Estimation of Corynebacterium diphtheriae antitoxin in human sera: a comparison of an enzyme-linked immunosorbent assay with the toxin neutralisation test

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Summary. Serum samples from 101 individuals were titrated for Corynebacterium diphtheriae antitoxin by an IgG-specific enzyme-linked immunosorbent assay (ELISA) and a neutralisation test in tissue culture (TC). In some of the sera, the concentrations of antitoxin determined by the two assays were different; antitoxin values in these sera were titrated again by neutralisation tests in guinea pigs (GNT). Antitoxin concentrations of >0·01 IU/ml by GNT partly corresponded to values obtained in both ELISA and TC. Only the values from TC agreed with lower GNT results. Heat inactivation of sera was investigated and rejected as a possible reason for the discrepancy in the results. ELISA can be used to detect levels of <0·1 IU/ml, although the accuracy below 0·01 IU/ml, often considered a protective level, is questionable. At higher levels ELISA was reproducible for the titration of diphtheria antitoxin in human sera and offers a useful alternative to both in-vivo assays and TC.

Introduction

The first published method for estimating the level of Corynebacterium diphtheriae antitoxin in human serum was the neutralisation test in guinea pigs (Romer and Somogyi, 1909). A modification of this test, in which rabbits were used (RNT), was later reported (Jensen, 1933). These two methods of titration in vivo are well established; therefore, results of any in-vitro methods proposed as alternatives must show good correlation with the results from these assays. An alternative assay should be sufficiently sensitive to detect antitoxin concentrations of <0·01 International Units (IU)/ml of serum, the level below which a positive reaction occurs in the Schick test (Wilson and Miles, 1975). Results obtained by TC correlate well with those from the RNT (Kriz et al., 1978) and TC has been accepted as a reliable in-vitro technique replacing GNT and RNT in many instances.

ELISA has been compared with the RNT for the measurement of diphtheria antitoxin in rabbit sera (Svenson and Larsen, 1977) and also human sera, although no reference serum was included in this study (Camargo et al., 1984). This assay has been recognised as a convenient technique for the estimation of diphtheria antitoxin in human sera (Ruuskanen et al., 1980; Cheng, 1984; Li, 1985), although in these studies results were expressed as titres and not as units/ml of serum. It is a valuable assay for use in laboratories where animals cannot be used and where there are limited tissue-culture facilities. We have investigated the sensitivity, reproducibility and accuracy of an ELISA that measures antibody of the IgG isotype and compared it with an established TC assay in routine use. The GNT has been used for further examination of any sera showing poor correlation between the assays.

Materials and methods

Serum samples

Serum samples submitted to the Public Health Laboratory, Leeds, for assays of diphtheria antitoxin were obtained from a cross-section of those most at risk from diphtheria, including nursing staff and immigrant children with an unknown history of immunisation. Sera from patients with suspected toxoplasmosis but found to have an insignificant serological profile for this disease, were also selected at random.

Assay methods

ELISA. The ELISA for the measurement of IgG antibodies to tetanus toxoid described by Melville-Smith et al. (1983) was modified for this study by employing
purified diphtheria toxoid at a protein concentration of 5µg/ml, assayed by the method of Schacterle and Pollack (1973), as the antigen. A human serum containing diphtheria antitoxin 0.8 IU/ml was used for reference purposes. This potency had been ascertained by titrations against the Third British Standard for Diphtheria Antitoxin (66/153, available from National Institute for Biological Standards and Control, Herts, EN6 3QG) by the GNT.

Neutralisation test in tissue culture (TC). The estimations of diphtheria antitoxin were performed in tissue culture by a modification of the method of Miyamura et al. (1974) but with MK2 cells and 66/153 as a reference serum.

Neutralisation test in guinea pigs (GNT). The method used was a modification of that described in the British Pharmacopoeia (1980), with 66/153 as a reference serum. The test levels used were the Lr/100, Lr/1000 or Lr/10 000 dose of diphtheria toxin, i.e., the smallest dose of toxin which, when mixed with 0·01, 0·001 or 0·0001 IU of the reference antitoxin respectively and injected intradermally into guinea pigs, causes a skin lesion on or before the second day.

Comparison of data

Results from all three assays were expressed in IU/ml. The results from ELISA and TC were compared and eight sera with antitoxin levels >0·01 IU/ml when titrated by ELISA but <0·01 IU/ml when titrated by TC, were screened at 0·01 IU/ml by the GNT and at a lower level when there was enough serum. Five sera whose antitoxin concentrations measured by the two assays differed by more than one two-fold dilution step were also titrated by the GNT. The results from the GNTs were compared with those from the other two assays. No further comparisons with the GNT could be made because of insufficient volumes of sera.

Tests of heat-inactivated sera

A further 17 serum samples were selected from normal adults and from non-immunised adults known to have little if any diphtheria antitoxin. The concentrations of diphtheria antitoxin were estimated by both ELISA and TC and the sera subsequently held in a 56°C water bath for 1 h. The inactivated sera were then assayed for diphtheria antitoxin by ELISA and TC, and the results from all assays investigated by analysis of variance.

Reproducibility studies

Eleven sera whose antitoxin concentrations were in the range 0·01–>10 IU/ml when titrated by TC were selected for a reproducibility study of ELISA, and eight sera were selected for a reproducibility study of TC. Four assays were performed by ELISA and five assays by TC as described above, over a period of 10 months. The geometric means (GM) and geometric coefficients of variation (GCV) (Kirkwood, 1979) were calculated for both methods.

Results

Initially, 101 sera were examined in the comparative study. Taking >0·01 IU/ml as the level of antitoxin indicating immunity, there was agreement in the immune status of the subject between the ELISA and TC in 83·2% (84 out of 101) of the patients studied. In all cases of disagreement, antitoxin concentrations were higher by ELISA and showed patients to be immune. Similar values for antitoxin concentrations, within one doubling dilution, were shown in the two assays for 49 (48·5%) of the 101 sera, rising to 72 (71·3%) for a range within two doubling dilutions. Discrepant results were obtained with 29 sera; 28 (97%) of these gave higher results in the ELISA system. The antitoxin concentrations obtained by ELISA (the technique under evaluation) are plotted against the corresponding TC results (the accepted technique) in the figure. The line of 100% agreement between the two assays has been drawn. There was no agreement between the results from the two techniques for sera with antitoxin concentrations <0·01 IU/ml by TC. When we excluded these sera from the analysis, the regression coefficient between the results from the two assays on the remaining sera was 0·94 by the method of Deming (1943).
Relationship between ELISA, TC and GNT

A total of 13 sera for which there was poor agreement in their results by ELISA and by TC were further tested by the GNT. Table I shows these antitoxin concentrations. The eight sera tested by GNT because of disagreement between the ELISA and TC assays on the immune status of the subject, had values by GNT that agreed with TC results. For the five sera showing discrepant values between the ELISA and TC, there was some correlation with the results from GNT and both in-vitro methods.

Table I. Concentrations of diphtheria antitoxin in sera measured by TC, ELISA and GNT

<table>
<thead>
<tr>
<th>Serum no.</th>
<th>TC</th>
<th>ELISA</th>
<th>GNT</th>
<th>TC v GNT</th>
<th>ELISA v GNT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>&lt;0.0006</td>
<td>0.02</td>
<td>&lt;0.005</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2*</td>
<td>0.001</td>
<td>0.03</td>
<td>&lt;0.001</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3*</td>
<td>0.003</td>
<td>0.02</td>
<td>&lt;0.01</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4*</td>
<td>0.003</td>
<td>0.03</td>
<td>&lt;0.01</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5*</td>
<td>0.005</td>
<td>0.02</td>
<td>&lt;0.01</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6*</td>
<td>0.005</td>
<td>0.03</td>
<td>&lt;0.01</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7*</td>
<td>0.007</td>
<td>0.05</td>
<td>&lt;0.01</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8*</td>
<td>0.009</td>
<td>0.03</td>
<td>&lt;0.01</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9†</td>
<td>0.02</td>
<td>0.10</td>
<td>&lt;0.06</td>
<td>(+)</td>
<td>-</td>
</tr>
<tr>
<td>10†</td>
<td>0.02</td>
<td>0.49</td>
<td>0.38</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>11†</td>
<td>0.02</td>
<td>0.31</td>
<td>0.10</td>
<td>-</td>
<td>(+)</td>
</tr>
<tr>
<td>12†</td>
<td>0.06</td>
<td>0.31</td>
<td>&lt;0.08</td>
<td>(+)</td>
<td>-</td>
</tr>
<tr>
<td>13†</td>
<td>0.08</td>
<td>1.50</td>
<td>0.63</td>
<td>-</td>
<td>(+)</td>
</tr>
</tbody>
</table>

* Discrepancies in determination of immune status between methods.
† Discrepancies in determination of antitoxin concentration between methods.
‡ + = Agreement within ±1 dilution; (+) = agreement within ±2 dilutions; - = disagreement between methods >2 dilutions.

Table II shows the concentrations of diphtheria antitoxin obtained by ELISA for the 27 sera which had non-protective levels (<0.01 IU/ml) when titrated by TC. Of these, 17 (63%) were considered to have protective levels by ELISA but none of the sera had levels ≥0.1 IU/ml. Using McNemar’s Test we found a significant difference in the determination of immune status between the TC and ELISA assays on all 101 sera (p<0.01).

A χ² significance test of the results obtained with sera from two donor groups ("normal", i.e., nurses and children, and "diseased", i.e., toxoplasmosis patients) showed that the differences between the results from ELISA and TC on the total set of 101 sera were not due to differences in the health status of the serum donors (p>0.05).

Heat inactivation of sera

The mean concentration of diphtheria antitoxin for the untreated sera was lower than that for the heat-inactivated sera when assayed by ELISA. However, there was no significant difference between these two means (F-test; p>0.01). There was no significant difference between the mean antitoxin concentrations of the sera before and after heat inactivation when assayed by TC.

Reproducibility studies

Tables III and IV show the concentrations of diphtheria antitoxin together with the GMs and GCVs of sera selected for the reproducibility study of ELISA and in TC. The average values for the
GCVs were 25·5 for ELISA and 17·3 for TC, with no dependence on antitoxin concentration. This shows that the two assays reproduce well, although only four ELISA assays were performed and five TC assays.

Discussion

This study has shown ELISA to be a reproducible assay for the estimation of the activity of diphtheria antitoxin in human sera. It is able to detect antitoxin concentrations as low as 0·003 IU/ml. This is less than the minimum concentration considered necessary for protection against diphtheria (0·01 IU/ml).

There was agreement between ELISA and TC results for sera with concentrations of diphtheria antitoxin >0·01 IU/ml. Although the ELISA was able to detect antitoxin concentrations <0·01 IU/ml it failed to detect 63% (17 out of 27) of individuals with antitoxin concentration <0·01 IU/ml. This shows that the two assays reproduce well, although only four ELISA assays were performed and five TC assays.

Table IV. Concentrations of diphtheria antitoxin determined in five TC assays on eight sera performed over several months

<table>
<thead>
<tr>
<th>Serum code</th>
<th>Antitoxin concentrations determined in assay</th>
<th>GM</th>
<th>GCV</th>
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<tr>
<td>L</td>
<td>0·005 0·005 0·019 0·009 0·019 0·019 0·019</td>
<td>19·5</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>0·009 0·019 0·005 0·002 0·002 0·005 0·005</td>
<td>26·5</td>
<td></td>
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<tr>
<td>N</td>
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<td>10·1</td>
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<tr>
<td>O</td>
<td>0·04 0·078 0·078 0·078 0·078 0·078 0·078</td>
<td>14·5</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>0·08 0·039 0·078 0·078 0·078 0·078 0·078</td>
<td>13·7</td>
<td></td>
</tr>
<tr>
<td>Q</td>
<td>0·08 0·039 0·156 0·156 0·156 0·156 0·156</td>
<td>20·0</td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>0·16 0·078 0·156 0·078 0·078 0·078 0·078</td>
<td>14·7</td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>0·30 0·31 0·62 0·62 0·62 0·62 0·47</td>
<td>14·8</td>
<td></td>
</tr>
</tbody>
</table>

Serum from all these had antitoxin concentrations <0·1 IU/ml, including 12 (70·6%) of 17 with concentrations ≤0·03 IU/ml.

ELISA has been used routinely on other sera from unimmunised infants to evaluate concentrations in the range <0·01–<0·001 IU/ml of diphtheria antitoxin (unpublished data), yet differences in the results obtained with the two methods were found in the current study at this level of antitoxin. No satisfactory explanation was found to account for these discrepancies. Heat inactivation at 56°C for 30 min is used routinely in the Toxoplasma Unit at Leeds to minimise the risk of infection from the human immunodeficiency virus (HIV). Heat inactivation has been shown to cause false positive results in other ELISA systems (Francis et al., 1987) but only minimal changes in ELISA absorbance readings were observed in the current study when sera were heat inactivated at 56°C for 1 h. The ELISA system appears to detect low levels of specific IgG that is unable to neutralise toxin in TC or GNT.

ELISA can be used with confidence to identify individuals with a serum diphtheria antitoxin concentration <0·1 IU/ml and it has been shown that there is little or no adverse reaction when a low dose diphtheria vaccine, developed for use in adults, is administered to individuals with such levels (Mortimer et al., 1986). Thus ELISA can be used as a method for monitoring the concentrations of diphtheria antitoxin in individuals at risk from exposure to diphtheria provided all values below 0·1 IU/ml are considered to indicate non-immunity. Moreover, it is a more rapid and convenient technique than either TC or the in-vivo assays.

We thank Miss J. Watkins for performing the in-vivo assays, Mr A. Curtis for the analysis of variance and Mrs I. Stephens for typing the manuscript.

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