Double-Antibody Sandwich Enzyme-Linked Immunosorbent Assay for Rapid Detection of Toxin-Producing *Corynebacterium diphtheriae*

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An enzyme-linked immunosorbent assay for determining the toxigenicity of *Corynebacterium diphtheriae* is presented. The assay uses hyperimmune horse diphtheria antitoxin as a capture antibody and mouse monoclonal diphtheria antitoxin as a detecting antibody. Growth of bacteria and capture of diphtheria toxin by antitoxin are carried out in one step. Toxin produced by as little as 100 toxin-producing corynebacteria is detectable, corresponding to a sensitivity of 10 ng of diphtheria toxin per ml. Demonstration of toxin after incubation of the bacteria for 4.75 h, as well as after 18 h, was in accordance with the modified Elek gel diffusion method and the guinea pig inoculation test. However, heavy inocula incubated overnight produced significantly lower optical density than did diluted inocula; thus, the higher optical density was used as an indicator of toxin production. A decrease in optical density was also seen by shortening the incubation time. For laboratory safety, ethanol was added to the microtiter plate wells before washing out of the bacteria. This resulted in a further decrease in optical density. Using 4.75-h incubation time gave a single false-negative result. No false-positive results were ever seen. Incubation for 18 h is suitable for large-scale screening, and 4.75 h of incubation is suitable for rapid identification of toxin-producing *C. diphtheriae*.

Diphtheria has almost disappeared in Western Europe and the United States. However, small outbreaks of diphtheria in unprotected populations still call for laboratory preparedness for making rapid identification of toxin-producing strains of *Corynebacterium diphtheriae* (1-3, 5, 7, 11, 15). Such occasional outbreaks often result in a demand for large-scale screenings, leading to an extreme strain on the laboratories involved (2, 9, 27).

We developed an enzyme-linked immunosorbent assay (ELISA) which, in less than 8 h, can demonstrate production of toxin from *C. diphtheriae*. The same ELISA is suitable for large-scale screenings. The present study demonstrates the use of this ELISA and discusses some technical problems.

MATERIALS AND METHODS

Toxin, toxoid, and antitoxin. Purified diphtheria toxin isolated from a culture of *C. diphtheriae* subsp. *intermedius* Park-Williams no. 8, used for vaccine production, was used (24). Diphtheria toxoid was produced from formaldehyde-treated toxin by conventional methods (30).

Hyperimmune horse diphtheria antitoxin was produced after repeated immunization of horses with toxoid adsorbed onto Al(OH)₃ partially purified on a DE-52 anion-exchange column. Mouse monoclonal diphtheria antitoxin from clone 17.6B9G6C9 (17.6) was produced by conventional methods, essentially as described by Köhler and Milstein (14), by fusion of the myeloma cell line X63 Ag8 6.5.3 with spleen cells from BALB/c mice immunized with diphtheria toxoid adsorbed onto Al(OH)₃. Monoclonal antibody 17.6 was of the immunoglobulin G1 (IgG1) subclass, with κ light chains. This antibody had diphtheria toxin-neutralizing activity when assessed in an in vitro neutralization system. The unfractionated culture supernatant was used. It contained 10 μ g of specific antibody per ml.

Conjugated secondary antibody. Peroxidase-conjugated rabbit anti-mouse IgG (code P161) was obtained from DAKOPATT, Glostrup, Denmark.

Culture medium. Linggood tryptic digest medium was used to grow bacteria in microtiter plate wells; the same medium is used to grow bacteria for vaccine production (16).

Bacteria. Three reference strains from the National Collection of Type Cultures, London, England, were used. Of two *C. diphtheriae* subsp. *gravis* strains, one (NCTC 10648) had strong and another (NCTC 3984) had light toxin production; the third strain, *C. diphtheriae* subsp. *mitis* (NCTC 10356), with no toxin production, served as a negative control. *C. diphtheriae* subsp. *intermedius* Park-Williams no. 8 was also tested, as were 24 *C. diphtheriae* subsp. *gravis*, 24 *C. diphtheriae* subsp. *intermedius*, and 42 *C. diphtheriae* subsp. *mitis* strains isolated during the epidemic outbreaks of diphtheria in Denmark in the 1940s.

All strains were examined for toxin production by animal inoculation (19), as well as in vitro, by using the Elek precipitation method (10, 12). Toxigenicity was found in 22 *C. diphtheriae* subsp. *gravis*, 23 *C. diphtheriae* subsp. *intermedius*, and 5 *C. diphtheriae* subsp. *mitis* strains.

Modified Elek gel diffusion method. After subculture of Loeffler serum medium (17) for 4 to 6 h, the strains were transferred to Elek base medium containing 16% horse serum. In the bottom of the dish was placed a horse diphtheria antitoxin-impregnated strip of Whatman no. 1 filter paper. Control strains *C. diphtheriae* NCTC 10648, NCTC 3984, and NCTC 10356 were always assayed simultaneously.

In vivo intradermal guinea pig inoculation test. The collection of *C. diphtheriae* was tested for toxin production during the epidemic in the late 1940s, and the recorded results were used in this study (19).

Bacterial culture. The bacteria were grown overnight on blood agar at 35°C. Colonies were transferred to Linggood medium for culture directly in microtiter plate wells. Titra-

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FIG. 1. Titration of capture antibody (A) and detecting antibody (B). Microtiter plates were prepared by incubation of horse diphtheria antitoxin in serial dilution (A) or diluted 1:1,000 (B), followed by incubation of diphtheria toxoid (1 µg/ml). The plates were then reacted with mouse monoclonal diphtheria antitoxin (17.6), 0.1 µg/ml (A) or various concentrations (B), followed by incubation with labeled anti-mouse IgG diluted 1:1,000 and containing 1% horse serum.

tion curves for measurement of toxin production were constructed by using various dilutions of bacteria.

When testing the collection of strains, a few bacterial colonies were suspended in 1 ml of Linggood medium, and 100 µl of this suspension was applied to each well in dilutions of 1:1 and 1:1,000.

ELISA technique. Each well of a polystyrene flat-bottomed microtiter plate was coated overnight at 4°C with 100 µl of horse antitoxin diluted in carbonate-bicarbonate buffer, pH 9.6, and then washed four times with phosphate-buffered saline containing 1% Triton X-100, pH 7.2. The toxin, the toxoid, or the bacteria were diluted in Linggood

medium and incubated at 20 or 35°C for various times. After washing, mouse monoclonal antitoxin was added for 1.5 h at 20°C. Another washing was followed by 100 µl of peroxidase-conjugated rabbit anti-mouse IgG (heavy chain specific; DAKOPATT no. P161) diluted 1:1,000 and containing 1% horse serum. After incubation for 1 h at 20°C and another wash, 100-µl volumes of freshly prepared substrate (0.4 mg of *o*-phenylenediamine per ml, 0.06% hydrogen peroxide in citrate-phosphate buffer, pH 5) were added. The reaction was allowed to proceed for 30 min at 20°C before addition of 150 µl of 1 M sulfuric acid. Optical density (OD) was measured at 492 nm in a photometer (Teknunc Immunoreader NJ-2000).

Statistical analysis. Levels of statistical significance were evaluated by using the median OD values and the confidence limits.

RESULTS

Capture ELISA for detection of diphtheria toxin. Horse diphtheria antitoxin was used for coating microtiter plates in various dilutions. A dilution of 1:1,000, corresponding to a protein concentration of 10 µg/ml, was found to be optimal (Fig. 1A). Binding of diphtheria toxin was detected by mouse monoclonal antibody to diphtheria toxoid (17.6), and here the optimal dilution was 1:100, corresponding to an IgG concentration of 0.1 µg/ml (Fig. 1B). Finally, the optimal dilution for detecting peroxidase-conjugated rabbit anti-mouse IgG was 1:1,000 (DAKOPATT no. P161), and 1% horse serum was added to remove cross-reactive antibody specificities.

Sensitivity of the ELISA. Purified diphtheria toxin was

FIG. 2. Sensitivity and specificity of the assay. Microtiter plates were prepared by incubation of horse diphtheria antitoxin (10 µg/ml) and then various concentrations of diphtheria toxin and toxoid. Incubation time was 18 h at 22°C. The plates were then reacted with mouse monoclonal diphtheria antitoxin (17.6), 0.1 µg/ml, followed by incubation with labeled anti-mouse IgG diluted 1:1,000. The assay is capable of detecting approximately 10 ng of purified diphtheria toxin or toxoid per ml, corresponding to dilution 5×10^{-6} . The concentration of undiluted toxin containing undiluted toxoid was 1 mg/ml.

FIG. 3. Titration of *C. diphtheriae* subsp. *gravis* NTCT 10648 (●) and NCTC 3984 (▲) and *C. diphtheriae* susp. *mitis* NTCT 10356 (○). Microtiter plates were prepared by incubation of horse diphtheria antitoxin (10 µg/ml). Various numbers of bacteria measured by CFU were grown in the microtiter plate wells at 35°C for 18 h (A) or 4.75 h (B). After washing, the plates were reacted with mouse monoclonal diphtheria antitoxin (17.6), 0.1 µg/ml, followed by incubation with labeled anti-mouse IgG diluted 1:1,000. The data are from a single experiment, with all incubations done in duplicate.

used to estimate the sensitivity of the ELISA. Figure 2 shows a titration curve in which undiluted toxin had a concentration of 1 mg/ml. This figure shows that a toxin dilution of 1:100,000, corresponding to a toxin concentration of 10 ng/ml, can be detected. For comparison, a purified diphtheria toxoid was also titrated, and also with this antigen a dilution corresponding to 10 ng/ml could be detected. The figure shows a somewhat better reaction with toxoid than with toxin. The explanation for this is most likely the fact that the monoclonal antibody was raised against toxoid and that the affinity is therefore higher when antibody reacts with toxoid than when it reacts with toxin.

We also assayed the influence of incubation for 4.75 or 18

FIG. 4. Determining the toxigenicity of 90 strains of *C. diphtheriae*. Microtiter plates were prepared by incubation of horse diphtheria antitoxin (10 µg/ml). Bacteria suspended in Linggood medium 1:1 or 1:1,000 were grown in the microtiter plate wells at 35°C for 18 h. After washing, the plates were reacted with mouse monoclonal diphtheria antitoxin (17.6), 0.1 µg/ml, followed by incubation with labeled anti-mouse IgG diluted 1:1,000. The higher OD values are given according to biotype. The median OD and the 99% confidence limits are given for the toxigenic strains.

h at 22 or 35°C. We found that 18 h of incubation at 22°C was optimal.

Measurement of production of diphtheria toxin in antibody-coated microtiter plate wells. *C. diphtheriae* was cultured on blood agar plates, and after incubation overnight, various numbers of bacteria were incubated in microtiter plate wells previously coated with horse diphtheria antitoxin. Incubation time varied from 4.75 to 18 h. Toxin production could already be demonstrated after 4.75 h and with a primary inoculum of 100,000 bacteria and after 18 h with a primary inoculum of 100 bacteria (Fig. 3). Culture of non-toxin-producing *C. diphtheriae* subsp. *mitis* (NCTC 10356) in Linggood medium containing toxin could be shown not to influence detection of toxin.

Figure 3 also shows that increasing the number of bacteria in the microtiter wells sometimes led to a lower estimated toxin value. The effect was only noticed after incubation for 18 h; the mechanism for this is unknown.

Toxin production by individual strains of *C. diphtheriae*. The ELISA was tested with 90 strains of *C. diphtheriae*. Bacterial colonies grown on blood agar plates were suspended in Linggood medium, and all of the strains were incubated in 1:1 and 1:1,000 dilutions. The 1:1,000 dilution of toxin-producing strains yielded a significant increase in median OD from 0.42 to 0.64 ($P < 0.001$); thus, the higher OD was used as an indicator of toxin production (Fig. 4). A

single strain which formerly was toxin producing by the guinea pig inoculation test and the modified Elek gel diffusion method had lost its ability to produce toxin. Retesting the strain confirmed the loss. Also during this study, a subculture of the Park-Williams no. 8 strain lost its ability to produce toxin as detected both by the Elek method and the ELISA. We found an overnight (18 h) incubation period suitable for a screening assay and a 4.75-h incubation period suitable for rapid demonstration of toxin production in a specific strain. The differences in OD and sensitivity are given in Fig. 5.

The ELISA would detect toxin when an OD of 0.050 was read. Using this OD as a cutoff, a 100% accordance among the 18-h ELISA, the modified Elek gel diffusion method, and the guinea pig inoculation test was found in demonstrating toxin production.

Different ways of applying the bacteria to the microtiter plate were assessed. We tried to let the bacteria dry out on the plate and also to cultivate the bacteria in tubes and then make a sterile filtrate of the medium. Neither method yielded better results than when the bacteria were cultured directly in microtiter plate wells.

To prevent laboratory infections when growing the bacteria directly in microtiter plates, we inactivated all of the bacteria by adding 150 μ l of absolute ethanol to the 100 μ l of medium. The ethanol was left for 5 min, and the control cultures from the wells showed no growth. A significant decrease in the median OD value from 0.62 to 0.54 was found ($P < 0.01$), but still all of the toxin-producing strains were found to be positive (Fig. 5). When ethanol was applied to the rapid variant of the assay with only 4.75 h of incubation time, the median OD value decreased significantly from 0.40 to 0.19 ($P < 0.001$), and the assay yielded a single false-negative result (Fig. 5). Every day, serial dilutions of diphtheria toxoid were assayed. The daily variation was less than 10%.

FIG. 5. Effect of ethanol on the measured OD values of 25 toxin-producing strains of *C. diphtheriae*. Microtiter plates were prepared by incubation of horse diphtheria antitoxin (10 μ g/ml). Bacteria were grown in the microtiter plate wells at 35°C for 18 or 4.75 h. Absolute ethanol was added to the wells for 5 min. Corresponding wells were without ethanol. After washing, the plates were reacted with mouse monoclonal diphtheria antitoxin (17.6), 0.1 μ g/ml, followed by incubation with labeled anti-mouse IgG diluted 1:1,000. The median OD and the 99% confidence limits are given.

DISCUSSION

Increased communication between industrial countries and developing countries, together with immigration of large numbers of refugees, increases the risk of reintroducing diphtheria into Western Europe (23, 25; Editorial, Lancet ii:949-950, 1983).

Refugees often give rise to special problems because they usually live in very crowded conditions before they are accepted as political refugees. During this period, conditions are optimal for creating an epidemic, and spreading carriers throughout the country.

Survey studies of the current vaccination policy have revealed that older people must be considered unprotected against diphtheria. In survey studies from Denmark and Canada, no protective antibodies were found in 19% of the population, and the need for revaccination of adults is under debate (1, 8, 13, 18, 22, 26, 28; Editorial, Lancet i:1081-1082, 1985). In the United States most outbreaks are characterized by a high attack rate for American Indians, poor socioeconomic conditions, and incomplete immunization (5). Two localized outbreaks of diphtheria in Gothenburg and Stockholm, Sweden, were connected with subpopulations of persons abusing drugs and alcohol (1).

Immunological methods used to identify *C. diphtheriae* have been described, but they have not yet been widely used (4, 6, 20, 21, 29). Immunofluorescence techniques do not always give satisfactory results on slides made directly from clinical specimens. Furthermore, the techniques do not discriminate between toxigenic and nontoxigenic strains. Cross-reactions may occur with strains of diphtheroids, mycobacteria, nocardia, and staphylococci. Slide agglutination is considered rather specific for *C. diphtheriae* (87%). However, spontaneously agglutinable or inagglutinable strains occur (4).

Suspected cases of diphtheria raise two major problems. First, does a suspected isolate produce toxin or not? Here our conventional methods—the modified Elek gel diffusion method and the guinea pig inoculation test—may take 1 to 3 days before a result can be obtained; furthermore, the guinea pig inoculation test is often difficult to interpret. The ELISA described here gives an answer after about 6 h. Second, a few cases or even a single case of diphtheria may be followed by a demand for large-scale screening, creating a heavy strain on the laboratories in question (2, 9, 27). During the outbreak in Sweden in 1984 and 1985, the number of specimens tested in the laboratory soon increased from 10 a year to 1,600 a day. Such large-scale screening will be facilitated by the described ELISA with automatic reading of the plates.

We have described an assay for diphtheria toxin production after cultivation of bacteria on blood agar. We believe that the method described will be useful as a standard one for fast and reliable diagnosis of toxin-producing strains of *C. diphtheriae*. Because the bacteria are not fixed to the solid phase in our assay, cross-reaction should not be possible.

Our ELISA combines growth of bacteria and capture of toxin by antitoxin in one step. Additionally, we do not need to subculture the bacteria on Loeffler serum medium. Detection of toxin is a qualitative rather than a quantitative test. As shown in Fig. 3 and 4, a high inoculum of bacteria may yield a lower OD value than does a smaller inoculum. This problem, however, is solved by inoculating two different dilutions of bacteria (e.g., 1:1 and 1:1,000). The higher OD values is used as an indicator of toxin production. We have never seen a false-positive OD value. Growth of *C.*

diphtheriae subsp. *mitis* (a non-toxin-producing strain, NCTC 10356) does not affect toxin detection. Growth of bacteria directly in microtiter plate wells gives rise to special problems concerning laboratory safety. However, addition of absolute ethanol to a final concentration of 60% was sufficiently bactericidal. Both incubation at 35°C rather than 20°C and addition of ethanol decreased the OD value, but because of the very high sensitivity of the assay, no false-negative strains were found after incubation for 18 h.

It would be of interest to investigate whether swabs from suspected individuals can be cultivated directly in microtiter wells. Such an investigation, however, is only possible in an area where diphtheria is still endemic or epidemic.

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