of a suppurative lymphangitis and lymphadenitis in Australian cattle comparable with the disease which occurs in African cattle.

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