Further Studies on *Corynebacterium* Species Capable of Producing Diphtheria Toxin (*C. diphtheriae*, *C. ulcerans*, *C. ovis*)

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SUMMARY

Lysogenization experiments showed that diphtheria phages can induce the production of diphtheria toxin in strains of *Corynebacterium ulcerans* and *C. ovis*.

In addition to diphtheria toxin, lysogenic *C. ulcerans* and *C. ovis* strains each produce their own specific toxin; they also maintain their specific morphology and urease production whilst *C. diphtheriae* strains lysogenized by *C. ulcerans* phages produce only diphtheria toxin. The majority of *C. ulcerans* phages are unable to induce the production of diphtheria toxin in strains of *C. ulcerans*.

Phage typing with *Corynebacterium ulcerans* phages is a practical possibility. Thirteen well-defined phage types were established by this technique in 172 *C. ulcerans* and 62 *C. ovis* strains with only one of the phage types (III C) occurring in both species. *C. ulcerans* can be distinguished from *C. ovis* by the pathogenicity test in mice. *C. ovis* kills mice by any route of inoculation whilst *C. ulcerans* strains cause only arthritis. *C. ulcerans* wild strains could be identified in this way and *C. ulcerans* and *C. ovis* strains from different type culture collections could be distinguished from each other by these well-defined characteristics.

INTRODUCTION

The somewhat surprising capacity of strains of *Corynebacterium ovis* to produce diphtheria toxin as a consequence of lysogenization with a diphtheria phage has led to further investigation of some of the biological characters of the three *Corynebacterium* species able to produce diphtheria toxin. These species are *C. diphtheriae* (C.D.), *C. ulcerans* (C.U.) and *C. ovis* (C.O.).

Earlier investigations of the characteristics of *Corynebacterium ulcerans* (Stănică *et al.* 1968; Maximescu, 1967; 1968) enabled us to collect a number of strains of this species during routine tests of diphtheria cases and carriers. According to many authors the differentiation between the species *C. diphtheriae*, *C. ulcerans* and *C. ovis* is not very precise, particularly as we found that they share some common characteristics, such as hydrolysis of cystine, sugar fermentations (according to either the *gravis*, the *mitis* or the *intermedius* patterns) and release of diphtheria toxin. Since we described the production of diphtheria toxin by lysogenized *C. ovis* strains (Maximescu, Pop, Oprişan & Potorac, 1968) it seemed important to investigate further the phage-host relationships in strains of *C. ulcerans*, *C. ovis* and *C. diphtheriae* in an attempt to improve the characterization of these species and their classification.

The four different aspects investigated were: (i) lysogenization; (ii) attempts at phage-typing strains of *Corynebacterium ulcerans* and *C. ovis* with a set of *C. ulcerans* phages;

(iii) evaluation of the mouse pathogenicity test for the differentiation of *C. ulcerans* from *C. ovis*; and (iv) a survey of a larger number of wild- and type-collection strains of *C. ulcerans* and *C. ovis* from an epidemiological point of view.

METHODS

Corynebacterium strains. Wild *Corynebacterium ulcerans* strains were isolated in Romania: 4 strains from human sore throats, 122 strains from human carriers, and 35 strains from the rhinopharyngeal flora of normal horses. Among these strains were 298G, 9304, 40c, A238 and 775, mentioned elsewhere in this paper, most of which were kindly supplied by our Romanian colleagues from Public Health Laboratories.

Collection strains of Corynebacterium ulcerans. The following strains were kindly supplied by Professor H. R. Carne of Cambridge (see Carne, 1968): u. 25908, u. 26346, and u. 26348, originating from N. S. Mair; no. 1 starch-fermenting and no. 2 non-starch-fermenting, originating from W. H. H. Jebb (see Jebb, 1948, and Jebb & Martin, 1965); and u₁. 2972/49, u₄. 1254/50, u₅. 1605/50, u₆. 1661/50 and u. 976/51, originating from S. D. Henriksen (see Henriksen, 1955). Strains u₃. 892/50, 389/53 and 355/53 were kindly supplied by Professor S. D. Henriksen, Oslo, Norway, and 16L supplied by the late Professor D. T. Robinson and Dr E. C. Armstrong, Liverpool.

Collection strains of Corynebacterium ovis. Strains 137c₂ (Australia), Cap 1, Cap 2, Cap 3 (Ayoub, Egypt), Bov 1, Bov 2 (Zaki, Egypt), 1133, 1134 (Australia), 1123, 1124 (U.S.A.), and 1129, 1130 (Kenya), were all kindly supplied by Professor H. R. Carne, Cambridge; *C. ovis* A (Australia) was kindly supplied by Dr M. Battey, Brisbane, Australia (see Battey, Tonge, Horsfall & McDonald, 1968); *C. ovis* T was received from Dr Afnan, Teheran, Iran (see Afnan, 1969); ten *C. ovis* strains were received from Dr M. Zaki, Assiut, Egypt; *C. ovis* 20 and 21 (Zaki) were supplied by Professor N. Stamatin, Bucharest, Romania; also a further 40 strains from collections.

All strains were also given registered numbers at our laboratory (see column 1, Table 1).

Corynebacterium diphtheriae strains. 1030₍₋₎^{tox-} *C. belfanti* (Maximescu, 1968) and 5056₍₋₎^{tox-} *C. D. intermedius*.

All strains were either lyophilized or kept at room temperature on Loeffler slopes.

Media. (i) For phage typing: 1.5 % heart-brain agar (Narbutovicz, 1955) with 0.001 % cystine and 10 % normal ox serum (Saragea & Maximescu, 1964). (ii) For phage multiplication: heart broth with 0.001 % cystine. (iii) For production of diphtheria toxin: Pope-Linggood broth with 0.6 % maltose and 1 % sodium acetate (Saragea & Maximescu, 1964); for production of ovis and ulcerans toxin: heart broth with 0.6 % maltose and 1 % sodium acetate.

Toxigenicity. The presence of diphtheria toxin was demonstrated by skin tests on rabbits (Fraser, 1931), guinea-pig inoculation by the Elek agar gel diffusion method as modified by Maximescu & Drăgoi (1971), and in monkey kidney tissue culture (Calalb *et al.* 1965).

Pathogenicity test. White mice (strain NMRI, 16 to 18 g weight, irrespective of sex) were injected intravenously with 0.1 ml of a 24 to 48 h broth culture of either *Corynebacterium ulcerans* or *C. ovis*.

Bacteriophages. (i) *Corynebacterium diphtheriae* phages: W (PW8 phage, Maximescu, 1968); nos. 2, 20, 21 and 23 from the set of phages used by Saragea & Maximescu (1964). (ii) *C. ulcerans* phages: 15542/238 (a); 213/40_c (b); 16259/40_c (c); 210/16L (d); 9304/984 (g) and 984/984 (h). (iii) Phage 76c is a *C. diphtheriae* phage (originated from the 76c *C. D. gravis* tox⁺ strain) restricted in strain *C. ovis* 21.

Table 1. *Corynebacterium ulcerans* and *C. ovis* collection strains*

Registered no.	Source	Diphtheria toxin	Phage type
<i>Corynebacterium ulcerans</i>			
978	u. 25908, N. S. Mair, Leicester, Publ. Hlth Lab., 1957	+	IVD
979	u. 26346, N. S. Mair, Leicester, Publ. Hlth Lab., 1957	+	IVD
980	u. 26348, N. S. Mair, Leicester, Publ. Hlth Lab., 1957	+	IVD
981	No. 1, starch-fermenting, W. H. H. Jebb, Publ. Hlth Lab., Oxford	+	VE
983	No. 2, non-starch-fermenting, W. H. H. Jebb, Publ. Hlth Lab., Oxford	+	IVD
982	u. 1, 2972/49, S. D. Henriksen, Oslo, Norway	+	IIB ₂
984	u. 4, 1254/50, S. D. Henriksen, Oslo, Norway	+	A ₀
985	u. 5, 1605/50, S. D. Henriksen, Oslo, Norway	+	IA
986	u. 6, 1661/50, S. D. Henriksen, Oslo, Norway	—	Phage-sensitive, non-typable
987	u. 8, 976/51, S. D. Henriksen, Oslo, Norway	+	A ₀
2506	u. 603/50, S. D. Henriksen, Oslo, Norway	—	IA
2507	u. 3, 892/50, S. D. Henriksen, Oslo, Norway	—	A ₀
2508	u. 389/53, S. D. Henriksen, Oslo, Norway	+	IVD
2509	u. 355/53, S. D. Henriksen, Oslo, Norway	+	A ₀
16L	<i>C. ulcerans</i> , D. T. Robinson and E. C. Armstrong, Liverpool	+	IIIC
9304	<i>C. ulcerans</i> , P. Maximescu, 1968	—	IA
775	u. 775, non-starch-fermenting, Romania	+	IVD
<i>Corynebacterium ovis</i>			
988	137 c2, Sydney, Australia, 1950	—	VIG ₂
989	Cap. 1, Dr Ayoub, Egypt, 1965	—	IIB ₁
990	Cap. 2, Dr Ayoub (from Egyptian goat), 1965	—	IIB ₁
991	Cap. 3, Dr Ayoub (from Egyptian goat), 1965	—	IIB ₁
992	Bov 1, Dr Zaki (from Egyptian buffalo)	+	IIIC
993	Bov 2, Dr Zaki (from Egyptian buffalo)	+	IIIC
994	No. 1133, lymph-node abscess in sheep, Australia, 1974	—	VIG ₂
995	No. 1134, lymph-node abscess in sheep, Australia, 1974	—	VIG ₂
996	No. 1123, pectoris abscess in horse, U.S.A., 1960	—	IIB ₀
997	No. 1124, pectoris abscess in horse, U.S.A., 1960	—	IIB ₀
998	No. 1129, ulcerative lymphangitis, Kenya, before 1950	—	IIB ₀
999	No. 1130, ulcerative lymphangitis, Kenya, before 1950	—	IIB ₀
A	C.O. A., Australia, human case	—	VIG ₂
20	C.O. 20, Dr Zaki, Professor Stamatin collection, Bucharest, Romania	—	VIG ₂
21	C.O. 21, Dr Zaki, Professor Stamatin collection, Bucharest, Romania	—	VIG ₂
T	C.O. Teheran, Dr Afnan	—	IIB ₀

* Strains quoted in the papers of: Afnan (1969); Battey, Tonge, Horsfall & McDonald (1968); Henriksen (1955); Howard & Jann (1954); Maximescu (1968).

Phage-typing of *Corynebacterium ulcerans* and *C. ovis* strains was performed by the technique described by Saragea & Maximescu (1964) for *C. diphtheriae* strains.

Throughout this paper diphtheria toxin is referred to as Di-toxin and the strains producing it as Di^{tox+}.

RESULTS

Lysogenization experiments

These were carried out with diphtheria phages in strains of *Corynebacterium ulcerans* and *C. ovis*, and with *C. ulcerans* phages in strains of *C. diphtheriae*.

Four *Corynebacterium ulcerans* strains (40c, A238, 298G and 9304) could be lysogenized

Fig. 1. The skin test in rabbit (by Fraser method) of the filtrates of the *Corynebacterium ovis* 21 (1) and C.O. 21₍₇₆₎^{tox+} (2, 3, 4), as compared with the diphtheria toxin (5). The filtrates were given before (b) and after (a) diphtheria antitoxin inoculation.

and converted with phage W and the diphtheria phages nos. 2, 20, 21 and 23. Strain *C. ovis* 20 produced Di-toxin (and was then labelled C.O. 21₍₇₆₎Di^{tox+}) when phage 76 was used. Di-toxin in lysogenized *C. ulcerans* and *C. ovis* cultures was detected by all four techniques described under Methods. As would be expected, considerably smaller amounts of neutralizable diphtheria toxin appearing in filtrates of Di^{tox+} cultures of *C. ulcerans* and *C. ovis* could be detected by assays in rabbit skin, in monkey kidney tissue cultures and in agar gel diffusion tests (see Figs. 1 to 3). Lysogenized strains of *C. ulcerans* and *C. ovis* retained their coccal morphology and ability to split urea and in addition to diphtheria toxin also produced their own specific toxins.

Corynebacterium ulcerans phage 'h' induced production of Di-toxin in two *C. ulcerans* strains (40 and A238). However, when the ulcerans phages 'b' and 'c' were used to lysogenize the same strains no induction of diphtheria toxin resulted.

When widely phage-sensitive *Corynebacterium diphtheriae* strains such as 5056₍₋₎^{tox-} *C.D. intermedius* and 1030₍₋₎^{tox-} *C. belfanti* were used, the ulcerans phages 'b' and 'h' lysogenized the strains and formed plaques. The 'b' phage, while lysogenizing, did not induce production of diphtheria toxin in strain 5056₍₋₎^{tox-} *C.D. intermedius*, but phage 'h' converted strain 1030₍₋₎^{tox-} *C. belfanti* to Di-toxigenicity without changing either its morphological or biochemical characteristics.

Fig. 2. Testing in monkey tissue culture of Di-tox⁺ *C. ovis* 21 culture. (a) Test, (b) control. In the control tube the cytopathogenic effect was prevented by diphtheria antitoxin.

Phage typing of *C. ulcerans* and *C. ovis* strains

No specific pattern emerged and only few strains proved to be phage-sensitive when the standard set of diphtheria phages for typing *Corynebacterium diphtheriae* strains was used.

When the set of the six ulcerans phages 'a' to 'h' together with the diphtheria phage 76 restricted in *Corynebacterium ovis* strain no. 21 were used on 172 *C. ulcerans* and 62 *C. ovis* strains, a provisional phage-typing scheme consisting of 13 sensitivity patterns could be worked out (see Table 2). Strains from collections 20 years old were typed in this way. In man and horses some identical phage types were found (see Table 2).

The experiments established that: (i) All *Corynebacterium ulcerans* and *C. ovis* strains available to us could be phage-typed by this method (see Table 3). (ii) Only the widely phage-sensitive *C. diphtheriae* strains 5056₍₋₎^{tox-} *C.D. intermedius* and 1030₍₋₎^{tox-} *C. belfanti* were sensitive to ulcerans phages. All other *C. diphtheriae* strains tested routinely were not susceptible to ulcerans phages. (iii) Phage types A₀, IA, IIB₂, IVD, IVD₁, VE, VIG and VIG₁ occurred only in *C. ulcerans* strains; phage types IIB₀, IIB₁ and VIG₂ were found only in *C. ovis* strains; phage type IIIC was present in both *C. ulcerans* and *C. ovis* strains (see Table 1).

Mouse pathogenicity test

Contrary to the findings of Zaki (1966), in our hands only *Corynebacterium ulcerans* (five strains) produced arthritis in white mice. By contrast, 20 *C. ovis* strains administered intraperitoneally, intravenously or intracerebrally resulted in death of white mice. It was possible

Table 2. *Provisional phage-typing scheme for Corynebacterium ulcerans and C. ovis*

Host strain	Pattern of sensitivity to phages							Phage type
	(a) 15542/238	(b) 213/40 _o	(c) 16259/40 _o	(d) 210/16L	(g) 9304/984	(h) 984/984	(76) 76c ovis 21	
<i>C. ulcerans</i>								
984	×	×	×	×	×	×	×	A ₀
9304	×	×	×	.	.	×	×	1A
5229	×	×	×	.	×	.	×	1A ₁
<i>C. ovis</i>								
996	×	×	.	×	×	×	×	11B ₀
990	×	×	.	×	.	×	×	11B ₁
<i>C. ulcerans</i>								
16259	×	×	.	.	.	×	×	11B ₂
16L	×	×	.	×	×	.	.	111C
210L	×	×	.	.	.	.	.	11VD
318c	×	×	.	.	.	.	.	11VD ₁
213L	×	.	.	×	×	.	.	VE
15542	.	×	.	.	.	.	.	VIG
4870	.	×	.	×	.	.	.	VIG ₁
<i>C. ovis</i>								
021	.	×	.	.	.	.	×	VIG ₂

Table 3. *Analysis of Corynebacterium ulcerans and C. ovis strains according to phage types*

Diphtheria toxin	A ₀	1A	1A ₁	11B ₀	11B ₁	11B ₂	111C	11VD	11VD ₁	VE	VIG	VIG ₁	VIG ₂	Total
<i>C. ulcerans</i> wild-type, human														
tox ⁺	.	.	.	.	.	.	15	15	8	.	4	1	.	43
tox ⁻	.	44	4	.	.	1	.	7	20	.	3	.	.	79
<i>C. ulcerans</i> , equine														
tox ⁺	.	.	.	.	.	.	6	.	.	.	.	.	.	6
tox ⁻	.	9	2	.	.	.	.	16	2	.	.	.	.	29
<i>C. ulcerans</i> collection														
tox ⁺	4	1	.	.	.	.	1	6	.	1	.	.	.	13
tox ⁻	.	2	.	.	.	.	.	.	.	.	.	.	.	2
<i>C. ovis</i>														
tox ⁺	.	.	.	.	.	.	2	.	.	.	.	.	.	2
tox ⁻	.	.	.	26	2	.	.	.	.	.	.	.	32	60

in this way to establish that the two strains Bov 1 (992) and Bov 2 (993) isolated from buffaloes in Egypt have to be regarded as *C. ovis*. Both these strains were found by us to be Di-toxigenic and to belong to phage type 111C.

Epidemiological implications of phage-typing of C. ulcerans and C. ovis strains, both wild-type and collection strains

Of the 157 wild-type *Corynebacterium ulcerans* strains isolated, 31.2 % were Di-toxigenic and in four cases of human tonsillitis the serum level of diphtheria antitoxin of 0.2 i.u. was adequate for protection against diphtheria toxin. Most strains isolated from man occurred in carriers, mostly children in rural areas, and only rarely was a sore throat or tonsillitis present. When the studies of Stănică *et al.* (1968) were extended it was found that 50 % of horses

Fig. 3. Gel-immuno-diffusion of toxin produced by *Corynebacterium diphtheriae* Park Williams No. 8 strains (Weissensee) (A) and Co 1113_(w)^{tox+} (B) against purified diphtheria antitoxin (C).

investigated carried *C. ulcerans*. Among wild *C. ulcerans* strains, ten were non-starch-fermenting and belonged to different phage types, but four were similar to those of Mair u.26.348 and W. H. H. Jebb no. 2 non-starch-fermenting.

Strain C.O. 21₍₇₆₎Di^{tox+} produced an accidental laboratory skin infection in one of us. Local and general symptoms were recorded and the Di-tox⁺ *Corynebacterium ovis* strain was isolated from the lesion.

DISCUSSION

The demonstration of phage-induced diphtheria toxin production in *Corynebacterium ulcerans* and *C. ovis* raises two questions. How far is it justified to consider diphtheria toxin to be an attribute only of the diphtheria species? What importance is attached to the capacity to produce diphtheria toxin in the characterization of C.D. species?

Corynebacterium ovis is known to differ sharply from *C. diphtheriae* by producing a filterable toxin which differs fundamentally from that of *C. diphtheriae*, although *C. ulcerans* and *C. ovis* share in common with this species characters recognized as biotypes.

The problems which arise are how much these species differ from each other (if it is justified to regard *Corynebacterium ulcerans* and *C. ovis* as species) and what causes the Di-tox⁺ gene to be expressed as a common character in such different hosts.

The present experiments confirm Howard & Jann's (1954) findings about the lack of activity of *C. ulcerans* phages on strains of *C. diphtheriae* and this favours Henriksen's (1955) view that *C. ulcerans* is a separate species of *Corynebacterium*.

However, in our experiments *Corynebacterium ulcerans* phages were able to form plaques in a few *C. diphtheriae* strains, but only in those strains possessing a wide phage-sensitivity. Our work raises yet another unanswered question, namely, do *C. ulcerans* strains carry both *ulcerans* and diphtheria phages?

Our earlier findings (Maximescu *et al.* 1968) on diphtheria toxin induction in *C. ovis* which

lead to the proof that the gene *tox*⁺ can be expressed in *Corynebacteria* other than *C. diphtheriae*, has been accepted (Barksdale, 1970). The present results confirm that the *Di-tox*⁺ character may be more widely induced among species of the genus *Corynebacterium*. From this, the question arises: is the genetically determined capacity to produce diphtheria toxin dependent also on the host bacterial cell, or only on the phage type inducing the production of diphtheria toxin?

Since these problems require further investigation, it seems possible that some of our findings, in particular the distinction between *Corynebacterium ulcerans* and *C. ovis* by means of phage typing and the mouse pathogenicity test, may prove of value.

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