The role of adherence in determining the site of infection by *Corynebacterium diphtheriae*

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SUMMARY

Twenty-nine strains of *Corynebacterium diphtheriae* isolated from throats and 29 strains from skin lesions, the latter mainly from communities of low socio-economic status in tropics and cold climates, have been examined for the property of adherence to human buccal epithelial cells. All throat strains showed adherence. In contrast, strains from skin lesions were predominantly poor adherers. These results indicate that strains of *C. diphtheriae* from throats must now be added to the important group of pathogens which possess the property of adherence to surface epithelial cells of mucous membranes, thus providing an essential first step in the process of colonizing their hosts. The possible role of this phenomenon of adherence to bucco-pharyngeal epithelial cells in the evolution of the host-parasite relationship of *C. diphtheriae* is discussed.

INTRODUCTION

In western 'developed' countries prior to the widespread use of prophylactic immunization with diphtheria toxoid, the diphtheria bacillus was responsible for one of the most serious and frequently fatal infectious diseases, especially of children. The mucosa of the nasopharynx is the usual site of infection producing the characteristic pseudomembrane. In addition to such clinical cases, *Corynebacterium diphtheriae* has also been shown to be present for long periods in the throats of symptomless carriers who can be the source of fresh outbreaks of the disease. Infection of the skin is infrequent and then usually appears to be the result of secondary infection of wounds of the skin by *C. diphtheriae* derived from the primary lesion in the throat.

Detailed studies on public health problems of isolated communities of various ethnic groups having low socio-economic standards and little contact with western people developed more slowly but have revealed that in many such communities classical diphtheria of the throat does not occur, nor is *C. diphtheriae* demonstrable in throat swabs but is present in chronic ulcers of the skin, especially of arms and legs of children. Schick tests reveal that the great majority of children and adults have significant levels of circulating diphtheria antitoxin. A growing number of reports indicate that this pattern of skin infection by *C. diphtheriae* with little or no evidence of clinical illness is widely prevalent throughout both wet and dry

tropical regions e.g. South Sea Islands (Liebow *et al.* 1946; Bacon & Marples, 1955; Marples & Bacon 1956; Markham & Stenhouse, 1959), New Zealand Maoris (McCarthy & Marples, 1954), Burma (Livingood, Perry & Forrester, 1946; Thaung *et al.* 1978), India (Ayyagari, Venugopalan & Ray, 1977), Colombia (Bennett, 1967), Trinidad (Bray *et al.* 1972), Uganda (Bezjak & Farsey, 1970). Reports (Dixon & Thorsteinson, 1969; Jellard, 1972, 1978) have revealed that a similar condition occurs amongst Eskimos, North American Indians and Metis in Northern Canada, and the question arises whether low socio-economic conditions and standards of personal hygiene rather than climate are the more important predisposing factors.

Where frequent and close contact and varying degrees of integration have taken place between 'developed' and more primitive ethnic races or socio-economically depressed groups, a mixed pattern of incidence of throat and skin lesions has been observed. Recent examples have been reported by Koopman & Campbell (1975), Belsey & LeBlanc (1975) and Pedersen *et al.* (1977).

The possibility that the property of specific adherence might be involved in determining the site of establishment of initial infection by *C. diphtheriae* and provide an explanation of the sharply contrasting clinical picture seen in advanced and more primitive communities led us to test and compare two groups of strains of *C. diphtheriae*, the first isolated from throats and the second from skin lesions, for the presence of the property of adherence to buccal epithelial cells.

The phenomenon of adherence has been extensively studied in recent years and has been comprehensively reviewed by Beachey (Beachey, 1980; 1981) and in the Ciba Symposium no. 80 (Ciba Symposium 1980). Specific adherence is mediated by complementary molecules, *adhesins*, on the bacteria which react with receptors on the surface of the epithelial cells. Adhesins are lectin-like molecules forming fimbriae or pili that bind to sugar residues on the receptors on the epithelial cells. It has been shown that such piliation leading to specific adherence may be transmitted by plasmids (Saunders, 1981; Shipley, Gyles & Falkow, 1978).

MATERIALS AND METHODS

Bacterial strains: We have examined 29 strains of *C. diphtheriae* isolated from throats (19 from patients with throat lesions, 10 from asymptomatic carriers) from the U.K. (24), Romania (4) and Canada (1). These strains included the following biotypes – 12 *gravis* (7 tox⁺, 5 tox⁻), 15 *mitis* (7 tox⁺, 8 tox⁻), 2 undetermined (see Table 1 for further details). Also examined were 29 strains from skin lesions (13 adults and 16 children); 12 strains from Trinidad, 2 from Colombia, 11 from Northern Canada and 4 from the U.K.. These comprised 7 *gravis* biotypes (all tox⁻) and 22 *mitis* (11 tox⁺, 11 tox⁻) (see Table 2). Biotype and toxinogenic status were determined by donors from recognized diagnostic laboratories. Stock cultures were maintained freeze-dried and subcultured on tryptic digest agar slopes as required. Bacterial suspensions were prepared from log phase cultures on digest agar slopes, washed off with phosphate buffered saline (P.B.S); transferred to a sterile bottle containing glass beads; shaken for 3 min in a Mickel high speed shaker and diluted with P.B.S. until bacterial density was approximately 7.5×10^8 bacteria/ml.

Buccal epithelial cells were obtained by lightly scraping the buccal surfaces of

both cheeks with a sterile wooden depressor, suspended in P.B.S. and shaken for 3 min in a Mickle high speed shaker to break up clumps. Cells were then freed from unattached oral bacteria by two successive washings in sterile P.B.S. Cell density was measured with a Neubauer haemocytometer and finally adjusted to 2×10^5 cells per ml.

Bacterial adherence was determined by a testing system similar to that of Gibbons and van Houte, 1971; 1 ml of bacterial suspension diluted from stock suspension with P.B.S. to give a final bacterial/cell ratio of 500/1 was added to 1 ml of cell suspension. A control of 1 ml of cell suspension and 1 ml of P.B.S. was included. Mixtures were incubated at 37 °C for 30 min while being rotated at 60 rev/min attached to the perimeter of a sloped circular disc 32 cm in diameter; centrifuged at 300 g for 5 min, the supernatant was removed and cells resuspended in 0.5 ml of P.B.S. Films were made on microscope slides marked with 3 squares of 2 cm², one control without bacteria and 2 test films for each bacterial strain, and stained by Gram's method. Counting was by light microscopy with $\times 1000$ oil immersion objective, the number of bacteria adhering to each of the first 50 intact and well-stained cells was recorded. Statistical analysis (see below) indicated that this was an adequate sample provided that cells were not badly damaged or poorly stained. The *adherence value* for each strain was calculated by taking the number of bacteria attached to 50 cells, subtracting the number of bacteria attached to control cells (representing the adherent indigenous flora), and dividing by 50 to give the average number of adherent bacteria per cell. Counting in each series was done by the same person, (K.A.S.) for throat strains, (S.J.D.) for cutaneous strains, and was performed 'blind', i.e. without knowledge of strain identity.

General adherence level: three classes were arbitrarily defined as:

'poor' < 5 bacteria per cell (mean value),

'moderate' 5–20 bacteria per cell (mean value),

'good' > 20 bacteria per cell (mean value).

Statistical analysis

Size of cell sample examined for adherence. In order to choose a suitable size of cell sample, results from a series of experiments using different strains of *C. diphtheriae* and one cell source were analysed when adherence to 10, 20, 30, 40 and 50 cells was counted. Consistently significant differences in levels of adherence of strains occurred with 40 and 50 cells, and hence 50 cells was selected as an appropriate sample.

Repeatability. Repeated tests using cells from a single donor (S.J.D.) showed that the general levels of adherence, i.e. 'poor', 'moderate' and 'good', remained approximately constant over a 4 month period. However chi (χ^2) *p* values showed that significant fluctuation in the numbers of adherent bacteria did occur over time (Table 1). Fluctuations were most marked for strains showing 'poor' adherence.

Effect of cells from different donors (Table 2). Differences in the level of adherence occurred between strains when tested with cells from each of four donors and these differences were found to be statistically significant, i.e. variation in adherence between strains was significant. However, although the same general trends of

Table 1. *Variation in adherence levels of C. diphtheriae strains with repetition of adherence experiments*

(Cells used were successive harvest taken at intervals from one donor (S.J.D.) over a 5 month period.)

Strain number	Adherence value*							Chisquared test (χ^2) p values†
	1 day	10 days	3 mths	4 days	8 days	4 weeks		
35	43.1	52.8	63.1	—	—	—	—	0.1-0.2
40	—	—	—	—	5.4	0.4	2.3	0.05-0.1
41	—	—	—	1.0	2.4	1.5	—	0.5-0.8
42	—	—	—	0.7	4.0	2.8	—	0.2-0.5
44	—	—	—	0.7	3.7	3.1	—	0.2-0.5
58	1.3	5.8	10.1	—	—	0.1	0.5	< 0.001
56	58.9	36.6	43.9	—	—	27.5	12.9	< 0.0001
C7 _s (-)	12.9	8.2	8.4	—	—	11.3	—	0.5-0.8
Date of adherence test	13/10 1977	14/10 1977	24/10 1977	27/1 1978	31/1 1978	8/2 1978	8/3 1978	

* Adherence value = number of bacteria attached to 50 cells, less the number of indigenous buccal bacteria attached to control cells, divided by 50.

† 'p' values represent probability that no significant differences occur in adherence levels for successive tests ('p' > 0.95 indicates 5% confidence level).

Table 2. *Variation in adherence of C. diphtheriae strains with different cell donors*

Cell source	Control‡	Strain adherence†			
		35	58	56	C7 _s (-)
1	0.3	63.1	10.1	43.9	8.4
2	27.9	27.5*	18.5*	66.3*	15.0*
3	27.1	44.1	7.9	Not done	Not done
4	12.1	34.2	2.2	Not done	Not done

* Asterisked values: control values not subtracted from experimental values.

† Unless otherwise denoted with asterisk, values for adherence are average number of bacteria per cell based on a 50 cell sample with control values subtracted.

‡ Control values: represent average number of adherent indigenous oral flora per cell.

adherence level were observed for cells from each donor, there was statistically significant variation according to cell donor, i.e. variation in cell source was significant. When both bacterial strain and cell donor were considered together, it was found that there was no consistent variation between degrees of adherence of the bacterial strains from cell donor to cell donor, i.e. strain/cell source interaction was not significant.

The results of these preliminary observations formed the basis of the design of the experiments carried out by S.J.D. in 1977-78 (with strains 30-58) and by K.A.S. in 1980-81 (strains 1-29). To eliminate the possibility of observer bias, K.A.S. carried out 'blind' adherence tests on three of the strains of *C. diphtheriae* isolated from skin lesions from the series examined by S.J.D. These strains were

Table 3. Comparison of adherence tests carried out on 'good', 'moderate' and 'poor' adherence strains by S.T.D. in 1977 and K.A.S. in 1981

Strain no.	Biotype	Adherence value*	
		S.J.D.	K.A.S.
35	<i>Gravis</i>	53.0	55.32
43	<i>Mitis</i>	10.7	11.74
47	<i>Mitis</i>	1.4	11.3

* Adherence value = number of bacteria attached to 50 cells, less the number of indigenous buccal bacteria attached to control cells, divided by 50.

chosen as representative of each of the three adherence categories as observed by S.J.D. The results are given in Table 3 and show that the specific adherence values are very similar to those obtained by S.J.D. for these organisms.

RESULTS

The results of adherence tests with 29 throat strains are given in Table 4 and with 29 skin strains in Table 5.

The significant feature is that all throat strains showed adherence, 27/29 (93 %) being good adherers, 2/29 (7 %) moderate adherers and there were no non-adherers. In contrast, strains from skin lesions were predominantly poor adherers (less than a mean value of 5 bacteria per cell); 20/29 (69 %) were poor, 6/29 (21 %) moderate, and 3/29 (10 %) good adherers. It was not possible to ascertain the source of infection of these good adherent skin strains; it is possible that they may have been derived from throat strains brought into these communities by carriers from developed countries. Correlation of adherence with biotype could not be satisfactorily studied because of the unequal numbers of different biotypes, but the mean adherence values of *gravis* strains tended to be higher than those of *mitis* strains. The presence or absence of toxin production did not have any significant influence on adherence level.

DISCUSSION

These results indicate that strains of *C. diphtheriae* from throats must now be added to the group of important pathogens e.g. enteropathogenic strains of *Escherichia coli*, and *Neisseria gonorrhoeae* which possess the property of adherence to surface epithelial cells of mucous membranes thus providing an essential first step in the process of colonizing their hosts (reviewed by Beachey, 1981). We have not determined the detailed mechanism of the adherence shown by our throat strains of *C. diphtheriae* to buccal epithelial cells but assume that it is probably similar to that responsible for adherence of pilated *Corynebacterium renale* which causes an ascending infection of the urinary tract in cattle (Takai, Yamagawa & Kitamura, 1980) and in mice (Honda & Yamagawa, 1978).

A brief consideration of the probable evolutionary history of the host-parasite relationship between *C. diphtheriae* and man suggests an explanation of the sharply

Table 4. *Adherence properties of strains of C. diphtheriae isolated from throats*

Strain no.	Toxino-genesis	Isolated from	Adherence value†	Culture from:	
Biotype: <i>Gravis</i>					
1	+	Throat lesion	48	PHLS Swansea, U.K.	1977
2	+	Throat lesion	42.4	PHLS Swansea, U.K.	1975
3	+	Asymptomatic carrier	72.7	PHLS Swansea, U.K.	1975
4	+	Asymptomatic carrier	47.7*	Romania	1962
5	+	Asymptomatic carrier	37.4	Romania	1963
6	+	Asymptomatic carrier	80.66	Romania	1962
7	+	Throat lesions	68.46	Romania	
8	—	Throat lesions	72.55	PHLS Swansea, U.K.	1980
9	—	Throat lesions	74	PHLS Swansea, U.K.	1980
10	—	Asymptomatic carrier	65	PHLS Swansea, U.K.	1980
11	—	Asymptomatic carrier	76.8	PHLS Swansea, U.K.	1980
12	—	Throat lesion	82.7*	Edmonton, Canada	1967
Biotype: <i>Mitis</i>					
13	+	Asymptomatic carrier	25.3*	PHLS Swansea, U.K.	1980
14	+	Throat lesion	31	PHLS Swansea, U.K.	1980
15	+		42.62	PHLS Swansea, U.K.	1980
16	+		36.7*	Colchester, U.K.	1970
17	+		16	Colchester, U.K.	1970
18	+		22	Colchester, U.K.	1970
19	+		32.5	Manchester, U.K.	1980
20	—		13 *	Colchester, U.K.	1970
21	—		38.5*	PHLS Cambridge, U.K.	1971
22	—		40.6*	Manchester, U.K.	1980
23	—	Carrier	32	PHLS Swansea, U.K.	1980
24	—	Throat lesion	68.9	PHLS Swansea, U.K.	1980
25	—	Carrier	48.9	PHLS Swansea, U.K.	1980
26	—	Carrier	30.7	PHLS Swansea, U.K.	1980
27	—	Throat lesion	67.5	PHLS Swansea, U.K.	1980
28	Undetermined	Throat	65	Manchester, U.K.	1970
29	Undetermined	Tonsil	67.5	Manchester, U.K.	1970

* Average of 2 or more counts.

† Adherence value = number of bacteria attached to 50 cells, less the number of indigenous buccal bacteria attached to control cells, divided by 50.

PHLS = Public Health Laboratory Service.

contrasting clinical picture of infections by *C. diphtheriae* seen in developed and less advanced countries. It seems probable that *C. diphtheriae* evolved from purely saprophytic soil corynebacteria which obviously were closely associated with evolving mammals including man. Minor wounds of the skin would have provided a rich nutritive environment favouring the growth of such organisms. *C. diphtheriae* has been observed to persist in such wounds over many months, many strains being non-toxinogenic; but the host-parasite relationship becomes complicated by the presence of various other types of micro-organisms. We have found, however, that many of such strains of *C. diphtheriae* from skin ulcers will persist for weeks as pure cultures in the tissue fluid in subcutaneous chambers in guinea pigs. Evidence is accumulating that the diphtheria bacillus may acquire a property of 'infectivity' involving the capacity of the micro-organism to establish a primary footing and

Table 5. *Adherence properties of strains of C. diphtheriae isolated from skin lesions*

Strain no.	Toxino- genesis	Isolated from	Adherence value†	Culture from:	
Biotype: <i>gravis</i>					
30	—	Finger, adult	6.0	PHLS Cambridge, U.K.	1973
31	—	Skin of ear, adult	8.3	PHLS Cambridge, U.K.	1973
32	—	Skin of foot, adult	29.9	Edmonton, Canada	1972
33	—	Skin of foot, adult	11.9	Edmonton, Canada	1973
34	—	Skin lesion, adult	19.1	Ft. McMurray, Canada	1973
35	—	Skin lesion, adult	53.0*	PHLS Cambridge, U.K.	1973
36	—	Skin lesion, adult	13.0	PHLS Cambridge, U.K.	1973
Biotype: <i>mitis</i>					
37	+	Skin lesion, child	2.5	Buena ventura, Colombia	1966
38	+	Skin lesion, child	0.0	Buena ventura, Colombia	1965
39	—	Scalp, child	0.2*	Ponoka, Canada	1970
40	—	Skin of face, adult	2.7*	Alberta, Canada	1972
41	—	Skin of finger, adult	1.6*	North West Territory, Canada	1972
42	—	Skin lesion, adult	2.5*	Frog Lake, Nth. Canada	1972
43	—	Skin lesion, adult	10.7	Boyle, Nth. Canada	1972
44	—	Skin of hand, adult	2.5*	Ponoka, Nth. Canada	1973
45	—	Skin lesion, adult	0.1	Edmonton, Canada	1967
46	—	Skin lesion, child	0.1	Trinidad	1969
47	—		1.4		1969
48	+		1.3		1969
49	+		0.0		1969
50	—		1.9		1969
51	—		2.2		1969
52	+		0.7*		1969
53	+		0.0		1969
54	+		0.4*		1969
55	+		3.3*		1969
56	+		36.0*		1970
57	+		0.9		1969
Biotype: <i>intermedius</i>					
58	+	Skin lesion, child	3.6*	Ft. McMurray, Canada	1970

* Average of 2 or more experiments.

† Adherence value = number of bacteria attached to 50 cells, less the number of indigenous buccal bacteria attached to control cells, divided by 50.

PHLS, Public Health Laboratory Service.

multiply in the living tissues of its host in spite of the normal cellular and humoral defence mechanisms (Barksdale, Garmise & Rivera, 1960; Barksdale, 1970). Our experimental evidence supporting this will be reported in a future communication. Further modification of some strains may then have occurred by the chance acquisition of two other significant properties: firstly, toxin production resulting from infection by a bacteriophage carrying the gene which codes for the characteristic diphtheria toxin protein. The amount of toxin produced by different strains varies between wide limits, but strains producing only small to moderate amounts would be favoured as colonizers of skin wounds in man as there would be selective pressure against highly toxigenic strains which killed their hosts. Infection of

skin wounds during early childhood by such moderately toxinogenic strains stimulates production of circulating diphtheria antitoxin which protects against severe intoxication following chance infection of wounds by highly toxinogenic strains. This would represent the host-parasite relationship in primitive human communities and still existing at present in the skin infections which are widespread amongst many peoples of low socio-economic status with poor standards of personal hygiene. In communities with higher socio-economic level and better personal hygiene, children's limbs are usually more protected from skin wounds by clothing and wounds are dressed with antiseptics or (now) antibiotics, thus preventing the establishment of chronic skin infection with *C. diphtheriae*.

Secondly, some strains of *C. diphtheriae* may acquire the property of adherence to epithelial cells of the fauces, possibly by plasmid transmission as occurs in *Corynebacterium renale*. Such adherence enables initial colonization of the fauces by strains which may or may not be toxinogenic. In developed countries, individuals and especially children, who have not received the antigenic stimulus of toxin from chronic diphtheritic ulcers of the skin leading to the production of circulating diphtheria antitoxin, nor have been prophylactically immunized with diphtheria toxoid, will be fully susceptible to faucial diphtheria with accompanying dangerous intoxication caused by adherent highly toxinogenic strains.

If the foregoing represents the true evolutionary history of the host-parasite relationship between *C. diphtheriae* and its human hosts as they evolved from primitive social groups towards modern 'developed' civilizations, the present widespread incidence in undeveloped countries of skin ulcers caused by *C. diphtheriae* with little disturbance of general health, should be regarded as a tolerated *modus vivendi* between host and parasite leading to spontaneous immunization against diphtheria toxin, and such skin ulcers constitute the reservoir of infection in these communities. However, it has been observed that, in developing countries in whose rural populations diphtheritic ulcers of the skin are the norm, as urbanization increases with progressive adoption of higher standards of living and better personal hygiene, but before prophylactic immunization with diphtheria toxoid has become general, typical acute faucial diphtheria may appear amongst the urban population. It would seem likely that two factors may be responsible, namely, improved standards of living and personal hygiene which reduce the incidence of skin ulcers and thus prevent the process of natural immunization against diphtheria toxin, and secondly, increasing contact with people from developed countries amongst whom are symptomless carriers who may introduce good adherent throat strains of *C. diphtheriae*. With this disturbance of the normal host-parasite relationship found in more primitive communities, there would be a selection pressure in favour of strains of *C. diphtheriae* possessing properties of specific adherence to the faucial epithelium. The spread of such strains would be favoured in highly susceptible populations, especially when aggregated in schools, etc. It is noteworthy that toxinogenic *C. diphtheriae* may be carried for long periods in the throats of clinically healthy individuals in western communities who have been fully immunized with diphtheria toxoid and can act as the sources of clinical diphtheria in young infants prior to immunization and older persons who have not been effectively immunized by prophylactic inoculation with diphtheria toxoid (Simmons *et al.* 1980).

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