Microtissue Culture Test for the Titration of Low Concentrations of Diphtheria Antitoxin in Minimal Amounts of Human Sera

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Received for publication 29 August 1972

A microtissue culture method for the assay of low concentrations of diphtheria antitoxin in human sera has been developed, using a monkey kidney cell (VERO) culture technique. Results obtained with sera from nonvaccinated children and with immune sera from children vaccinated with three and four injections of diphtheria pertussis tetanus vaccine were in agreement with antitoxin levels considered necessary to denote immunity to diphtheria. The use of microplates and organic buffer for culturing the animal cells improved the stability of the tissue culture system. The described method is sensitive, economical, and applicable for the titration of antitoxin in human sera particularly from infants and children from whom a minimum amount of serum is available.

Since Levaditi and Mutermilch reported in 1913 (4) that chicken embryo tissue cultures were susceptible to diphtheria toxin and that the cytopathic effect of the toxin was prevented by homologous antitoxin, several investigators have successfully used tissue culture techniques to determine the specific antitoxin activity of human sera (3, 5, 9). Different animal cells were used, and the results obtained in vitro were comparable to those obtained by the rabbit intradermal test (1, 7, 10). Although tissue culture methods have been an important development for assays of low concentrations of diphtheria antitoxin in human sera, they require a substantial amount of blood, often more than 1 ml.

Recently, microtissue culture techniques have been regarded as sensitive, economical, and applicable to the study of infectious diseases (2, 8, 11, 12).

The present study was undertaken to examine the possibility of developing a routine, reproducible, microtissue culture method for the titration of diphtheria antitoxin in minimal amounts of human sera from infants and children.

MATERIALS AND METHODS

Tissue culture. Monkey kidney cells (VERO), established from primary African green monkey kidney cultures, were obtained from Flow Laboratories Inc., Rockville, Md. For our studies, VERO cells were subcultured weekly in 75-cm² Falcon plastic flasks containing tissue culture medium. After trypsinization, the monolayer was dispersed, and 0.1-ml samples of VERO suspensions (3.2 x 10⁵ cells per ml) were dispensed in microplates and incubated at 36.5 C in a humidified incubator. Confluent monolayer culture was obtained after overnight incubation.

Tissue culture media. Planting media consisted of Eagle minimum essential medium (MEM) (Grand Island Biological Co., N.Y.) supplemented with 10% (v/v) fetal calf serum. Maintenance media consisted of MEM with only 1% (v/v) fetal calf serum. All media were prepared in Earle balanced salt solution buffered with a mixture of 24 mm sodium bicarbonate and 30 mm HEPES (N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid) (6, 13).

Diphtherial reagents. Diphtheria toxin (IMH lot number 246-1) stabilized in gelatin borate buffer at pH 7.6 (lethal dose = 1/40 as measured in guinea pigs) was used in all experiments. U.S. standard antitoxin, 6 antitoxic units (AU) per ml, was used as a reference in our experimental procedures.

The effect of diphtheria toxin was prevented by homologous antitoxin at very low concentrations, whereas tetanus antitoxin did not protect the tissue culture against the destruction by diphtheria toxin.

Human sera. All sera were derived from infants and children admitted in a local hospital for other reasons than infectious diseases. The medical files indicated their status of vaccination with diphtheria pertussis tetanus (DPT) and the number of injections (Table 1).

Microtitration equipment. Microdiluters (capacity of 0.025 ml) and micro-pipettors (0.025 ml or 0.05 ml of fluid per drop) (Cooke Engineering Co., Alex-
andria, Va.) were used. Disposo-trays FB-96TC with covers were employed (Linbro Chemical Co., New Haven, Conn.).

RESULTS AND DISCUSSION

The initial step in the tissue culture method for antitoxin titration was the determination of the minimal cytotoxic dose (MCD) of diphtheria toxin. The MCD was defined as the greatest dilution of diphtheria toxin causing complete destruction of tissue culture cells in 4 days. This MCD value is obtained with 0.025 ml of our standard toxin diluted 1:10⁸ (1:400,000,000 final concentration) and did not change during the course of repeated titrations. We then determined the lethal toxic concentration (Ltc dose) at the levels of Ltc/100, Ltc/1,000, and Ltc/10,000 toxin dilutions. In the preliminary titration to determine the Ltc/100 (the dilution of toxin which, when mixed with 0.01 AU, will give at least a 3 to 4+ cytotoxic reaction [75 to 100%] in the tissue culture, whereas the same dilution mixed with 0.011 AU will show monolayer destruction of 25% or less), equal volumes of various dilutions of toxin were mixed with antitoxin so that the final concentration of antitoxin was 0.01 AU in 0.2 ml.

In our studies, the Ltc/100 determination was performed by mixing 0.1 ml of toxin diluted 1/110 and 0.1 ml of standard antitoxin diluted to 0.01 AU/ml and by incubating the mixture at room temperature during 1 hr. Samples of 0.025 ml were distributed in each of four cups (Disposo-trays) containing the tissue culture monolayers. The microplates were then incubated at 36.5 C in a humified incubator. Readings of the cytotoxic effect were made after 4 days of incubation. In our experimental conditions, the Ltc/100, Ltc/1,000, and Ltc/10,000 were established and corresponded to the toxin’s dilutions of 1/110, 1/1,200, and 1/12,000, respectively.

The titration of the serum was made by diluting it at 1:10 (0.025 ml of serum + 0.225 ml of tissue culture medium) and then mixing 0.1 ml of subsequent tenfold dilutions with 0.1 ml of toxin diluted to the Ltc/10,000 (i.e., 1/12,000 dilution of toxin).

The present data demonstrate the high susceptibility of the monkey kidney cells (VERO) to diphtheria toxin. Complete destruction of monolayer was consistently observed with 0.025 ml of our standard toxin diluted 1:10⁴. A 4 + destruction of the monkey kidney cells was obtained with a 1:12,000 dilution of toxin mixed with 0.01 AU/0.1 ml.

The cytotoxic effect caused by diphtheria toxin was easily seen (Fig. 1). After 1 day of incubation, the cytopathic effect was estimated as 25% or 1 + monolayer destruction (Fig. 1B). Small, rounded refringent cells and holes were observed in the monolayer. The number of rounded cells and holes in the cell sheet increased after 48 hr of incubation and affected 50% or 2 + of the tissue culture (Fig. 1C). Finally, complete destruction of the cell sheet occurred invariably after 4 days of incubation (Fig. 1D). The degree of cytopathic effect observed in each cup incubated with the same dose of toxin or of toxin-antitoxin mixture was consistently identical in all four cups.

The stability of the monolayer is known to be affected by the pH of the medium. Alkaline reactions as well as acid reactions which could injure the monolayer were never observed in our tissue culture system. These undesirable side reactions were prevented by employing an organic compound (HEPES) in our buffer system. The use of such an organic buffer allowed us to perform our tests in unsealed microplates and to incubate them in an ordinary humidified incubator.

Twelve sera from infants and children were tested with the microtissue culture test, and the results are summarized in Table 1. Data are presented according to age and indicate the state of immunity after three or four injections of DPT vaccine. The vaccination is made according to the following schedule: three serial injections at 2, 4, and 6 months, and one booster dose at 12 to 18 months of age.

One can see that child MB and child IE, 2 and 3 years of age, respectively, who were not vaccinated, presented less than 0.01 AU/ml in their serum. Three out of four children that have been vaccinated with three injections had

| Serum identification | Age      | DPT vaccination | Antitoxin units per ml |
|----------------------|----------|-----------------|------------------------|
| BL                   | Newborn  | Nil             | 0.01-0.1               |
| DL                   | 7 months | x3              | 0.1-1                  |
| AS                   | 12 months| x4              | 0.1-1                  |
| ID                   | 16 months| x3              | 0.01-0.1               |
| YT                   | 18 months| x3              | 0.01-0.1               |
| MB                   | 2 years  | Nil             | <0.01                  |
| NM                   | 2 years  | x4              | 0.1-1                  |
| CS                   | 2.5 years| x4              | >1                     |
| IE                   | 3 years  | Nil             | <0.01                  |
| DT                   | 6 years  | x4              | 0.1-1                  |
| PN                   | 9 years  | x4              | 0.1-1                  |
| JA                   | 12 years | x3              | 0.01-0.1               |
Fig. 1. Cytopathic effect of diphtheria toxin on VERO: monolayer cell control (A), cell affected with 25% or 1+ destruction (B), 50% or 2+ destruction (C), complete destruction or 4+ (D). Magnification, x100.

ACKNOWLEDGMENTS

We thank J. H. Joncas for making the human sera available and H. Durand and N. Rocque for their excellent technical assistance.

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