Rapid, Direct Tissue Culture Test for Toxigenicity of *Corynebacterium Diphtheriae*

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A method for testing toxigenicity of *Corynebacterium diphtheriae* in tissue culture is described. The technique, called the colony overlay test (COT), involves inoculating material from an isolated colony of *C. diphtheriae* to a small area on the surface of an agar medium which overlays a monolayer of toxin-susceptible HeLa cells. If toxin is produced during incubation at 37 C, it diffuses to the tissue monolayer and destroys the cells below the inoculation site. Twenty-four hours after inoculation, organisms are killed and tissue cells are fixed with formaldehyde. The agar overlay is then removed, and the monolayer is stained with crystal violet. Toxin-affected areas fail to stain or stain poorly. A second plate with antitoxin incorporated in the overlay serves as a control for specificity. Forty-eight strains of *C. diphtheriae* were tested by the COT, guinea pig, and in vitro, gel diffusion tests. The COT is as specific as the other two tests, is easy to read, and can be used to test large numbers of isolates for toxin action more conveniently than by animal inoculation.

After isolation of a strain of *Corynebacterium diphtheriae*, determination of toxigenicity must be made. The guinea pig intracutaneous test and the in vitro gel diffusion method are most commonly used (1). After Lennox and Kaplan’s demonstration (7) of the action of diphtheria toxin on cell cultures, a few attempts were made to develop a cell culture method for toxigenicity testing. Andre et al. (2) described a technique in which material from a throat swab, or from a broth culture of an isolated strain, was tested for cytotoxic activity on monolayers of monkey kidney cells in tube cultures. Schubert et al. (9) added a small amount of a broth culture of a strain of *C. diphtheriae* to monolayers of primary rabbit or monkey kidney cells in tube culture. In both cases observations for cytotoxicity were made over a 24- to 96-h period. Destruction of 50% or more of the cells in the monolayer in the experimental tube and absence of cytotoxicity in the control tube containing diphtheria antitoxin were evaluated as a positive test for toxigenicity.

We wish to report a new procedure for the detection of toxigenicity in strains of *C. diphtheriae*. In this procedure, which we shall call the colony overlay test (COT), material from an isolated colony of suspected *C. diphtheriae* is inoculated directly on a small area of the surface of an agar medium which overlays a monolayer of toxin-susceptible tissue culture (HeLa) cells. When a toxigenic strain is present, the toxin produced diffuses to the tissue cell layer and kills the cells within 18 to 24 h after incubation. At that time, the tissue monolayer is fixed and then is stained with crystal violet. Toxin-affected areas fail to stain or stain poorly compared with the unaffected areas. A second plate with antitoxin incorporated into the agar provides the necessary control. In this study, we compared the guinea pig intracutaneous test (1), the gel diffusion test (6), and the COT.

**MATERIALS AND METHODS**

**Cultures.** Forty-eight strains of classified and unclassified *C. diphtheriae* were taken from our collection (Table 1). The strains were maintained on heart infusion slants.

Human heteroploid cells (HeLa) kindly supplied by George Kenny of this institution were employed throughout the study.

**Cell culture media.** HeLa cells were grown in Eagle minimum essential medium (MEM) with 10% fetal calf serum (5) (Grand Island Biological Co.). The basic ingredients of MEM are an amino acid mixture, vitamin mixture, glutamine, and Hanks basic salt solution (BSS). MEM was buffered with 20 mM N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES, Calbiochem) per liter, and the medium was titrated to a final pH of 7.8 with sterile
TABLE 1. Comparison of toxigenicity tests on various strains of Corynebacterium diphtheriae

| Strain designation | No. tested | Guinea pig, intracutaneous | Toxicity test | Colony overlay test |
|--------------------|-----------|---------------------------|---------------|-------------------|
| Mitis              | 8         | -                         | -             | -                 |
| Gravis             | 5         | +                         | +             | +                 |
| Intermedius        | 5         | +                         | +             | +                 |
| Unclassified       | 8         | -                         | -             | -                 |

NaOH. Penicillin and streptomycin were added to a final concentration of 100 U and 100 µg/ml, respectively.

The overlay medium employed in the test was prepared as follows. Medium 2X MEM was made by doubling the concentration of amino acids, vitamins, glutamine, TES, and fetal calf serum in MEM and then bringing them to volume with BSS. Antibiotics were omitted from this medium. Agarose (Difco; 0.8%) was prepared in BSS at pH 7, autoclaved, and then stored at room temperature. The pH was not adjusted to 7.8 at this time, because a precipitate is formed on autoclaving if the pH is above 7.2. To prepare the final overlay medium, the agar was melted, held at 44 C, and mixed with an equal volume of 2X MEM warmed to the saline temperature. The final pH of the overlay medium was 7.8 because of the buffering capacity of 2X MEM. A pH of 7.8 is critical for the growth of the HeLa cells and for preventing destruction of the monolayer by acid produced by bacterial metabolism. Diphtheria antitoxin (National Drug Co., Philadelphia, Pa.) was added to the control overlay to a final concentration of 1 U/ml.

HeLa cells were maintained in 900-g (32-ounce) prescription bottles as follows. Confluent monolayers containing approximately 2 × 10^5 cells were washed twice with BSS and then were trypsinized with 6 ml of 0.5% trypsin dissolved in Ca^2+ - and Mg^2+ free BSS. Two bottles were then seeded with 3 ml of trypsinized cells, and 50 ml of MEM was added to each. In general, the monolayer became confluent within 2 days, and the medium became acidic on the 3rd day. When cells were to be used in the COT, monolayers were fed with fresh MEM 12 to 18 h before they were used. In this case, feeding was done approximately 1.5 days after transfer. At this time, the monolayers were about 80% confluent, but the medium had not become acid. The reason for this precaution is discussed later.

Toxicity tests. The gel diffusion (in vitro) test was performed as described by Hermann et al. (6), and the intracutaneous test in guinea pigs was by the method of Fraser and Weld (1).

The colony overlay test (COT) was performed in the following manner. Shortly before the test, the recently fed monolayer was washed three times with 6 ml of BSS (pH 7.8) and then was harvested by treatment with trypsin. The suspended cells were then diluted to a final concentration of 2.5 × 10^4 cells/ml with antibiotic-free MEM medium. Each 15 by 60-mm tissue culture dish received 5 ml of cell suspension for a total of approximately 1.2 × 10^5 cells per dish, and each was then placed in a 37 C incubator for 4 h. During this time the cells attached to the dishes. The medium was then poured off, and each monolayer was carefully washed twice with 1 ml of antibiotic-free MEM medium. At this stage the monolayer was almost confluent. Overlay medium (6 ml plus or minus antitoxin) was then poured as an overlay on the cell layer. After the overlay hardened at room temperature (after about 10 min), the dishes were placed in a 37 C incubator for about 2 h in order to dry the surface of the plates, after which they were inoculated with the bacterial strains. During drying the lids were slightly open.

Cultures of C. diphtheriae to be tested for toxigenicity were streaked from heart infusion slants to Tinsdale medium (8) approximately 24 h before testing. This medium was chosen in order to simulate the procedure employed in the isolation of C. diphtheriae from the throat; inocula for the test can also be grown on heart infusion broth or agar. The tissue culture with overlay was then inoculated with material from a single, well-isolated colony on the Tinsdale medium, as would be the case following primary isolation from a patient. The tip of a sterile, cylindrical applicator stick was used to transfer material from the colony to the surface. This agar was spread and gave an area of inoculation approximately 2 mm in diameter. Care was taken not to break the agar overlay. An antitoxin-containing agar overlay plate was inoculated in an identical fashion from the same colony. The plates were then incubated at 37 C for 18 to 24 h. By proper spacing, a positive, negative, and two unknown strains could be tested on each 15 by 60-mm dish.

After incubation, 5 ml of 10% Formalin was poured onto each plate and allowed to remain for 20 min. The Formalin and overlay were then shaken out of the dish, and the monolayer was stained for 30 s with 1 ml of 1% crystal violet in 20% ethanol. The excess stain was then gently rinsed off with tap water, and the experimental and control monolayers were compared visually.

RESULTS

The results of a typical test are shown in Fig. 1. A positive test is one in which the area on the monolayer immediately below the zone of bacterial growth is completely or partially destroyed. Under these circumstances the area fails to stain or is stained less intensively than the surrounding, unaffected area. In the antitoxin control, the area below the zone of bacterial growth should be indistinguishable from the remainder of the monolayer.
Tests were performed on 48 known strains of *C. diphtheriae*, of which 32 were toxigenic and 16 were nontoxigenic. All strains were also tested by the gel diffusion technique and by intracutaneous inoculation of a guinea pig. All tests were in agreement. The tissue culture test was completed in 18 to 24 h after inoculation. The gel diffusion tests were positive for the known toxigenic strains in 48 to 72 h, and the guinea pig skin tests were positive in 48 h.

The COT has also been performed with HeLa cells grown in spinner culture. However, unless large numbers of tests are to be performed, maintenance of spinner cultures requires more attention and material than bottle-grown cells. We have also used large, 25 by 150-mm plates for this test. Thirty-four tests can be performed on a single plate in addition to the controls. The only changes in the technique are to increase the HeLa cell inoculum to $8 \times 10^6$ cells/plate, to increase the agar overlay to 55 ml, and to fix with approximately 50 ml of Formalin per plate.

**DISCUSSION**

The tissue culture method we have described for toxigenicity testing (COT) can be performed with comparative ease wherever sensitive cell culture lines are maintained. The use of TES buffer eliminates the need for a CO$_2$ incubator. Within the limits of our tests, the technique is as specific for diphtheria toxin as are the gel diffusion test or guinea pig skin test. The COT requires no special skill or training to read and poses virtually no problems in interpretation. This is in contrast to the guinea pig test and, in
some cases, the gel diffusion test, where critical judgment may be required.

There are a few sources of error in the COT which must be guarded against. HeLa cells to be used in the test should not be allowed to grow beyond the time a confluent monolayer is established and must not be allowed to incubate in a medium which has turned acidic. Cells which have done so appear to lose their sensitivity to diphtheria toxin. We assume that protein synthesis is shut down in these cells. It is for this reason that cells to be used in the COT are grown and fed in the manner described. One must also be certain that the inoculum of each bacterial strain tested is adequate and that it has grown well on the overlay medium. The MEM medium is very rich and has supported all of the strains tested thus far, but it conceivably might require further fortification for other strains. Finally, it is important that the inoculated monolayers are not incubated to the point where the medium becomes acidic, a stage generally reached after 24 h of incubation. Acid produced by the bacteria after depletion of the buffer will damage the monolayer. Evidence that monolayer damage is due to acid rather than toxin is provided by the control plate which, under these circumstances, will also exhibit cell destruction. A similar response can be anticipated if acid-producing organisms which are not C. diphtheriae are accidentally selected. Obviously, the test for toxigenicity does not eliminate the need for other criteria in identifying C. diphtheriae.

The convenience and speed of the present test over the guinea pig inoculation test in the diagnosis of diphtheria is apparent. One advantage of the tissue culture test over the gel diffusion test is that the mode of action of toxin in tissue culture is probably the same as that in the susceptible animal (4). Furthermore, we have found, up to now, that the tissue culture method has not been affected by various lots of the components employed in the medium, a problem experienced with some components of the medium used in the “in vitro” gel diffusion test (3).

Clearly, extensive tests with a larger number of strains are required to establish the reliability of the COT, and there are many variations in procedure that can be visualized. If further testing confirms its reliability, the COT then could be used to run many tests for toxigenicity in a convenient manner. This would make it useful in assessing the epidemiological situation during an outbreak of diphtheria or in undertaking large scale surveys.

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