Quantitation of *Clostridium perfringens* Type A Enterotoxin by Electroimmunodiffusion

CHARLES L. DUNCAN AND EILEEN B. SOMERS

Food Research Institute and Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706

Conditions for quantitation of *Clostridium perfringens* type A enterotoxin by electroimmunodiffusion are described. As little as 0.01 µg of enterotoxin could be detected. Electroimmunodiffusion was more sensitive than either single gel diffusion or quantitation based on erythemal activity of the toxin in guinea pig skin.

Clostridium perfringens type A enterotoxin can be readily detected on the basis of its biological activity. The toxin induces fluid accumulation in ligated ileal loops of rabbits and lambs (1, 4), is lethal in mice (3, 7), and produces erythema in the skin of guinea pigs or rabbits (2, 7). For quantitative purposes, the assay based on erythemal activity is more sensitive than the loop technique or the measurement of lethality in mice (3, 7). All of these techniques suffer from the variations in sensitivity of the animals being used, the requirement for sufficient controls to eliminate the activity of other toxins or nonspecific responses, and the cost of the animals involved.

This study was initiated to determine the feasibility of using immunochemical procedures for specific enterotoxin quantitation. The technique of electroimmunodiffusion was chosen for investigation. This technique has been used for accurate and sensitive quantitation of a variety of proteins including immunoglobulins and albumin (5) and type A botulinum toxin (6). The procedure involves use of a high-voltage electrical field to induce rapid migration of a protein sample from a well into gel containing antibody prepared against the protein. A cone of protein antigen is precipitated along the path of antigen migration wherever antigen-antibody equivalence is reached. The cone length is proportional to the amount of protein in the sample. Conditions to be considered in adapting this method to the measurement of any protein soluble in low-ionic-strength electrolyte solutions have been discussed (5). The results reported here demonstrate the usefulness of the method for quantitating *C. perfringens* type A enterotoxin. The method is also compared with quantitation of enterotoxin by single gel diffusion and by the skin test.

MATERIALS AND METHODS

Enterotoxin. Crude enterotoxin was obtained from extracts of sporulating cells of *C. perfringens* NCTC 8239 that had been grown for 8 hr at 37 C. The enterotoxin was purified by chromatography on Sephadex G-100 and diethylaminoethyl Sephadex. The enterotoxin used was 97% pure and had a specific activity of 3,230 erythemal units/mg of protein. Dilutions of enterotoxin for electroimmunodiffusion were prepared in the barbital-acetate buffer of μ 0.025 described below.

Antisera. Antiserum was prepared against partially purified enterotoxin and against crude enterotoxin as previously described (7). At the dilutions used, the antiserum against partially purified toxin was specific only for enterotoxin antigen. Approximately 17,000 erythemal units (7) could be neutralized by 1.0 ml of this antiserum. The antiserum prepared against crude enterotoxin was rendered specific for the enterotoxin by absorption with crude cell extract of an enterotoxin-nonproducing, nonsporulating mutant (8-1) of strain NCTC 8798.

Electroimmunodiffusion buffer. Barbital-acetate buffer, pH 8.6, was prepared in distilled, deionized water. The composition of the stock buffer was 3.09% sodium barbital, 0.552% barbital, and 1.23% sodium acetate. The buffer solution was heated at 55 C for 4 hr to dissolve the buffer salts. A continuous buffer system was used for electrophoresis. The electrode vessels contained buffer of μ 0.1, prepared by diluting stock buffer with two volumes of water, and the gel solutions were prepared with μ 0.025 buffer obtained by diluting the stock buffer with 11 volumes of water.

Preparation of electroimmunodiffusion slides. Projector slide cover glasses (Kodak), 8.25 by 10.17 cm, were used for support of the agar gel. A thin coating of 2.0% Ionagar no. 2 (Oxoid) was applied to the glass slides with a brush and allowed to harden for 15 min. The slide was then layered with 15 ml of agarose-antiserum mixture. Unless indicated otherwise, the agarose used was obtained from Fisher Scientific Co. The agarose-antiserum mixture was prepared by mixing equal amounts of antiserum

(diluted in μ 0.025 buffer) and agarose, both warmed to about 50 C. Except where indicated, the final dilution of antiserum was 1:100 and the final concentration of agarose was 0.6%. The slides were allowed to harden for at least 15 min. The slides may be conveniently prepared ahead and held in a humidity chamber prior to use.

Sample wells 3 mm in diameter were punched along the longer dimension of the slide and at the cathode end. The wells were positioned 20 mm from the end of the slide and 8 mm apart. A wick of Whatman no. 40 filter paper was sealed to each end of the slide with 2.9% Ionagar no. 2.

Conditions of electroimmunodiffusion. An electrophoresis chamber with a cooling platform for holding gel plates was constructed from Plexiglas acrylic sheets. The chamber size was sufficient to accommodate six slides. An ethylene glycol solution circulating through the cooling platform was maintained at 4 C.

Sample wells were filled with 10 μliters of appropriately diluted enterotoxin immediately prior to electrophoresis. Electrophoresis was conducted for 1 to 8 hr at a constant current of 10 ma/slide. The initial potential gradient was 12 v/cm of gel. After electrophoresis, slides were removed to a humidity chamber and allowed to develop for 16 to 24 hr. This period of time was necessary for optimal demonstration of cones of precipitate, especially at higher antiserum dilutions. Cone length was determined by measuring from the top of the sample well to the tip of the cone. When desired, the slides were overlaid with moist filter paper, dried at 37 C, and stained with Coomassie brilliant blue. The slides could then be stored indefinitely.

Single gel diffusion. Quantitation of enterotoxin in single gel diffusion tubes was used for comparison with electroimmunodiffusion. The buffer used for preparing agarose gels (Fisher Scientific Co.) and for dilutions of enterotoxin was 0.02 M, pH 7.4, phosphate-buffered saline containing 0.04% thimerosal. The buffer was prepared according to Weirether et al. (8). Immunodiffusion tubes (Corning Glass Works) were 6 by 50 mm. A total of 3.0 ml of agarose-antiserum mixture was added to each tube. The final concentration of agarose was 0.5%. The maximal dilution of antiserum used was 1:40. The hardened gels were overlaid with 3.0 ml of appropriately diluted enterotoxin. The tubes were covered with Parafilm and allowed to develop at 37 C. The length of precipitate bands was measured at 24-hr intervals for a total of 120 hr.

Erythemal activity. Erythemal activity was determined as described previously (7).

RESULTS AND DISCUSSION

Determining the proper antiserum concentration for electroimmunodiffusion. The effect of antiserum concentration on cone height of various concentrations of enterotoxin is shown in Fig. 1. The cone height is a function not only of the concentration of enterotoxin antigen, but also of the concentration of an-

tiserum in the agarose gel. As the concentration of antiserum is decreased in the agarose gel, the enterotoxin antigen migrates further towards the anode. As the antigen continues to migrate, precipitation occurs on the sides of the cone, thus reducing the amount of migrating antigen. Eventually, equivalence of antigen and antibody is reached at the top of the cone and precipitation occurs. Although the cone height can be increased by decreasing the antiserum concentration, a resulting decrease in clarity of the cone occurs, owing to less specific immune precipitation. Cone heights were most easily determined within a range of about 0.5 to 4.5 cm. The antiserum concentration necessary for producing cones of this height must be determined for each different batch of antiserum that may be employed.

Antiserum prepared against crude enterotoxin and made specific by absorption with extract of an enterotoxin-nonproducing mutant also was used for enterotoxin quantitation. The results were similar to those obtained with antiserum prepared against purified enterotoxin. Thus, purified enterotoxin is not an absolute necessity in preparing specific antiserum for quantitation of enterotoxin by electroimmunodiffusion.

Effect of duration of electrophoresis on cone height. As the concentration of enterotoxin increased, the electrophoresis time required for maximal cone height was also increased (Fig. 2). If electrophoresis is continued until all enterotoxin antigen has been consumed, a linear relationship exists between
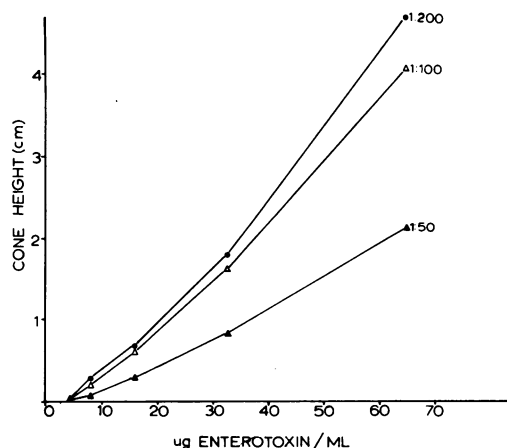

FIG. 1. *Effect of antiserum concentration on cone height of various concentrations of enterotoxin. Antiserum dilution is indicated on the curves. Electrophoresis was for 8 hr. Antiserum was prepared with purified enterotoxin.*
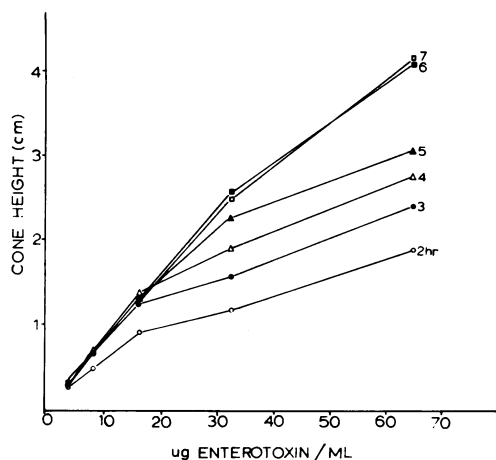
FIG. 2. *Effect of duration of electrophoresis on cone height at various concentrations of enterotoxin. Antiserum was diluted 1:100.*

cone height and enterotoxin concentration. Cones in which migration is complete are sharp and pointed, whereas in those in which migration is not complete the cone tends to be rounded. When the conditions of antiserum concentration, duration of electrophoresis, and concentration of enterotoxin are arranged to achieve a linear relationship between concentration and cone height, a minimum of two standards may be used to establish a calibration curve for each plate. The cone height of unknown concentrations should be equal to or less than that obtained for the highest concentration of standard used in establishing conditions for a linear standard curve. For routine quantitation, we have used an antiserum dilution of 1:100, electrophoresis for 6 hr, and two standard enterotoxin samples containing 8.135 and 32.5 μg/ml, respectively. As little as 1 μg of enterotoxin/ml could be detected. This concentration of enterotoxin produced a cone height of 0.1 cm under these conditions. This was considered a minimal measurable cone height. However, for routine quantitation purposes, a minimal height of 0.5 cm was used.

Choice of suitable agar for gel preparation. A variety of agar gels were tested for their suitability in electroimmunodiffusion. The choice of agarose versus Noble agar for an electrophoresis gel is dependent on the charge of the protein antigen at the pH of the buffer to be used in the agar (5). The isoelectric point of the enterotoxin protein is 4.3. At the buffer pH used in these experiments, the protein is negatively charged. Agarose is an uncharged gel and gives a relatively low electroosmosis. Thus, proteins with a negative charge migrate toward

the anode in an agarose gel. Noble agar possesses charged groups and gives a relatively high electroosmosis which may affect the otherwise normal direction of migration of a protein.

When Noble agar (Difco) was used as the support gel, retrograde migration of the antigen towards the cathode occurred. Considerable drying of the agar layer at the cathode end of the slide also occurred. Reversing the polarity resulted in retrograde migration of the antigen towards the anode and drying at the cathode end of the slide.

Agarose obtained from Fisher Scientific Co., Bio-Rad Laboratories, or Gallard-Schleisinger Chemical Co. served equally well as a support medium in allowing normal migration of the enterotoxin toward the anode. However, agarose obtained from Calbiochem was unsatisfactory in that extreme drying occurred at the cathode end, as it did with Noble agar. Very little migration of the antigen occurred when this gel was used. The Calbiochem agarose used
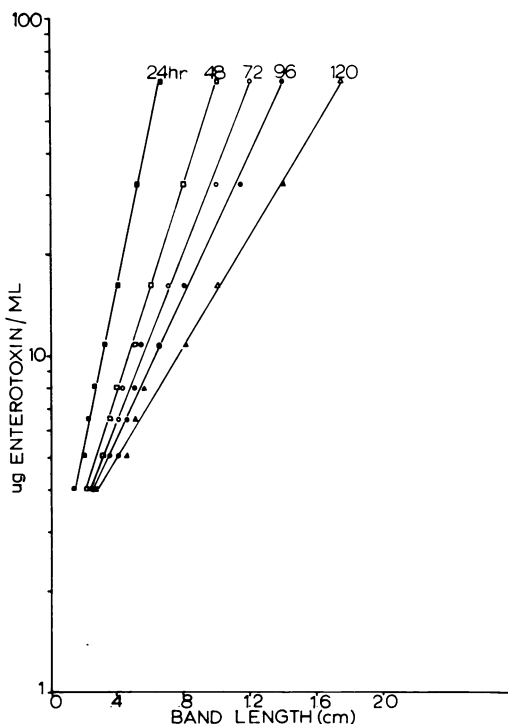

FIG. 3. *Effect of incubation time on the calibration curve for enterotoxin quantitation by the single gel diffusion technique. Precipitation band lengths were measured after the time intervals indicated on each curve. Antiserum was diluted 1:40. Extrapolation of the curve obtained at 24 hr allows determination of a minimal concentration of 3.0 μg/ml with the use of a lower band length limit of 0.1 cm.*

in the present experiments was apparently sufficiently charged to result in high electroosmosis during electrophoresis.

Quantitation of enterotoxin by single gel diffusion. A linear relationship existed between the length of the band of precipitate occurring in single gel diffusion tubes and the logarithm of the concentration of enterotoxin antigen (Fig. 3). Increasing the incubation time from 24 to 120 hr increased the length of the bands without appreciably affecting the sensitivity of the assay. The maximal dilution of antiserum that would allow the production of a visible band of precipitate over the range of enterotoxin concentrations tested was 1:40.

Comparison of assay procedures. A comparison of the sensitivity of the erythemal activity skin test, electroimmunodiffusion, and single gel diffusion techniques for quantitation of *C. perfringens* enterotoxin is presented in Table 1. Electroimmunodiffusion was the most sensitive technique and the skin test was the least sensitive. On a weight basis, as little as 0.01 µg of enterotoxin could be detected by

TABLE 1. *Comparison of sensitivity of erythemal activity test, electroimmunodiffusion, and single gel diffusion techniques for quantitation of C. perfringens enterotoxin*

Quantitation technique	Enterotoxin detected[a]	
	Wt (µg)	Concn (µg/ml)
Erythemal activity	0.19	3.80
Electroimmunodiffusion	0.01	1.0
Single gel diffusion	—	3.0

[a] The lower end point for measurement of cone height in electroimmunodiffusion at an antiserum dilution of 1:100 was 0.1 cm. The lower end point for measurement of band length in single gel diffusion at an antiserum dilution of 1:40 was 0.1 cm. The values for erythemal activity are based on 3.23 erythemal units/µg of protein. A lower limit of 0.625 erythemal unit (skin reaction 0.5 cm in diameter) was considered the minimal detectable level.

electroimmunodiffusion as compared with 0.19 µg by the skin test.

Either electroimmunodiffusion or the single gel diffusion technique may conveniently be used in lieu of the biological activity test in the skin of guinea pigs for quantitation of enterotoxin. Electroimmunodiffusion is the more desirable of the two techniques because of the considerably smaller sample required, the lower concentration of antiserum needed, and its greater sensitivity.

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