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Variation among strains of *Corynebacterium diphtheriae* during an outbreak in a restricted environment

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SUMMARY

Strains of *Corynebacterium diphtheriae* isolated from a small outbreak in the restricted environment of a Mental Hospital were examined. All belonged to one serotype, but there was marked variation in diphthericin type, in sensitivity to bacteriophages and in the minor antigens possessed. One strain was non-virulent and laboratory-produced variants of this non-virulent strain showed changes in some of the characteristics used in the identification and typing of the organism, such as diphthericin type, sensitivity to bacteriophages and diphthericins, virulence, starch fermentation and, to a lesser extent, in antigenic structure. The epidemiological and experimental findings are consistent with the hypothesis that the strains isolated, both in the hospital and in the laboratory, were derivatives of a single parent and the mechanism of some of the variations could be related to changes in some structural component such as the cell membrane or the cell wall.

INTRODUCTION

Bacteriological studies of strains of *Corynebacterium diphtheriae* isolated in Victoria and New South Wales demonstrated that a number of strains existed which did not conform to the 'classical' definition based on cultural, biochemical and serological characteristics (Gibson, Cooper, Saragea & Maximescu, 1970). It was argued that the large number of varieties found were derived from one parental type, but the mechanism of the variation was not understood although involvement of bacteriophage was considered possible.

In January 1970, an outbreak of diphtheria occurred in the restricted environment of a Mental Hospital and the strains isolated were examined for their cultural, biochemical and serological characteristics. In addition, they were studied for sensitivity to bacteriophages of the typing scheme and for their ability to produce diphthericins. During the course of diphthericin typing of these strains it was noticed that, on incubation of the culture plates for 5 days or more, colonies quite frequently developed within the zone of diphthericin activity when certain producer-indicator combinations were examined. It was decided, therefore, to test

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whether variants of a strain of *C. diphtheriae*, selected for insensitivity to the action of diphthericin produced by another strain, exhibited concomitant changes in other characteristics used in the identification and typing of the organism. This latter approach seemed especially relevant in view of the fact that pleiotropic changes had been observed in colicin-tolerant mutants of *Escherichia coli* (Holland *et al.* 1970; Onodera, Rolfe & Bernstein, 1970) and in aeruginocin-tolerant mutants of *Pseudomonas aeruginosa* (Holloway, Krishnapillai & Stanisich, 1971).

METHODS AND MATERIALS

Bacteriological techniques

The methods of examining strains of *C. diphtheriae* for their cultural and biochemical characteristics, antigenic reactions, bacteriophage sensitivity and virulence were described previously (Gibson *et al.* 1970). Since certain bacteriophages were not possessed by the author, some of the Mental Hospital strains were sent to Romania where they were tested by Dr Alice Saragea for their sensitivity to the bacteriophages numbered 25 to 35. Additional tests and the scheme for typing strains by their production of diphthericins were also described elsewhere (Gibson & Colman, 1973). Because of the large number of variants to be tested for virulence, the intradermal (Romer) method as described in *Medical Microbiology* (Cruickshank, 1965) was employed, but a few variants and all the Mental Hospital strains were tested by subcutaneous inoculation.

Isolation of diphthericin-insensitive variants

Producer strain and sensitive strain

The Ferris (1950) stock strain of serotype Nadjarian, designation FN, was used as the producer of diphthericin in these experiments. It differed in many characteristics (Table 2) from strain 201742 which was isolated from one of the Mental Hospital patients and was the parent of the diphthericin-insensitive variants (Tables 1 and 2).

Method of selection

An overnight broth culture of strain FN was used to inoculate Tryptone Soya Horse Blood Agar (T.S.H.B.A.) in glass Petri dishes. The inoculum was applied in a thin circle whose radius was approximately two-thirds that of the dish; in addition, the inoculum was applied to the centre of the circle. This technique ensured that the surface of the agar was thoroughly impregnated with diphthericin after incubation at 30° C., for 60 hr. The growth was removed and the remaining cells killed with chloroform vapour.

An overnight broth culture of strain 201742 was diluted 1/10 and 1/100; 0·1 ml. of each dilution and of the broth itself were spread over the surface of the diphthericin impregnated plates which, after drying, were incubated at 30° C., for 5–7 days. Colonies which developed on the plates were assumed to have arisen from cells which were insensitive to the diphthericin of strain FN and in one such

experiment the selection rate was 0·01 %. All colonies were plated on Potassium Tellurite Horse Blood Agar (P.T.H.B.A.) for purity. One colony from each P.T.H.B.A. plate was used to inoculate a Loeffler slope and the growth from the Loeffler slope was stored at −20° C., in 1·0 ml. horse serum (free of antibodies against diphtheria toxin and diphtheria bacilli) before their being re-tested for diphthericin insensitivity and other bacteriological characteristics.

Method of adsorption

The bacterial cells could be insensitive to the action of diphthericin by either of two mechanisms, namely tolerance and resistance, and these two states can be distinguished because tolerant cells will adsorb bacteriocin, but the resistant strains will not (Bhattacharyya, Wendt, Whitney & Silver, 1970). It was thus necessary to establish whether or not the derivative strains of 201742 would adsorb diphthericin, although they were insensitive to its action.

Attempts to induce the production of large amounts of diphthericin, in broth cultures of strain FN by ultraviolet irradiation or by mitomycin C, have not been successful and so a semi-quantitative adsorption experiment was performed. Strain FN was inoculated diametrically across T.S.H.B.A. in the manner used for diphthericin typing (Gibson & Colman, 1973). However, in order to standardize the method as much as possible, 20 ml. of medium was poured into glass dishes whose internal diameter was 8·8 cm. and depth was 1·6 cm. Strain FN was applied to the agar by means of a cylindrical swab stick whose diameter and length were 2·0 mm. and 8·6 cm. respectively. The swab stick was thoroughly seeded with culture and, after excess culture was allowed to drain, the stick was placed across the agar and then removed after 30 sec. In this way, the width of the growth of strain FN was always between 5·0 and 6·0 mm. After incubation at 30° C., for 48 hr., the growth was scraped off and the remaining cells killed with chloroform vapour. An overnight broth culture of adsorbing strain was spread across half of the T.S.H.B.A. at right angles to the original diametric streak. This culture was incubated at 41·5° C., for a further 48 hr., since preliminary experiments had shown that, although diphthericin produced at 30° C. is still active at the higher temperature, its actual formation, at 41·5° C., is inhibited.

After 48 hr. at 41·5° C., the growth was removed and the remaining cells were killed as before. The entire surface of the T.S.H.B.A. was then inoculated with 0·2 ml. of an overnight broth culture of the sensitive strain 201742. After overnight growth, at 30° C., or 37° C., the width of the zone of diphthericin activity was measured with calipers and the reduction in width, between that half of the plate which had been adsorbed and the other half of the plate which had not, was regarded as a measure of the amount of adsorption.

RESULTS

The mental hospital outbreak

Forty isolates were obtained from 16 cases and 19 apparently healthy carriers one of whom was bacteriologically diagnosed after a second swabbing and another

Table 1. *Characteristics of strains of* C. diphtheriae *isolated during an outbreak of diphtheria in a Mental Hospital*

No. of isolates	Diphthericin type	Bacteriophage* Type, or sensitivity	Minor† antigens	M.D.U.‡ strain no.
7	L3	XVI	–	.
3	L3	3, 10, 925/944, 951/950	–	.
3	L3	Resistant	–	.
1	L3	11, 12, 25, 26, 28, 34, 951/950	–	200790
1	L3	11, 12, 25, 26, 28, 34	–	201637
2	L3a	Resistant	–	.
1	L3a	11, 12, 25, 26, 28, 34	–	201642
1	L3a	11, 12, 25, 26, 28, 34	McLean	201389
4	L4	Resistant	–	.
1	L4	Resistant	McLean	.
1	L4	11, 12, 25, 26, 28, 34	2, McLean	200585
10	L5	IV	–	.
1	L5	IV	McLean	.
3	L5	Resistant	–	.
1§	L5	34, 35	2, McLean	201742

* Resistant signifies that the isolates were resistant to bacteriophages numbered 1–24, inclusive, and those agents numbered 925/944, 951/936, 951/939, 951/950 and 951/956.

† The serotype (major antigen) for all strains was 6387-Greenwood.

‡ The bacteriophage-sensitivity pattern of each of these strains was provided by Dr Alice Saragea.

§ This strain produced only a transient illness in the guinea-pig, i.e., non-virulent. The other 39 isolates were virulent.

Table 2. *Characteristics of the stock producer strain* FN, *the diphthericin-sensitive strain* 201742 *and the diphthericin-insensitive derivative groups*

Strain	Reaction to diphthericin of strain FN	Diphthericin type	Bacteriophage type or sensitivity	Major antigen (serotype)	Minor antigens	Colonial* appearance	Acid from starch	Virulence
FN (producer)	Insensitive	L2	XIV	Nadjarian	–	Daisy-head	+	+
201742† (wild type)	Sensitive	L5	34, 35‡	6387-Greenwood	2, McLean	Small daisy-head	–	–
201742FN/A (derivative)	Insensitive	L3	11, 12, 25,‡ 26, 28, 34	6387-Greenwood	–	Small daisy-head	–	+
201742FN/B (derivative)	Insensitive	L2	XIV	6387-Greenwood	2, McLean	Daisy-head	+	+

* For a description of the colloquial terms see Wilson & Miles (1964).

† Isolated during the Mental Hospital outbreak.

‡ Results from Dr Alice Saragea (personal communication, 1971).

only after the third attempt. Four of the cases were aged 21, 23, 29 and 31 respectively and the ages of the remaining cases ranged from 9–18 years. Six of the cases had received the complete primary course of three injections of 'triple antigen' within the first 4 years of life (three of the six had received the primary course plus one booster injection) and ten had not been given any prophylactic inoculation (personal communication from the Hospital Authorities, 1970).

Two isolations of *C. diphtheriae* were made on different days from three of the clinical cases. The first strain from the throat swab of one patient was characterized as diphthericin type L4, bacteriophage resistant and serotype 6387-Greenwood but the second isolation, made a few days later from a second swab, was diphthericin type L3, was sensitive to bacteriophages 3, 10, 925/944 and 951/950 yet was the same serotype as the first strain. In the second patient both strains were serotype 6387-Greenwood but the first was diphthericin type L5 and bacteriophage type IV whereas the second was diphthericin type L3a and was resistant to the bacteriophages of the typing system. Both strains from the third patient were serotype 6387-Greenwood and diphthericin type L3 but the first obtained was bacteriophage resistant while the second was bacteriophage type XVI.

The characteristics of all 40 strains are presented in Table 1. All strains were serotype 6387-Greenwood but five strains cross-reacted with antiserum McLean and two of them also cross-reacted with antiserum 2. Much variation was observed in their diphthericin types and more so in their sensitivity to bacteriophages. Eighteen strains were placed into two of the well-established bacteriophage types in that seven were grouped into type XVI, being sensitive to agents 10–24 inclusive, and eleven were sensitive to bacteriophages 7, 23 and 24 (type IV). Thirteen strains were bacteriophage resistant and the remaining nine exhibited patterns of sensitivity not yet recognized as specific bacteriophage types.

The strain 201742 differed from all the others in two characteristics; it did not kill the guinea-pig in the subcutaneous virulence test and it was sensitive to the diphthericins produced by those strains classed as diphthericin type L3 or type L3a.

The derivative groups

The bacteriological characteristics of producer strain FN, sensitive parent strain 201742 and the variants derived from strain 201742 are presented in Table 2. Variants selected for their insensitivity to the diphthericin of strain FN were placed in one of two 'derivative groups'. Strains of the group designated 201742 FN/A were selected at a rate of 1 in 10^4 and differed from the parent strain 201742 in four characteristics, namely diphthericin activity, bacteriophage sensitivity, serological reaction and virulence; whereas strain 201742 was capable of producing, in the guinea-pig, some of the pathological effects of diphtheria without actually killing the animal, the virulence of the derivative group 201742FN/A appeared fully restored.

The second group 201742FN/B was selected at a rate of 1 in 15×10^4. They were similar to the parent strain in their antigenic type, including serological cross-reactions, but they differed in their ability to ferment starch, in virulence, in the

Table 3. *Adsorption of diphthericin by strains of* C. diphtheriae

Adsorbing strain	Width of the zone of diphthericin activity (mm)		% reduction in zone size
	Before adsorption	After adsorption	
201742 (wild type)	23·0–25·0	13·0–15·0*	40·0
201742FN/A (derivative)	23·0–25·0	0·0	100·0
201742FN/B (derivative)	22·0–24·0	0·0	100·0
FN (producer)	23·0–25·0	0·0	100·0

* After two adsorptions with strain 201742, the zone of diphthericin activity was almost completely eliminated.

size of colony formed on P.T.H.B.A., in bacteriophage type and in diphthericin type. In fact, their characteristics were almost identical with those of the producer strain FN, but they were distinguished by their serological reaction since the producer strain was serotype Nadjarian.

A few colonies were selected from the impregnated T.S.H.B.A. plates which, on retesting for diphthericin insensitivity, were still sensitive to the diphthericin of strain FN. It was assumed that they had arisen from cells which had survived the various procedures taking place on the plate hence they were useful in that they served as controls in the subsequent bacteriological tests since they proved to be identical, in every characteristic, with the parent strain 201742.

Adsorption of diphthericin

Table 3 shows the results of adsorption using strain 201742, derivative strains 201742FN/A and 201742FN/B and producer strain FN as the adsorbing cultures. Using the indicator strain 201742 as the adsorbing strain, the width of the zone of diphthericin activity was 13·0–15·0 mm. in the region treated with adsorbing culture compared with a width of 23·0–25·0 mm. in that half of the plate which had not been inoculated with adsorbing culture. Thus, the zone of diphthericin activity was reduced by approximately 40 %.

Using the derivative strains 201742FN/A or 201742FN/B, or the producer strain FN for adsorption, diphthericin activity was completely eliminated since no lysis of the indicator culture was observed in the region where the adsorbing strain had grown, whereas in the other half of each plate a large zone of diphthericin activity was seen in the lawn culture. It is notable that the producer strain FN adsorbs its own diphthericin.

It is likely that the indicator strain 201742 achieved no more than 40 % reduction in zone size because the only cells available to adsorb diphthericin were those cells which constituted the inoculum, whereas the derivative strains and the producer strain were able to grow and multiply in the presence of diphthericin with the result that an ever increasing number of cells was available for adsorption. In

fact, if two adsorptions with strain 201742 were performed before adding the indicator lawn culture, the zone of diphthericin activity was almost completely eliminated.

The size of the zone of diphthericin activity appears to be a constant, reproducible characteristic for the producer strain/sensitive strain combination. Thus the zone of activity between producer strain FN and sensitive strain 1281 (Gibson & Colman, 1973) is always 35·0–37·0 mm., whereas the zone size formed between producer strain 201742FN/A and sensitive strain 201742 is 9·0–10·0 mm. It is notable that the zone size obtained with strain 201742FN/B as the producer and strain 201742 is the same size as the zone produced by strain FN and strain 201742. The 1·0–2·0 mm. range in zone size for a particular combination may be a reflection of the variation in width of the growth of the producer strain described in 'Method of Adsorption'.

DISCUSSION

The findings of Gibson & Colman (1973) indicated that an association existed between the diphthericin type of *C. diphtheriae* and other bacteriological characteristics used in the identification and typing of the organism. Even in the small outbreak which occurred in a Mental Hospital, some association was observed in that those strains which were more active in their production of diphthericins were, in general, sensitive to more bacteriophages, whereas of the 21 strains, which were relatively inactive in their diphthericin activity, eight were bacteriophage-resistant, 12 were sensitive to no more than three bacteriophages and only one was sensitive to six bacteriophages.

A more obvious feature of Table 1 is the wide variation in the characteristics of the strains isolated during an outbreak which occurred in the highly restricted environment of a hospital. Nineteen strains were diphthericin type L3 or L3a and exhibited five bacteriophage-sensitivity patterns; five were diphthericin type L4 and displayed two bacteriophage-sensitivity patterns; and the fifteen diphthericin type L5 strains showed three sensitivity patterns and one of them was non-virulent. The characteristic common to all strains was their serotype; the major antigen was 6387-Greenwood but five of the strains possessed antigen McLean and, in addition, two reacted with antiserum 2. The constancy of the major antigen was again apparent in the varieties derived from the Mental Hospital strain 201742; both derivative groups were serotype 6387-Greenwood, but the group 201742FN/A had lost minor antigens 2 and McLean. This group, however, displayed other characteristics similar to some of the Mental Hospital strains. For instance, 200585 possessed the same cross-reacting antigens and was sensitive to the same six bacteriophages. Again, derivative group 201742FN/A and strains 201389 and 201642 were sensitive to these six bacteriophages, and in addition, strain 201389 cross-reacted with antiserum McLean. Finally, the strain numbered 201637 was similar, in every characteristic examined, to this derivative group, being diphthericin type L3, serotype 6387-Greenwood and exhibiting the same bacteriophage sensitivity.

Two explanations can be offered for the variation of characteristics observed in

the Mental Hospital strains. The first is that there existed in the Hospital a number of varieties of the diphtheria bacillus, each of them acting independently in producing a similar disease. The second explanation is that the different forms or varieties described represented phases of a parent strain as it passed from host to host. The 'one-parent' concept seems the more likely explanation of both the epidemiological and experimental findings. Thus (*a*) wide variation in characteristics was found in strains isolated during a small outbreak which occurred in a highly restricted environment, yet the strains were related by their serotype; (*b*) two strains, again similar in serotype but differing in other characteristics, were found in the one individual; and (*c*) diphthericin-insensitive variants produced in the laboratory differed from the parent strain in some characteristics but retained their serotype antigen.

Although the mechanism of this variation is not understood, the simplest explanation for some of the differences observed, especially in the light of the variants derived experimentally, is that the derivatives had undergone alteration in some structural component such as the cell membrane or the cell wall. Such alterations explained the changes observed in colicin-tolerant mutants of *Escherichia coli* (Holland *et al.* 1970; Onodera *et al.* 1970) and aeruginocin-tolerant mutants of *Pseudomonas aeruginosa* (Holloway *et al.* 1971). Thus, the diphthericin-insensitive variants of *C. diphtheriae* strain 201742 were able to kill guinea-pigs and were sensitive to more bacteriophages. Changes in these characteristics could be associated in some way with the surface layers of the cell; for instance, toxic activity is correlated with a 'cord-factor' found at the cell surface (Kato, 1970) and the toxin itself may be synthesized at the cell membrane (Uchida & Yoneda, 1967); again, a substance which inhibits adsorption of bacteriophage appears to be derived from a surface component of the cell (Groman & McCormick, 1961).

It would be tempting to correlate the diphthericin-insensitive groups with the classes of mutants proposed by Bhattacharyya *et al.* (1970) for *Escherichia coli*, namely bacteriocin-tolerant and bacteriocin-resistant, but resistant (non-adsorbing) variants of *C. diphtheriae* were not isolated. However, a better understanding of the nature of diphthericin-insensitivity will be obtained when stable, high-titre preparations of diphthericin have been made in order to investigate their physical and chemical nature.

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