The study of agglutination of trypsin-treated sheep red cells by *Corynebacterium diphtheriae* strains

N.N. Kostyukova a, S.R. Karas b and D.Ya. Kacimova b

*The Gamaleya Institute of Epidemiology and Microbiology of Academy of Medical Sciences of Russia, Moscow, Russia, and* Research Institute of Epidemiology, Hygiene and Occupational Diseases, Baku, Azerbaijan

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1. SUMMARY

620 *Corynebacterium diphtheriae* strains from 472 sick and healthy persons were studied for their adhesive activity (AA) in direct agglutination of trypsin-treated sheep erythrocytes. Toxigenic strains had more active AA than non-toxigenic ones which was not dependent on the presence of toxin in the culture. Neither biotype nor serotype of the strains correlated with their AA. Several lysotypes among toxigenic and non-toxigenic strains were more active than others. Toxigenic strains from patients had higher AA than those from carriers. Both toxigenic and non-toxigenic strains isolated from the prolonged carriers possessed the highest AA. It was concluded that AA measured in this way was an important colonization factor for all diphtheria strains and a pathogenicity factor for toxigenic strains.

2. INTRODUCTION

Colonization of epithelium cells by *Corynebacterium diphtheriae* has not previously been investigated. The first step in diphtheria infection is the colonization of the throat mucosa. Colonization of epithelium surfaces by many bacteria has been shown to depend on the process of adhesion. Deacock et al. [1] studied the adhesion of 58 strains of *C. diphtheriae* to human buccal epithelium cells and showed that there were no significant differences between adhesive activity (AA) of strains with different biological properties, but strains isolated from the skin lesions were less active than those from the throat. Yanagawa and Honda [2] described fimbriae in *C. diphtheriae* and other *Corynebacteria* which might be responsible for adhesion onto cell surfaces. Later, Kostyukova and Pereversev [3] failed to confirm these findings.

It has been suggested [4,5] that trypsin-treated sheep erythrocytes might be used to study the adhesive property of *C. diphtheriae*. The aim of the present study was the investigation of AA of
different strains of *C. diphtheriae* isolated from various sources and having different biological properties, using this method.

3. MATERIALS AND METHODS

3.1. Strains and cultures

During the years 1970–1980, 602 strains of *C. diphtheriae* from 472 people in 30 families and communities were isolated and investigated. 47 had throat diphtheria, 5 had cutaneous diphtheria and 420 were carriers with or without throat inflammation. Some strains were isolated repeatedly from the same person during illness.

All strains were investigated for their toxin formation and biochemical type. 381 strains were lysotyped according to the Romanian lysotyping schemes \[6,7\]. 354 strains were investigated for their serotype according to the scheme of serological classification devised by Robinson and Peeney [8]. Two genetically related strains, N. Groman’s C₄ tox + and C₄ tox −, were used (obtained from Professor S.B. Henrikes, Sao Paulo, Brazil).

Every culture was investigated immediately after isolation or after being revived from storage in the lyophilized state; strains were cultured in Hottinger meat digest agar (1.5 g NH₃ per litre), with 10% of normal horse serum; cultures were grown for 22–24 h at 37°C.

3.2. Trypsinised sheep erythrocytes

Fresh sheep erythrocytes (0.5% suspension) were added to 0.1% trypsin (SPOFA) in PBS, pH 7.3. The mixture was shaken and incubated at 37°C for 30 min, washed three times in PBS and resuspended in PBS to a final concentration of 0.5%.

3.3. Direct hemagglutination (DHA) test

After incubation as described earlier, cultures were plated onto agar slant tubes and after 22–24 h incubation at 37°C the suspension was prepared, at a density corresponding to 10⁹ cells per ml (according to an Enterobacteria density standard).

Microtiter plates with U-wells were used; 0.05 ml of serial 2-fold dilutions of the initial culture suspension in PBS were inserted into each well of the range. Control wells contained 0.05 ml of PBS only. Then 0.05 ml of 0.5% erythrocyte suspension was added to each well. The plates were stored at 37°C for 2 h and left overnight at 4°C. A positive test was recorded as an agglutination of at least 50% of erythrocytes. The AA of the culture was the reciprocal of the highest dilution which provided a positive result. To compare different groups of tests, geometric mean titers (GMT) were calculated.

4. RESULTS AND DISCUSSION

The study revealed that the DHA of *C. diphtheriae* was mannose independent, as was previously shown for other *Corynebacteria* [2].

The sensitivity of DHA to heating was studied. Thus, heating the cultures at 60°C for 30 min was without effect on DHA, but it decreased rapidly at 90°C and disappeared after boiling the culture for 1 s.

All strains investigated caused DHA but the degree of AA varied. Toxigenic strains were more active than non-toxigenic; GMT of the former was 54.25 (range 32–128) while of the latter only 6.77 (range 2–64) (P < 0.001). The DHA test of two genetically related strains C₄ tox + and C₄ tox − revealed no differences in their AA. Both strains had a DHA titer 32, suggesting that diphtheria toxin production is not responsible for the elevated AA of the toxigenic strains. No difference between strains belonging to different biovars (gravis or mitis) was noticed.

Most of the toxigenic strains belonged to serotype 2; the others were non-typable. Among non-toxigenic strains only 6.1% were typable and belonged to the serotypes 3, 5 and 6. Most toxigenic strains also belonged to the lysotype IX (59.3%) and XVI (29.6%); the others (16.1%) were non-lysable by the set of 20 phages. The strains of lysotype IX were significantly more active (GMT = 69.80) than those of the lysotype XVI (GMT = 54.37), but the non-lysable, five strains, were the most active (GMT = 128).

Among non-toxigenic strains 55% were lysable and belonged to the following lysotypes: J (23.0%),
Table 1

Adhesive activity (AA) of toxigenic diphtheria strains isolated from different sources

<table>
<thead>
<tr>
<th>Source of the strain</th>
<th>Number of strains *</th>
<th>Number of strains with AA-titers</th>
<th>GMT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 2 4 8 16 32 64 128 256</td>
<td></td>
</tr>
<tr>
<td>Patients</td>
<td>47</td>
<td>0 0 0 0 0 0 5 40 2</td>
<td>122.4</td>
</tr>
<tr>
<td>(100%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carriers:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>healthy</td>
<td>55</td>
<td>0 0 0 0 0 0 46 9 0</td>
<td>34.1</td>
</tr>
<tr>
<td>(100%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>with acute tonsillitis</td>
<td>38</td>
<td>0 2 0 0 0 0 34 2 0</td>
<td>28.7</td>
</tr>
<tr>
<td>(100%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>with chronic tonsillitis</td>
<td>32</td>
<td>0 0 0 0 0 0 32 0 0</td>
<td>32</td>
</tr>
<tr>
<td>(100%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* One strain.

G (20.8%), L and I (10.2%). Lysotype J was more adhesive (GMT = 9.60) than the other lysotypes (GMT = 5.3, P < 0.05) and the non-lysable strains (GMT = 6.63, P < 0.05).

The varying AA of different lysotypes of *C. diphtheriae* suggests that the adhesin of some lysotypes may serve also as a receptor for the appropriate phage, as occurs in *Klebsiella pneumoniae* [9].

The AA of a strain was found to depend upon its origin. Thus toxigenic strains isolated from patients had a higher AA (GMT = 122.46) than those isolated from carriers (GMT = 34.1; P < 0.01). The AA of carrier strains were similar whether the throat was normal or inflamed (Table 1). This indicates that AA of toxigenic diphtheria strains is not responsible for the inflammation. Difference in AA among toxigenic strains isolated from patients and carriers allows speculation that this property is an important pathogenicity factor for *C. diphtheriae*. Thus disease in a susceptible person requires not only production of diphtheria toxin, but also AA. There may be an increase in AA of bacteria resting in a toxic focus, but this has not been directly investigated.

Previously it was shown that toxigenic strains with a similar toxigenic activity were isolated both from patients and healthy carriers [10]. Dudley et al. [11] reported isolation of toxigenic strains from Shick-positive, but healthy, persons and considered this phenomenon as 'paradoxical' since they did not develop diphtheria. We also observed ten such paradoxical cases, when highly toxigenic strains were isolated from susceptible, but healthy, children. This might be due to infection by strains of moderate AA, resulting in a short period of carriage rather than disease.

Table 2

Carrier duration and adhesive activity (AA) of the diphtheria isolates

<table>
<thead>
<tr>
<th>Toxigenicity</th>
<th>Carrier duration</th>
<th>Number of strains</th>
<th>Percent of strains with AA titers:</th>
<th>GMT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 2 4 8 16 32 64</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>short</td>
<td>52</td>
<td>0 3.8 0 0 0 0 96.2 0</td>
<td>28.8</td>
</tr>
<tr>
<td></td>
<td>middle</td>
<td>25</td>
<td>0 0 0 0 0 0 96.0 4.0</td>
<td>33.0</td>
</tr>
<tr>
<td></td>
<td>long</td>
<td>48</td>
<td>0 0 0 0 0 0 12.5 87.5</td>
<td>58.3</td>
</tr>
<tr>
<td>-</td>
<td>short</td>
<td>116</td>
<td>12.1 19.8 6.9 44.8 15.5 0.9 0</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>middle</td>
<td>36</td>
<td>0 8.3 22.0 5.6 55.6 8.3 0</td>
<td>10.1</td>
</tr>
<tr>
<td></td>
<td>long</td>
<td>31</td>
<td>0 0 6.5 0 0 93.5 0</td>
<td>53.5</td>
</tr>
</tbody>
</table>


we confirmed previous work with buccal epithelium cells [1] that cutaneous strains (1 toxigenic and 4 non-toxigenic) are weakly adhesive (titers 4–8).

The possible pathogenic role of AA was demonstrated when we compared the AA titers of diphtheria strains isolated from carriers with different duration of carriage. All carriers were classified in three groups according to carriage duration: (i) short carriage (cultured only once); (ii) medium duration carriage (no longer than 4 weeks); and (iii) long lasting carriage (over a month, average 2–3 months). Table 2 demonstrates that AA of both toxigenic and non-toxigenic strains correlated with the duration of the carriage. The difference between strains of groups I and II was significant \((P < 0.001)\) for both toxigenic and non-toxigenic strains. Thus AA could be an important factor in the pathogenicity for the toxigenic \(C.\ \text{diphtheriae}\) by promoting colonization, as well as being responsible for prolonged carriage. It is notable that AA of toxigenic and non-toxigenic strains of group III was the same \((P < 0.1)\).

Analysis revealed that in contrast to toxigenic, non-toxigenic strains from persons with acute or chronic throat inflammation possessed AA (GMT = 18.37) higher than non-toxigenic strains from healthy persons (GMT = 6.17, \(P < 0.0001\)). Thus AA is an important colonization factor of non-toxigenic diphtheria strains, and may play a role in their pathogenicity.

There is much evidence that a high level of diphtheria antitoxin fails to prevent carriage by toxigenic strains [12–15]. For optimal protection against diphtheria and infection with toxigenic \(C.\ \text{diphtheriae}\), immunity to both diphtheria toxin and colonization factors is needed. The defence against the latter is probably provided by secretory IgA directed towards adhesin antigens. The prevention of carriage by toxigenic \(C.\ \text{diphtheriae}\) should lead to complete eradication of infection as well as disease.

In practice the determination of the AA of carrier toxigenic strains should reveal the most epidemiologically dangerous carriers, who could be strictly isolated.

REFERENCES