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Numerical analysis of the characteristics of *Corynebacterium diphtheriae* strains isolated in Victoria from 1962 to 1971

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SUMMARY

A study of the incidence of diphtheria in the State of Victoria, Australia, was carried out. Numerical analysis of the characteristics of 264 strains of *Corynebacterium diphtheriae* isolated between 1962 and 1971 placed them into 18 varieties plus six strains which were unique in their combination of reactions to the characteristics examined. During the 10-year period, some varieties appeared intermittently and were recognized by certain defining characteristics but exhibited a gradual change in their antigenic structure. In contrast, when the outbreaks were examined over shorter periods of time, a number of varieties and single strains were found which differed greatly from each other yet possessed the same major serotype antigen. These findings are discussed in terms of a 'one-parent' concept in which the varieties and single strains represent phases of a common ancestor.

By inspection and analysis of the characteristics of the strains, certain associations were apparent. For instance, a correlation was found between the antigenic structure of the organism and the colonial appearance on tellurite blood agar. Similarly, correlation was observed between bacteriophage type, diphthericin type and biochemical activity in that a strain which was highly active in one of the properties was also very active in the other two.

INTRODUCTION

Grouping of *Corynebacterium diphtheriae* into *gravis*, *intermedius* and *mitis* on the basis of colonial morphology on tellurite blood agar, ability to ferment starch and severity of disease has been in use for more than 40 years (Anderson, Cooper, Happold & McLeod, 1933). It was soon obvious, however, that such correlation did not always hold true as exemplified by investigations carried out in Scotland (Christison, Wright, Shearer & Pearson, 1936), and in Australia (Anderson, Goldsworthy & Ward, 1936). Indeed, it was considered possible by mutation for a strain of the *gravis* group to give rise to strains of the *intermedius* group or *mitis* group during the process of evolution (Hewitt, 1947). In fact, mutants that displayed variation in colonial morphology, starch fermentation and virulence were described by Oeding (1950).

A significant advance in the epidemiology of diphtheria in Australia was made with the application of methods such as serological typing (Ferris, 1950), bacteriophage typing (Gibson, Cooper, Saragea & Maximescu, 1970) and, more recently, diphthericin typing (Gibson & Colman, 1973). Using all three typing techniques in the investigation of an outbreak in the restricted environment of a mental hospital, Gibson (1973) believed his results strongly supported the evolution theory during the passage of the organism from host to host.

In order to substantiate the mutation concept of the diphtheria bacillus as it passed from one individual to another, the epidemiological study of the disease was extended to include all strains of *C. diphtheriae* isolated in Victoria over a period of ten years. Numerical analyses were carried out on the characteristics of the strains in order to study the changes which occurred during the 10-year period. In addition, the numerical analyses were used to determine whether or not associations existed between the characteristics observed, since Gibson & Colman (1973) found an apparent association between diphthericin type and some of the other characteristics used to identify and type the organism.

MATERIALS AND METHODS

Source of strains. All strains of *Corynebacterium diphtheriae* were either isolated from nose and/or throat swabs submitted to the Microbiological Diagnostic Unit by medical officers of the State Health Department and by general practitioners, or received as pure cultures from hospitals throughout the State of Victoria. Those strains isolated in the early years of this study were maintained in the lyophilized state and subsequently re-examined by the current methods.

Bacteriological techniques. Methods of isolation, identification and typing of strains have been described elsewhere (Gibson *et al.* 1970; Gibson & Colman, 1973), but, for the purpose of the analysis, some results were excluded from the examination because the tests had not been applied to all strains. For instance, sensitivity to the bacteriophages, numbered 1–24, was known for all strains but all of the strains had not been tested against the more recently isolated bacteriophages, 925/944, 951/936, 951/939, 951/950 and 951/956.

Numerical analysis. The characteristics of the majority of the strains were analysed by Miss Betty Laby and Professor E. J. Williams in the Department of Statistics at the University of Melbourne and the characteristics of the strains isolated in 1969, 1970 and 1971 were analysed by the author.

RESULTS

Strains of the diphtheria bacillus exhibited variable reactions to some of the characteristics examined and these included acid from starch, acid from maltose, colonial appearance, virulence, antigenic structure, bacteriophage sensitivity and diphthericin production. For convenience, the strains were numbered, in chronological order, 1–264 and strains which were identical in all characteristics recorded, or which differed in only one of the variable characteristics, were placed into the same group. By this procedure, the 264 strains were allocated to one of 18 groups

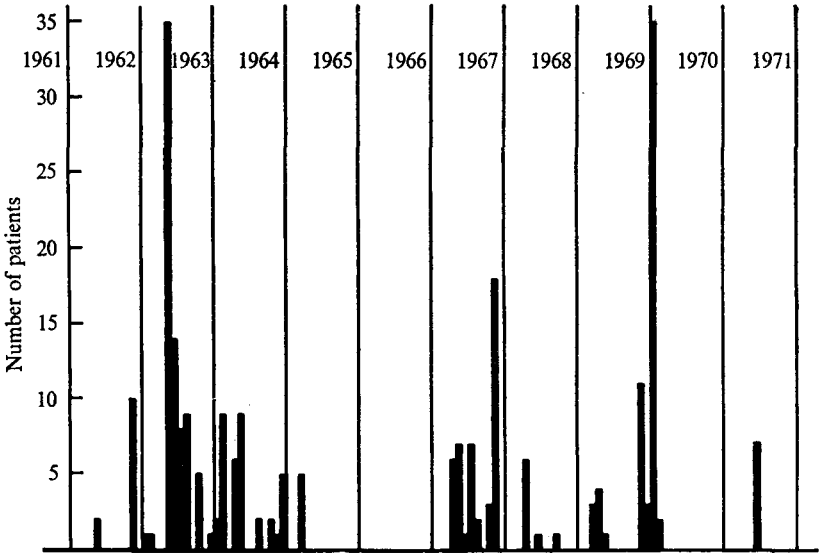

Fig. 1. Monthly incidence of diphtheria in Victoria, 1961–71.

which, for the purpose of the analysis, were designated 'varieties', together with six single strains each one of which was unique in its combination of characteristics A variety was numbered when a strain of that particular variety was first encountered, hence the varieties similarly were numbered in chronological order.

The month-by-month occurrence of diphtheria in Victoria between 1961 and 1971 is shown in Fig. 1. Isolations were made mainly during summer and/or autumn, but no outbreaks occurred and no strains were isolated in 1961, 1966 or 1972. In some instances, more than one strain was isolated from one patient whereas the strains isolated in May 1962 and in January 1963 were non-viable on reopening the ampoules, hence they were not included in the analysis. One outbreak was distinguished from another by an interval of at least one month and accordingly, in the period under consideration, 16 outbreaks of the disease were recorded. Fig. 1 clearly shows five intervals when neither clinical cases nor carriers were detected for periods greater than 4 months. Only two outbreaks are shown for 1967 but, in the early part of the year, some of the patients were found in Central and Northern Victoria and the others were found in Melbourne; contact between the two outbreaks was not established, thus it is likely that one additional outbreak would have been recorded if location had been considered in the analysis. The mental hospital strains mentioned earlier (Gibson, 1973) were isolated in January and February of 1970.

Varieties occurring in more than one outbreak between 1962 and 1971 are presented in Table 1 and the characteristics which define each variety are given. The characteristic which varies within each variety is also shown. Thus, variety 1 was characterized as diphthericin type L 2, bacteriophage type XVI, and possessed the cultural and biochemical characteristics of a 'classical' *gravis*. This variety appeared intermittently throughout the period of study and displayed a gradual change in antigenic structure from the highly specific serotype Nadjarian to auto-

more than one outbreak between 1962 *and* 1971

Variety	Year of isolation	Strains isolated	Antigenic type*	Diphthericin type	Bacteriophage type or sensitivity pattern	Colonial appearance†	Biochemical activity‡ v	m	s
1	1962	1– 9	Nadjarian	L 2	XVI	Daisy-head	+	+	+
	1963	11– 18 20– 26 30– 37 40– 52							
		77– 81	Untypable	L 2	XVI	Daisy-head	+	+	+
	1964	88– 91 93– 99	Nadjarian	L 2	XVI	Daisy-head	+	+	+
		63– 86 92 100–107 109–110	Nadjarian, 2	L 2	XVI	Daisy-head	+	+	+
	1965	121 123–125	2	L 2	XVI	Daisy-head	+	+	+
	1967	126	Nadjarian, 2	L 2	XVI	Daisy-head	+	+	+
	1969	197–201 203–208 210–212	6387–Greenwood, 2	L 2	XVI	Daisy-head	+	+	+
		202 209	6387–Greenwood	L 2	XVI	Daisy-head	+	+	+
2	1962	10	Johnson	L 5	Resistant	Poached-egg	+	–	–
	1964	113							
	1968	181 182 188	Johnson	L 4	Resistant	Poached-egg	+	–	–
3	1963	19 56– 68 71 74– 76	Nadjarian	L 5	Resistant	Daisy-head	+	–	–
		69, 70 72, 73	Nadjarian	L 5	Resistant	Daisy-head	–	–	–
	1967	157, 158 160–166	Nadjarian	L 5	Resistant	Daisy-head	+	–	–
		159	Untypable	L 5	Resistant	Daisy-head	+	–	–

Table 1 (*cont.*)

Variety	Year of isolation	Strains isolated	Antigenic type*	Diphthericin type	Bacteriophage type or sensitivity pattern	Colonial appearance†	Biochemical activity‡ v	m	s
4	1963	27– 29 38, 39 53– 55	Nadjarian	L 5	Resistant	Daisy-head	+	+	–
	1964	87							
5	1964	112 114, 115 117, 118	McLean	LU	VI	Small daisy-head	+	+	–
	1971	256§ 258–264§							
6	1964	116 119, 120	McLean	LU	Resistant	Small daisy-head	–	+	–
	1971	254	Untypable	LU	Resistant	Small daisy-head	–	+	–
		255	McLean	LU	Resistant	Small daisy-head	–	+	–
		257							
11	1967	147, 151	Untypable	L 5	Resistant	Poached-egg	–	–	–
		167–180‖	2	L 5	Resistant	Poached-egg	–	–	–
	1968	183–187¶							
	1969	189‖	2	L 5	Resistant	Poached-egg	–	+	–
		191‖	2	L 5	Resistant	Poached-egg	–	–	–
		192‖							
		194‖							
		196‖							

* Ten strains were untypable by serological methods because they were autoagglutinable in suspension. The other strains reacted with the respective antisera to a titre of $\geqslant 1/160$.

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

‡ v+ = virulence for guinea-pig; m+ = acid from maltose; s+ = acid from starch. s(±) in Tables 2 and 3 indicates that the acid reaction observed after 18 hr. incubation reverted to alkalinity after 48 hr.

§ These strains cross-reacted with antiserum 6387–Greenwood to a titre of 1/40 to 1/160.

‖ These strains cross-reacted with antiserum Johnson to a titre of 1/40 to 1/80 and with antiserum McLean to a titre of 1/80 to 1/320.

¶ Three of these strains cross-reacted with antiserum Nadjarian to a titre of 1/320 to 1/640.

Table 2. *Characteristics of the varieties of* Corynebacterium diphtheriae *which occurred in only one outbreak between* 1962 *and* 1971

Variety	Year of isolation	Strains isolated	Antigenic type*	Diphthericin type	Bacteriophage type or sensitivity pattern	Colonial Appearance	Biochemical activity		
							v	m	s
7	1967	127–129	2	L 5	Resistant	Poached-egg	+	+	−
		130, 131	2	L 5	Resistant	Poached-egg	+	−	−
8	1967	132–136	6387–Greenwood	L 1	Resistant	Poached-egg	+	+	−
		137–143	6387–Greenwood	L 1	Resistant	Poached-egg	+	−	−
		154–156	Untypable	L 1	Resistant	Poached-egg	+	−	−
9	1967	144, 145	Untypable	L 4	Resistant	Poached-egg	+	−	−
10	1967	146, 148, 149, 150, 152, 153	Untypable	L 5	11, 13, 18, 19	Poached-egg	+	−	−
12	1969	190, 193	Johnson	L 4	3, 10	Poached-egg	+	−	−
13	1970	219, 221, 232, 234, 239, 246, 253	6387–Greenwood	L 3	XVI	Small daisy-head	+	−	+
14	1970	216, 217, 235, 237, 248	6387–Greenwood	L 3	Resistant	Small daisy-head	+	−	−
		224,† 231, 240, 247	6387–Greenwood	L 3*a*	Resistant	Small daisy-head	+	−	−
15	1970	215, 243, 249	6387–Greenwood	L 3	3, 10	Small daisy-head	+	−	−
16	1970	214†	6387–Greenwood	L 4	Resistant	Small daisy-head	+	−	−
		218, 220,† 223, 245, 252	6387–Greenwood	L 4	Resistant	Small daisy-head	+	−	(±)
17	1970	222,† 225, 227, 228, 229, 230, 233, 236, 238, 242, 244	6387–Greenwood	L 5	IV	Small daisy-head	+	−	−
18	1970	226, 241, 251	6387–Greenwood	L 5	Resistant	Small daisy-head	+	−	−

* Eleven strains were untypable by serological methods because they were autoagglutinable in suspension. The other strains reacted with the respective antisera to a titre of $\geqslant 1/160$.

† These strains cross-reacted with antiserum McLean to a titre of 1/80 to 1/320. Strain 214 also reacted with antiserum 2 to a titre of 1/320. See also footnotes † and ‡ to Table 1.

agglutinable, to the doubly antigenic serotype Nadjarian/2 and, in 1965, to the highly specific serotype 2. It finally reappeared in November 1969, as the doubly antigenic serotype 6387–Greenwood/2 and the highly specific serotype 6387–Greenwood.

Variety 3 and variety 4, especially the latter, were similarly encountered throughout the period of study and, in fact, strains of these two varieties were associated with strains of variety 1 in that they were isolated from children of the same classroom at school and even isolated from children of the same family (Gibson, 1971). All strains which possessed antigen Nadjarian, especially those in varieties 1, 3 and 4, formed the daisy-head colony on tellurite agar. However, three strains of variety 11 isolated in 1968, formed the poached-egg colony, but the Nadjarian antigen was present as the minor antigen, the major one being antigen 2.

Varieties which were encountered only once between 1962 and 1971 are presented in Table 2. The serotype 6387–Greenwood strains of variety 8 were isolated from seven members of one family who lived on a remote farm in Central Victoria and the other three autoagglutinable strains of variety 8 were isolated in Northern Victoria from contacts of the family. Although the strains were resistant to bacteriophages 1–24, all were sensitive to bacteriophages 951/936, 951/939 and 951/956. Serotype 6387–Greenwood strains of varieties 13–18 inclusive were found in the mental hospital outbreak mentioned previously (Gibson, 1973) and occurred in the same outbreak as those variety 1 strains which also possessed antigen 6387–Greenwood.

Table 3 lists the six strains which individually possessed a combination of characteristics not found in any other strain isolated during the period under consideration. Strain 108 was the only member of serotype Wallis isolated during the period of study; however, four strains of this serotype, also similar in the other 'variable' characteristics, were isolated in 1960 and, in fact, this serotype was the dominant non-virulent serotype before 1960 (Gibson *et al.* 1970). Strain 111 was associated with the 1964 outbreak in which varieties 5 and 6 occurred but differed from these varieties in serotype and virulence respectively. On the two occasions when varieties 5 and 6 have been found, they have occurred in the same outbreak; in 1964 both varieties were isolated from patients who slept in the same dormitory of a boy's remand home. Again, in 1971 both varieties were isolated from young pre-school children who attended the same kindergarten.

Strain 122 was obtained from a child who lived in the same household as the other individuals infected during the outbreak of March 1965 but differed from the other strains in being sensitive only to bacteriophage 19, whereas the others were sensitive to bacteriophages 1–24 inclusive. Strain 250 was found in the mental hospital outbreak and was the only example of diphthericin type L 6. Strain 213 was the only example of diphthericin type L 1 and bacteriophage type XIV C to be isolated in Victoria; however, this combination of characteristics was found more frequently in New South Wales (Gibson & Colman, 1973).

Association between characteristics can be seen by inspection of Tables 1–3. The correlation between the daisy-head colony and Nadjarian antigen has already been mentioned, but the antigen 6387–Greenwood also seems to be associated with the

Table 3. *Characteristics of six strains of* Corynebacterium diphtheriae *isolated only once between* 1962 *and* 1971

Year of isolation	Strain number	Antigenic type*	Diphthericin type	Bacteriophage type or sensitivity pattern	Colonial appearance	Biochemical activity		
						v	m	s
1963	82	Untypable	L 5	Resistant	Daisy-head	−	+	(±)
1964	108	Wallis	L 5	13, 14, 15, 16, 17, 18	Poached-egg	−	+	−
1964	111	Edmonston	LU	Resistant	Poached-egg	+	+	−
1965	122	2	L 2	19	Daisy-head	+	+	+
1969	213	6387–Greenwood	L 1	XIVC	Daisy-head	+	+	−
1970	250	6387–Greenwood	L 6	Resistant	Daisy-head	—	—	(±)

* Strain 82 could not be serotyped because it was autoagglutinable in suspension. The other strains reacted with the respective antisera to a titre of $\geqslant 1/640$. Strains 213 and 250 cross-reacted with antiserum McLean to a titre of 1/160 and 1/80 respectively, and both reacted with antiserum 2 to a titre of 1/320.

See also footnotes † and ‡ to Table 1.

small or large daisy-head colony. However, notable exceptions were found in those strains isolated in Central Victoria during May 1967. All strains possessing major antigen Johnson formed the poached-egg colony and this correlation has been found for strains of this serotype isolated in New South Wales and South Australia (Gibson, 1971).

In general, non-virulent strains were resistant to the bacteriophages of the typing set with one exception – strain 108 being sensitive to six (Table 3) – and were relatively inactive with respect to diphthericin production, being active against only one indicator strain, hence diphthericin type L 5 (Gibson & Colman, 1973). A complete association of characteristics appears to exist in that a strain which is more active biochemically is sensitive to more bacteriophages and is more active in its production of diphthericins. The association between diphthericin activity and other characteristics has been discussed elsewhere (Gibson & Colman, 1973).

DISCUSSION

From their Greenwich Hospital School studies in 1934 Dudley, May & O'Flynn (1934) concluded that the number of variations in the group of bacteria defined as *Corynebacterium diphtheriae* was so large that most of them were temporary phases of one organism. They believed that variants were easily transformed from one phase into another and, consequently, these transformations were important in determining the morbidity and clinical type of diphtheria at different times and places. Furthermore, they thought that the *C. diphtheriae* group had a common ancestor and that virulence and other parasitic qualities exhibited short periods of existence in nature as the organism migrated from host to host.

The epidemiological findings presented would appear to substantiate this concept. The long-term study of the disease has shown that, in the case of one variety in particular, the antigens are subject to gradual replacement undergoing some form of 'antigenic drift' but, if the disease is studied over shorter periods, the antigen is the most stable of the characteristics. Thus, variety 1, which was defined by bacteriophage type, diphthericin type and biochemical/cultural type, showed a gradual change in its antigenic structure over a period of 10 years. However, in the shorter periods of time a number of varieties were encountered, two or more of which were isolated from one household and, in a few instances, two were isolated from the one individual. These varieties showed differences in some characteristics but possessed a common major antigen; for example, varieties 1, 3 and 4, which possessed antigen Nadjarian, were isolated during the same outbreaks of 1963 and 1964. Similarly, variety 1 strains, which possessed antigen 6387–Greenwood, were found during late 1969 and early 1970 in the same outbreak as varieties 13–18, also serotype 6387–Greenwood, but the latter varieties showed marked differences in bacteriophage sensitivity, diphthericin production, and fermentation of starch. Again, varieties 5 and 6 with common antigen McLean, which occurred in the same outbreaks of 1964 and 1971, showed variation in virulence and bacteriophage type; even with these two varieties some antigen drift was apparent in that, among the strains isolated in 1971, one was autoagglutinable

and the others possessed small amounts of antigen 6387–Greenwood, whereas those isolated in 1964 were highly specific.

It has been pointed out (Barksdale, 1970) that the colonial morphology of corynebacteria is dependent on the way the individual cells pile up and this, in turn, is a reflexion of the different surface antigens possessed by the cell. The results in Tables 1–3 would indicate that it is the relative proportion of antigens which determines the type of colony. For instance, strains with antigen McLean formed the small daisy-head colony, but, when antigen 2 was present, the poached-egg colony was formed. A similar correlation seems to exist between the Nadjarian antigen and antigen 2 in that the type of colony formed was dependent on the relative amounts of the respective antigens. However, five strains possessing antigen 2 were able to form the daisy-head colony; in the agglutination system employed, a 'background level' of reaction (titre of 1/20 or less) was usually observed and it may be that antigens present even at this low titre were able to influence the formation of the colony. This may explain why the strains of serotype 6387–Greenwood, isolated in 1967, formed the poached-egg colony.

It would appear that as an outbreak is prolonged, for example in 1963/4 and in 1969/70, an ever-increasing proportion of strains will be found which show decreased biochemical activity, increased resistance to bacteriophage and decreased diphthericin production. A similar conclusion was reached by Liebow, MacLean, Bumstead & Welt (1946) with respect to the property of virulence in that the continued existence of *C. diphtheriae* in the diphtheritic ulcer led to the formation of non-virulent variants, and Gunatillake & Taylor (1968) pointed out that, before the introduction of antibiotic therapy, it was possible to isolate from convalescent patients toxigenic and non-toxigenic strains which belonged to the same serological type. However, they considered it possible that the non-toxigenic strains were not descendants of the toxigenic forms, since the non-toxigenic strains displayed a bacteriophage sensitivity pattern which was different from that of the virulent organisms. In view of the results presented for strains isolated in Victoria, this apparent contradiction was probably a function of the relative stability of the typing systems in that the sensitivity to bacteriophages altered more dramatically than the serotyping antigens. The mechanisms by which these changes are brought about in nature are not entirely understood but, in the laboratory, lysogenic conversion by bacteriophage has brought about antigenic modification (Barber, Meitert, Saragea & Podhorski, 1966), a change in colonial morphology (Barksdale, Garmise & Rivera, 1961) and conversion from non-virulence to virulence simultaneously with a change in bacteriophage type (Meitert, Saragea & Bica-Popii, 1969); furthermore, a second mechanism, involving diphthericins, may bring about changes in some characteristics (Gibson, 1973).

Certainly, the epidemiology of diphtheria is a complex problem involving many interacting factors and the results have emphasized that three or four identifying and typing systems must be employed if a true picture of the disease is to be obtained. It would be very interesting to know what happens to the organism when the disease is not reported for intervals as long as 18 months (Fig. 1). The organism may exist at a very low level of incidence in the community; also, present methods

of primary culture may not be sensitive enough for isolation from the individual if only a few bacilli are present in the throat. It was at one time believed that certain domestic animals were concerned in the spread of diphtheria but much doubt has been cast on the evidence to support this (Wilson & Miles, 1964*b*); thirdly, the organism may exist in another form which is easily isolated and identified but to which little significance is attached with respect to diphtheria. Such a form could be *Corynebacterium belfanti* since lysogenic conversion from this organism to *C. diphtheriae* has been carried out in the laboratory (Gundersen & Henriksen, 1959); unfortunately, the incidence of the former species in the community has not been reported.

Further study of diphtheria in skin ulcers may help to solve some of these problems, since this form of the disease appears to be more prevalent in tropical countries (Bezjak & Farsey, 1970). Examination of the various characteristics of the organism in skin ulcers of individuals who are unlikely to be treated with antibiotics or immunoprophylactic injections would assist greatly in the understanding of the variation of *C. diphtheriae* during its prolonged association with the individual and during its continued existence in the herd.

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