Tissue Culture Method for Toxigenicity Testing of Corynebacterium diphtheriae

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A method for toxigenicity testing of Corynebacterium diphtheriae in tissue cultures was developed. Results were obtained by comparing destruction of the monkey kidney or, preferably, rabbit kidney monolayer by 0.1 ml of the C. diphtheriae culture in Elek's broth containing 20% rabbit serum with the appearance after the addition of 0.2 ml of a mixture of the C. diphtheriae culture and diphtheria antitoxin. The mixture of C. diphtheriae broth culture and 10 antitoxin units per ml was incubated for 1 hr at room temperature before it was added to the tissue cultures which were then incubated as long as 5 days; most results, however, were read in 72 hr. Elek's broth medium was superior to heart infusion broth for toxin production by C. diphtheriae. Addition of 20% rabbit serum improved toxin production in either broth. Numerous toxigenic and atoxigenic C. diphtheriae cultures were tested for toxigenicity in primary rabbit and monkey kidney tissue cultures. If properly controlled, this in vitro method appeared to have an advantage over the in vitro gel method; its results were comparable with the rabbit intradermal test. With the wider use of tissue cultures in most laboratories, we believe that the tissue culture method for toxigenicity would be more economical and easier to perform than the animal intradermal method.

Lennox and Kaplan (4) and Straus and Hendee (9) have shown that diphtheria toxin will destroy various tissue culture cells. Only in the last few years has this phenomenon been used for diagnosis. Ormay and Ujhelyi (5), Stanica et al. (8), and Schubert, Wiggins, and Taylor (7), have investigated tissue cultures as a method for titrating the antitoxin levels in human sera. Monkey kidney tissue culture cells were used by André, Audebaud, and Chambon (1) to obtain presumptive evidence of Corynebacterium diphtheriae directly from throat swabs for the rapid diagnosis of diphtheria. These findings suggested that tissue cultures might be used to determine toxigenicity of C. diphtheriae strains.

The purpose of this study was to develop the tissue culture technique as a procedure for differentiating toxigenic and atoxigenic C. diphtheriae cultures. Comparisons were also carried out to determine the value of these procedures as an alternate method for the animal intradermal and in vitro tests (3) in those laboratories in which such tests may be difficult to perform.

Materials and Methods

Cultures. Tests were carried out with 462 strains of C. diphtheriae, of which 299 were freshly isolated and 163 were obtained from storage. These included 170 toxigenic and 292 atoxigenic strains and were a random mixture of gravis, intermedius, mitis, and indeterminate types. The C. diphtheriae cultures were cultured to Pai medium (6) from single-colony isolation and were identified (3) morphologically by methylene blue smears and by biochemical reactions. The cultures were verified for toxigenicity by the in vitro plate (2) or the animal intradermal method (3).

In addition, 43 Corynebacterium strains other than C. diphtheriae received from the Bacterial Reference Unit, National Communicable Disease Center, were studied. Two of the strains were C. ulcerans; six were C. haemolyticum; four were C. aquaticum; and the remainder were not speciated.

Media. Broth cultures for the toxigenicity test were inoculated from the Pai medium (6). Heart infusion broth (HIB), which is the recommended broth for the rabbit intradermal test, was the initial culturing medium for this study. A broth modification of Elek's (2) original formula was compared with HIB. Elek's broth was prepared by combining 500 ml of distilled water, 10 g of Proteose Peptone (Difco), 1.5 g of maltose, 2.5 g of NaCl, 0.35 ml of lactic acid, and 0.75 ml of NaOH (40%). This mixture was heated to boiling with constant agitation and was filtered through Whatman no. 2 filter paper. After the pH in the mixture was adjusted to pH 8.0, the medium was tubed in 3-ml amounts, and it was autoclaved at 115 C for 10 min.

The rabbit serum for the Elek's broth and HIB was obtained from a commercial source. A C. diphtheriae, intermedius type, control was checked for satisfactory
toxin production in the in vitro test with the medium supplemented with this serum.

Reagents. The reagents consisted of one lot of Lederle toxin no. 5721 (100 Lf/ml), U.S. standard antitoxin, 6 antitoxin units per ml (au/ml), and Wyeth's antitoxin lots 20101-20104 (20,000 au/ml). (The Lf as used here is the amount of toxin in a mixture of toxin and antitoxin which causes optimal flocculation with diphtheria antitoxin.) Sterile phosphate buffer (pH 7.4) with 0.2% gelatin was used as the diluent for toxin and antitoxin.

Tissue cultures. The primary rabbit kidney and monkey kidney tissue cultures were prepared by the Tissue Culture Unit, National Communicable Disease Center, as described by Schubert et al. (7). In addition to these tissue cultures, 50 tubes of rabbit kidney cell preparations were obtained from two commercial sources.

The tissue cultures were prepared for use by discarding the growth medium and replacing it with Earle's balanced salt solution with 0.5% lactalbumin hydrolysate as the maintenance solution. The tissue cultures were incubated overnight and maintained at room temperature until the test culture mixture was added. The commercial tissue cultures were treated in the same manner.

Methods. The procedure for toxigenicity testing in tissue cultures consisted of adding 0.1 ml of the broth culture to each of two tissue culture tubes. The control was prepared by adding equal parts of broth culture and antitoxin containing 10 au/ml. This control was incubated for 1 hr at room temperature, and then 0.2 ml of the control was added to duplicate tissue culture tubes. Tissue cultures were then incubated at 37°C and read after 24, 48 hr, etc. The manner of reading cell destruction is given in detail by Schubert et al. (7). Tests were read so that 2 to 4+ (50% or more monolayer destruction) was positive.

The rabbit intradermal procedure was carried out by the classical method (3). The in vitro tests were performed according to the method of Elek (2).

RESULTS

Preliminary tests performed with commercial antitoxin (Wyeth, 20,000 au/ml, merthiolate preserved) indicated that it was cytopathogenic for tissue cultures, probably because of the concentration of merthiolate. No cell destruction was obtained when the antitoxin was diluted to contain 20 au/ml or less. The amount of antitoxin necessary to neutralize the toxin produced by random strains of *C. diphtheriae* in 48-hr Elek's broth cultures was tested with 0.01, 0.1, 1.0, and 10 au/ml. Throughout this study, 10 au/ml was selected for use because this amount of antitoxin was adequate to neutralize the toxin produced by each of the strains tested.

*C. diphtheriae* toxigenicity tests were performed in tissue cultures by adding the whole broth culture, the supernatant fluid of the broth culture after centrifugation, or the broth culture with antibiotics. Of these, the whole broth culture was selected for the test because no differences were observed among these inocula. The tissue cultures were affected adversely if more than 0.1 ml of the broth culture was used.

In initial tissue culture studies, HIB was used to culture the *C. diphtheriae*, since this medium was used for the rabbit intradermal test. Results using this medium gave only 2 to 3+ tissue cell destruction in 48 hr, but the antitoxin control occasionally showed as much as 1+ cell destruction. Other broth media, such as that of Elek, were prepared and evaluated to improve toxin production by the diphtheria cultures. This evaluation was needed to obtain a greater contrast of cell destruction between the test and the control.

The effect of adding 1, 5, and 20% serum, respectively (to the Elek broth medium), on toxin production by *C. diphtheriae* is shown in Table 1. The degree of toxin production was determined by mixing equal volumes of broth culture with antitoxin containing 0.001, 0.01, 0.1, and 1.0 au/ml. After incubation for 1 hr at room temperature, 0.2 ml of each mixture was added to the tissue culture and was incubated at 37°C. The results are expressed in terms of the amount

### Table 1. Antitoxin needed to neutralize *C. diphtheriae* toxin produced in Elek's broth with rabbit serum

<table>
<thead>
<tr>
<th>C. diphtheriae (culture no.)</th>
<th>Elek's media</th>
<th>No serum</th>
<th>(1%) Serum</th>
<th>(5%) Serum</th>
<th>(20%) Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.001-0.01</td>
<td>0.01-0.1</td>
<td>&gt;0.1</td>
<td>&gt;0.1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.01-0.1</td>
<td>0.01-0.1</td>
<td>0.01-0.1</td>
<td>&gt;0.1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.01-0.1</td>
<td>&gt;0.1</td>
<td>&gt;0.1</td>
<td>&gt;0.1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>&gt;0.1</td>
<td>&gt;0.1</td>
<td>&gt;0.1</td>
<td>&gt;0.1</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.01-0.1</td>
<td>0.01-0.1</td>
<td>&gt;0.1</td>
<td>&gt;0.1</td>
<td></td>
</tr>
</tbody>
</table>

* Tested in rabbit kidney tissue cultures.

* Amount of antitoxin (au/ml) needed to neutralize toxin.

### Table 2. Antitoxin units per milliliter required for neutralization of toxin produced by *C. diphtheriae* in Elek's broth and HIB in 48 hr

<table>
<thead>
<tr>
<th>C. diphtheriae (strain no.)</th>
<th>Elek's broth with rabbit serum</th>
<th>HIB with rabbit serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.1-1.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2</td>
<td>0.01-0.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>3</td>
<td>0.1-1.0</td>
<td>0.001-0.01</td>
</tr>
<tr>
<td>4</td>
<td>1.0/10</td>
<td>0.001-0.01</td>
</tr>
</tbody>
</table>

* Tested in rabbit kidney tissue cultures.

* These broths showed destruction when no antitoxin was added, but were neutralized by 0.001 au/ml.
of antitoxin in units per milliliter which did not neutralize the toxin compared to the next higher amount of antitoxin which did neutralize the toxin. An increase in the amount of antitoxin required for neutralization indicated more toxin formation. Generally, the amount of the toxin produced by *C. diphtheriae* increased with the addition of serum to the Elek broth (Table 1). These data, compiled with 5 strains, were representative of 20 strains tested.

A comparison of the effect of 20% rabbit serum on toxin production by *C. diphtheriae* in Elek's broth and HIB is given in Table 2. The tests were performed as described for Table 1 with the difference that antitoxin diluted to contain 0.001 to 10 au/ml was used. Broth cultures (0.1 ml) were added to tissue cultures without antitoxin as a control for the presence of toxin which could be neutralized by 0.001 au/ml. *C. diphtheriae* cultures produced 10- to 100-fold more toxin in Elek's broth than in HIB (Table 2).

Since more toxin was produced in Elek's broth, we examined it as a substitute for the HIB cultures used in intradermal tests. Tests with 148 toxigenic *C. diphtheriae* cultures and 9 toxigenic strains showed complete agreement between the two broth media.

Since the *C. diphtheriae* broth cultures were incubated 48 hr for the rabbit intradermal test, cultures of this age were used for preliminary studies of the tissue culture method. Later, both 24- and 48-hr incubations of the broth culture were compared.

A comparison of toxin produced after 24- and 48-hr incubation of *C. diphtheriae* in HIB and Elek's broth with and without 20% serum is presented in Table 3. The results show that Elek's broth with serum was satisfactory for toxin production after 24 as well as after 48 hr of incubation. When incubated for 48 hr, cultures grown in Elek's broth without serum or HIB with 20% serum showed sufficient cell destruction. After incubation for 5 days, the tissue cultures inoculated with HIB without serum failed to give the minimal 2+ destruction.

All the initial studies described were performed on rabbit kidney cells. Even though the rabbit kidney cells were more sensitive to destruction by *C. diphtheriae* toxin, a comparison was made of monkey kidney cells (generally on hand in most laboratories) with the rabbit kidney cells. Results with 38 strains of toxigenic *C. diphtheriae* are given in Table 4.

Readings (24 hr) of rabbit tissue cultures inoculated with the strains grown in Elek's broth with and without serum, Elek's broth with 20% serum, and HIB with 20% serum showed that 97, 100, and 42% of the cultures, respectively, were positive. Similar readings at 24 hr of monkey kidney cells inoculated with the same broth cultures were negative. At 48 hr, all 38 cultures in these broths were positive in the rabbit cells, and 92, 92, and 24% of the cultures were positive in monkey kidney cells when Elek's broth with no serum, Elek's broth with 20% serum, and HIB with serum, respectively, were used as the culturing medium for the *C. diphtheriae* strains. At 72 hr, all the monkey kidney tissue cultures which had been inoculated with Elek's broth with and without serum were positive. Even at 5 days, only 47% of the monkey kidney cells were positive with the HIB.

Elek's (2) in vitro gel toxigenicity test was compared with the tissue culture toxigenicity test inoculated with Elek's broth prepared with four different lots of Proteose Peptone. All the strains used gave satisfactory toxigenicity tests.

### Table 3. Rabbit kidney cell destruction in 48 hr by *C. diphtheriae* in broth cultures

<table>
<thead>
<tr>
<th>C. diphtheriae (strain no.)</th>
<th>HIB</th>
<th>Elek's broth</th>
</tr>
</thead>
<tbody>
<tr>
<td>No serum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 hr</td>
<td>48 hr&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24 hr</td>
</tr>
<tr>
<td>1</td>
<td>±&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>2</td>
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<tr>
<td>3</td>
<td>±&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>1-2</td>
<td>2-3</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>±&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Rabbit serum.
<sup>b</sup> Incubation of broth before addition to tissue culture.
<sup>c</sup> Tissue culture readings remained negative after 5 days, while all others became positive.

### Table 4. Percent of *C. diphtheriae* cultures<sup>a</sup> cytotoxic for monkey vs. rabbit kidney cells

<table>
<thead>
<tr>
<th>Broth culture</th>
<th>Rabbit kidney</th>
<th>Monkey kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hr&lt;sup&gt;a&lt;/sup&gt;</td>
<td>48 hr</td>
</tr>
<tr>
<td>Elek, no serum, 48 hr.</td>
<td>97&lt;sup&gt;c&lt;/sup&gt;</td>
<td>100</td>
</tr>
<tr>
<td>Elek, 20% serum, 24 hr.</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>HIB 20% serum, 48 hr.</td>
<td>42</td>
<td>100</td>
</tr>
</tbody>
</table>

<sup>a</sup> Toxigenic strains, 38.
<sup>b</sup> Incubation of tissue cultures in hours.
<sup>c</sup> Per cent of 38 cultures cytotoxic for tissue cultures.
<sup>d</sup> After 5-day incubation, 47% became cytotoxic.
with both media with different lots of Proteose Peptone.

Monkey and horse sera were compared to rabbit serum in Elek's broth medium, and, when tested in tissue cultures, were satisfactory for toxin production. Serum from 4 of 12 different rabbits tested in the in vitro gel toxigenicity test failed to give precipitin lines with some of the toxigenic C. diptheriae cultures used. These 4 sera, as well as the remaining 8, were added to Elek's broth for tissue culture testing. Although the C. diptheriae cultures were all toxigenic with the 12 different rabbit sera, the 4 sera unsatisfactory for the in vitro method gave a somewhat lesser amount of tissue cell destruction than the 8 remaining sera.

This study was generally performed with tissue cultures that required 4 days of growth before the monolayer developed in the growth medium, after which maintenance medium was added. The age at which rabbit tissue cultures would be satisfactory for toxigenicity testing was determined by testing the same cells at four time intervals: fresh (that is, when maintenance medium was added), and after 1 week, 2 weeks, and 3 weeks. One set of rabbit cells was satisfactory only when fresh. Two other lots were less sensitive to the toxin at 2 and 3 weeks of age than when at 1 week of age. No variation in sensitivity was obtained with three sets of fresh or 2-week-old monkey kidney cells.

Satisfactory toxigenicity tests were performed with rabbit kidney cells prepared by two commercial laboratories. The cells from one of the laboratories gave positive reactions in 24 hr, whereas the other required 72 hr. This difference in reactivity to the diphtheria toxin was occasionally observed in various tissue cell preparations obtained from our own laboratories.

The action of Corynebacterium other than C. diptheriae on the tissue cultures was tested with 43 Corynebacterium strains. Only negligible amounts (±) of cell destruction were obtained on rabbit or monkey kidney cells. Toxigenic C. ulcerans, on the other hand, is known to produce a toxigenic factor in addition to dipherthera toxin, caused 4+ cell destruction in the control with antitoxin.

A large comparison was carried out with rabbit kidney cells, the in vitro agar gel, and the rabbit intradermal test for toxigenicity. Complete agreement was obtained with the three tests using 138 toxigenic and 191 atoxigenic C. diptheriae strains. Tests with monkey kidney cells also gave results which were comparable to the in vitro and intradermal toxigenic test on 135 toxigenic and 114 atoxigenic strains.

**DISCUSSION**

Our results with rabbit kidney cells of different ages indicated that older cells become less sensitive to the toxin. Rabbit kidney cells beyond 1 week of age should not be used for toxin testing. Monkey kidney cells were usable for a longer time period.

During the time of this study, some lots of tissue cultures were not usable because of unsatisfactory monolayer formation. Other lots were somewhat more resistant to toxin, as was one commercial lot. These variations, however, can be controlled by using a known minimal toxin-producing culture. A control culture may be selected by using 0.01 au/ml prepared from the standard National Institutes of Health antitoxin (6 au/ml). A C. diptheriae culture which destroys the cell monolayer, but not the cell monolayer to which 0.01 au/ml was added, can be used for the control.

Although many laboratories, especially state health laboratories, have used the in vitro test to determine toxigenicity of C. diptheriae, variable results have occurred because of unsatisfactory rabbit serum or peptone (2). Rabbit serum used in the broth in which C. diptheriae is grown for toxin testing was a less variable factor in tissue cultures than it was when used in the in vitro toxigenicy method.

All toxin produced by C. diptheriae strains tested was neutralized by the addition of 10 au/ml with the exception of that from C. ulcerans. Since the endotoxin of C. ulcerans also destroys the tissue culture control containing antitoxin, it would be necessary to use Seitz filtrates of the culture for toxin testing.

The optimal method for determining toxin formation involves the use of rabbit kidney cells inoculated with the 24- or 48-hr broth culture prepared with Elek's formula with 20% serum. However, if only monkey kidney cells are available, they can be used if they are inoculated with cultures grown for 48 hr in Elek's broth with 20% serum.

All the C. diptheriae strains that were tested gave comparable reactions in the tissue culture and the rabbit intradermal test. Some atoxigenic strains which gave clearcut negative results in tissue culture gave equivocal reactions in the rabbit intradermal test. These strains were found to be atoxigenic by the guinea pig subcutaneous method. If a minimal positive reaction of 2+ is obtained in the test proper in tissue cells and if the control is negative, it is recommended that this strain should be confirmed by using the guinea pig subcutaneous tests.
Literature Cited


