Highly Sensitive Assay for *Clostridium perfringens* Enterotoxin That Uses Inhibition of Plating Efficiency of Vero Cells Grown in Culture

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A highly sensitive and reproducible biological assay for *Clostridium perfringens* enterotoxin is described that uses Vero (African green monkey kidney) cells grown in tissue culture. Very small doses of the enterotoxin inhibited the plating efficiency of the cells. This inhibition of plating efficiency could be used to detect as little as 0.1 ng (1 ng/ml) of enterotoxin, and a linear dose-response curve was obtained with 0.5 to 5 ng (5 to 50 ng/ml). A nonlinear, but reproducible, curve was obtained with a dose range from 0.1 to 100 ng (1 to 1,000 ng/ml). A new unit of biological activity, called the plating efficiency unit, was defined as that amount of enterotoxin that caused a 25% inhibition of the plating of 200 cells inoculated into 100 μ l of medium in a microwell culture system. One milligram of highly purified enterotoxin contained about 400,000 plating efficiency units. Additional studies demonstrated that the biological and serological activities of the enterotoxin molecule were not equally labile.

The enterotoxin responsible for *Clostridium perfringens* food poisoning can be detected by both serological and biological assays (8). Serological assays, such as microslide immunodiffusion (14), electroimmunodiffusion (1), counter-immunoelectrophoresis (11), and reversed passive hemagglutination (16), that have been developed for assay of the enterotoxin are, in general, highly sensitive and reproducible. For example, reversed passive hemagglutination can be used to detect as little as 0.05 ng (0.5 ng/ml) of the enterotoxin. The numerous biological assays, such as the ileal loop test in rabbits (2, 5, 6) and chickens (12), the erythema skin test (14), and the mouse lethality test (5, 14) are, in general, not very sensitive, reproducible, or reliable for quantitative assays. Quantitation with any reliability can be made only in the range of 1 to 5 μ g with the existing biological assay. Comparisons among these different biological assays have been made by Genigeorgis et al. (3) and Niilo (13).

A sensitive, reproducible, and reliable assay is needed to detect the biological activity of *C. perfringens* enterotoxin. There are at least two reasons why the sensitive serological assays do not suffice for studies of the mode of action of *C. perfringens* enterotoxin. First, the specific activity of the enterotoxin varies immensely from one preparation to another. Activities that range

from 1,500 to 5,000 erythema units (14) per milligram of purified enterotoxin have been reported in the literature. In our experience, aliquots of enterotoxin from one purification batch vary by ± 200 to 300 erythema units per mg. We believe that this is due in part to the insensitivity and variability of the assay used (guinea pig skin test) and to the inherent differences in the enterotoxin preparations themselves. Second, there is no clear evidence that biological and serological activities are equally labile in this molecule. Therefore, the biological activity may change while serological activity remains constant. This could be due, in part, to the enterotoxin molecule's propensity for aggregating into less biologically active forms that may retain their preaggregation serological activity. Furthermore, nothing is known about the relationship, if any, between biological activity and serological activity sites on the molecule. Generally speaking, enterotoxin dosages to be used in biological systems that are expressed only as units of mass have very limited meaning (9).

In this report we describe the development of a highly sensitive and reproducible assay for biological activity of *C. perfringens* enterotoxin that solves the problems outlined above for existing assays. The system described is based on the observation (7) that the enterotoxin inhibits the plating efficiency of Vero (African green

monkey kidney) cells grown in tissue culture. The assay is relatively simple, rapid, and inexpensive.

MATERIALS AND METHODS

Enterotoxin. Enterotoxin was prepared as described previously (15), and the biological activity (in erythral units) was determined by the guinea pig skin test method as described by Stark and Duncan (14). The specific activities of the enterotoxin preparations varied from 1,700 to 2,500 erythral units per mg of purified protein. Enterotoxin was dissolved in medium 199 (Flow Laboratories) without serum and stored at -20°C until used. Enterotoxin was inactivated by heating it in solution at 60°C for 10 min.

Culture of Vero cells. Vero cells were cultured in 75-cm^2 tissue culture flasks (Corning Glass Works) with medium 199 supplemented with 5% fetal calf serum (GIBCO) and 0.75% sodium bicarbonate. When monolayers reached confluency, they were removed by trypsinization with 0.25% trypsin in calcium- and magnesium-free Hanks balanced salts solution and reseeded into 75-cm^2 flasks or counted for inoculation into test wells as described below.

Determination of plating efficiency. Plating ef-

iciency was determined as described by McClane and McDonel (7). Confluent monolayers were trypsinized, and an aliquot was removed to determine cell concentration. After appropriate dilution, a $100\text{-}\mu\text{l}$ aliquot of a cell suspension (2,000 cells per ml) and the desired concentration of enterotoxin (0.1 to 1,000 ng) were added to each well of a Microtest II (Falcon Plastics) tissue culture plate. To determine the control plating efficiency of the Vero cells, a series of wells were inoculated with the omission of enterotoxin or addition of heat-inactivated enterotoxin. The plates, inoculated with 200 cells per well, were incubated for 18 h after inoculation. The supernatant medium, containing non-adherent cells, was then discarded, and the remaining cells were washed with 0.85% saline solution. Adherent cells were stained for 1 min with a 1% methylene blue solution to aid in counting. An alternate method used for staining cells was that described by Gurr (4) in which cells were stained with acridine orange. Individual wells were divided into quadrants by etching with a razor to aid in counting. The number of cells per well was determined by direct counting under the light (methylene blue) or the ultraviolet (acridine orange) microscope. Plating efficiency of enterotoxin-treated cells was expressed as a percentage of control plating efficiency (which was usually about 80%). In any one experiment, four replicate wells were inoculated for each enterotoxin dilution or control. Sample sizes given represent the numbers of experiments, each of which was performed with the four replicates at each dilution given.

Determination of serological activity. Immu-

FIG. 1. Effects of enterotoxin on Vero cell plating efficiency over a wide range of concentrations. Inhibition is expressed as a percentage of control plating efficiency. Values are means $\pm$ standard errors of the means ($n = 8$). The three regions of the curve (A, B, and C) represent areas of high, medium, and low enterotoxin concentrations, respectively. These regions were studied more closely with small increments of enterotoxin concentrations within each range (see Fig. 2 and 3).

FIG. 2. Effects of enterotoxin on Vero cell plating efficiency and the resultant curve over a range of medium concentration (range B). Values are means $\pm$ standard errors of the means ($n = 6$).

FIG. 3. Effects of enterotoxin on Vero cell plating efficiency and the resultant curve over a range of low concentration (range C). Values are means $\pm$ standard errors of the means ($n = 6$).

noreactivity of the enterotoxin with antiserum prepared against highly purified (>99% pure) enterotoxin (15) was determined by counterimmunoelectrophoresis as described by Naik and Duncan (11).

Determination of labilities of biological and serological activities. Aliquots of enterotoxin were stored in closed microcentrifuge tubes at various temperatures. At timed intervals, samples were taken for determination of biological activity (plating efficiency inhibition) and serological activity (counterimmunoelectrophoresis). Some samples were frozen in ethanol-dry ice and thawed in water (room temperature) various numbers of times and assayed for activities. The samples to be assayed for biological activity contained 6 ng of enterotoxin (aliquots from the temperature-treated samples [250 ng/ml]) per test well (60 ng/ml). The samples to be assayed for serological activity contained between 1,250 ng (low dilution) and 40 ng (high dilution) of enterotoxin (aliquots from the temperature-treated samples [250 μ g/ml]). Samples for counterimmunoelectrophoresis were diluted serially through seven dilutions. The results were read as the number of the highest dilution that gave a visible precipitin line. Standards were prepared from aliquots that were freeze-dried in the microcentrifuge tubes and stored at -20°C . Preliminary studies have shown that the enterotoxin will retain its biological and serological activities for an indefinite period of time under these conditions.

FIG. 4. Effects of enterotoxin on Vero cell plating efficiency and the resultant linear dose-response curve when concentrations are used that fall within the linear portions represented in Fig. 2 and 3. Values are means $\pm$ standard errors of the means ($n = 6$).

RESULTS

Cells plated in the presence of enterotoxin exhibited a marked reduction in plating efficiency (Fig. 1), as has been described previously (7). Significant inhibition occurred with as little as 0.1 ng of enterotoxin, whereas doses above 100 ng caused little increase in inhibition. To determine if a linear curve could be obtained when the data were plotted on a linear scale, separate experiments were performed with concentrations of enterotoxin in three dose ranges indicated in Fig. 1 as A, B, and C, for high, medium, and low dose ranges, respectively. Each range was constructed to overlap another. When dilutions within region A (high doses) were plotted on a linear scale, the percentages of inhibition obtained were consistent from one experiment to another, but an irregular curve was obtained (data not shown). This region was of less importance, because large amounts of enterotoxin were required and an insignificant increase in inhibition of plating efficiency occurred over the entire dose range. Therefore, sensitivity was minimal.

Figure 2 shows the results of dilutions from

FIG. 5. Typical example of an assay with three standards of known activity and six different samples (a through f) of "unknown" concentrations. Standards are indicated by closed circles and consisted of the concentrations given in Table 1. Arrows indicate where the unknowns are located on the standard curve.

range B (medium doses). There was a break in the curve between 5 and 10 ng of enterotoxin per well, but a linear dose-response curve was obtained between 0.6 and 5 ng. The linear portion of the curve from range B extended into range C (low doses) until less than 0.3 ng was used, at which point another break occurred (Fig. 3). The break represented another linear portion of the curve with a greater slope in the range of 0.08 to 0.6 ng.

These observations led us to decide that the best range for a linear dose-response curve should have been between about 0.5 and 5 ng. A dilution series was constructed in this range, and the results (Fig. 4) showed that excellent linearity existed in this range. With the enterotoxin samples we used, 25% inhibition occurred with 2.5 ng of enterotoxin. This value of inhibition was chosen to be the arbitrary unit of activity, which we call the plating efficiency unit (PEU)

because it allows the greatest latitude for various sample values to fall above and below this point while remaining within the linear range. It can be calculated that the enterotoxin used in these experiments contained, on the average, 400,000 PEU/mg of protein, compared with 2,000 erythral units per mg (the activity of most of the enterotoxin samples used in these studies). The PEU enables a 200-fold increase in sensitivity of detection of enterotoxin over the erythral unit (200 PEU per erythral unit). We found that specific activity as determined by the PEU assay varied far less than the activity as calculated by the erythral unit assay.

To test the reliability of this assay, we prepared enterotoxin samples at concentrations unknown to another worker who then performed the assay and determined the biological activities of the unknowns. The results are given in Fig. 5 and Table 1. The reproducibility of the linear standard curve (three standards) was excellent (Fig. 5). The standard curve shown (from one experiment) was typical of the plots obtained from many repetitions of the assay. Several unknowns were tested that were within the linear concentration range without dilution (unknowns a and b), and several had to be diluted in order to fall within the linear range (c through f). At times, the reader was given several identical concentrations that were labeled differently. The values obtained for identical unknowns were indistinguishable. Variation was usually less than 0.1 to 0.3 ng. The most reliable values were obtained at higher concentrations of enterotoxin (Table 1). We attribute the majority of variation to two factors: dilution errors and significant loss from very dilute solutions due to nonspecific binding to diluent vials. We have noted nonspecific binding to vials of ^{125}I -enterotoxin in previous studies (10).

When values obtained with methylene blue staining were compared with values from cells stained with acridine orange, no significant difference was noted. We prefer the acridine orange stain because it did not adhere nonspecifically to the culture wells as readily as did the methylene blue. Also, the cells stained with acridine orange were much easier to see and identify as cells rather than debris and the assay was much less strenuous and time-consuming when the acridine orange stain was used. However, the methylene blue stain, which did not require an ultraviolet light microscope, gave equally reliable results.

The effects of various temperatures on the biological activity of the enterotoxin are shown in Fig. 6. Biological activity decreased during the first 24 h at 37°C and was eradicated by day

TABLE 1. Use of the assay to determine unknown concentrations of enterotoxin^a

Enterotoxin	% Inhibition ^b	Aliquot value ^c		Calculated value ^d		Actual value ^e	
		ng	PEU	ng	PEU	ng	PEU
Standard	45.0					5.0	2.0
Standard	15.2					1.25	0.5
Standard	7.5					0.31	0.13
Unknown:							
a	10.8	1.0	0.4	1.0	0.4	1.3	0.5
b	17.1	1.8	0.7	1.8	0.7	2.5	1.0
c	13.8	1.2	0.5	9.2	3.7	10.0	4.0
d	22.2	2.2	0.9	17.4	7.0	20.0	8.0
e	29.8	3.1	1.2	25.0	10.0	25.0	10.0
f	45.0	5.0	2.0	40.0	16.0	40.0	16.0

^a Samples were analyzed as unknowns, though their activity had been previously determined.

^b Value calculated as percentage of control plating efficiency.

^c Value read directly from the standard curve.

^d Some samples had to be diluted to fall within the linear range of the standard curve. The values given in the "aliquot" column have been multiplied by the dilution factor.

^e The actual concentrations (nanograms per μ l) were determined from stock solutions that were then diluted to the concentrations given. "Actual" PEU values were calculated with the assumption that 2.5 ng contains 1 PEU.

FIG. 6. Effects of various temperatures on biological activity with time. Aliquots were incubated at 37°C (■), 24°C (□), 4°C (★), and -20°C (●). Bars represent means $\pm$ standard errors of the means ($n = 4$ at each point). Absence of bar indicates a standard error too small to represent.

7. The rate of decrease was more gradual at 24°C, with no activity remaining by day 14. Activity seemed actually to increase at 4°C during the first 3 days, but then decreased to no activity by day 21. The enterotoxin proved to be labile to frozen storage (-20°C) beyond day 14, with no activity remaining at 28 days.

Repeated freezing and thawing of the enterotoxin caused a decrease in biological activity (Table 2). With 25 freezings and thawings, no activity remained. Since enterotoxin is often stored frozen for various experimental procedures, if it is refrozen for future use, the activity could be expected to decrease by as much as

TABLE 2. *Effect of freezing and thawing on biological activity of enterotoxin*

No. of treatments ^a	% Inhibition of plating efficiency
0	24.0 ± 1.5
1	21.5 ± 0.5
2	20.2 ± 0.1
5	17.0 ± 0.1
10	12.0 ± 10.0
15	6.0 ± 1.5
25	0
40	0

^a A treatment represents one cycle of freezing with ethanol-dry ice and thawing in water (room temperature).

TABLE 3. *Effect of heating at 56°C on serological activity of enterotoxin*

Time (min)	Titer ^a
0	6
1	6
3	5
5	5
10	2
15	0

^a Titer represents the number of the highest dilution that produced a visible precipitin line.

20% upon thawing the second time.

When enterotoxin solutions were treated in a manner similar to that for determination of biological activity, serological activity was unaffected. At all temperature except 56°C, serological activity was unaltered within the times given. At 56°C the serological activity decreased to zero within 15 min (Table 3). Repeated freezing and thawing (up to 40 times) also had no detectable effect on the serological activity.

DISCUSSION

In this paper we have described a highly sensitive and reproducible biological assay for *C. perfringens* enterotoxin. A linear dose-response curve is obtainable over a range of less than 0.5 to 5 ng of purified enterotoxin. The response of the Vero cells used in this assay is consistent enough from one assay to the next that we have been able to define a unit of plating efficiency inhibitory activity. We have named this arbitrary unit the PEU and have defined it as that amount of enterotoxin that causes a 25% inhibition of the plating of 200 cells inoculated into 100 µl of medium in a microwell culture system. The specific activity of the enterotoxin we have been using is 400,000 PEU/mg.

In the experiments described above we have calculated the concentration of enterotoxin in unknowns in order to test the reproducibility

and reliability of the assay. However, it is not recommended that this assay or any other assay of biological activity for *C. perfringens* enterotoxin be used to calculate enterotoxin mass, because the specific activity of the enterotoxin varies from one preparation to another and the activity of any one preparation tends to reduce with time, when in solution, for reasons not fully understood at this time. Instead, it is recommended that this highly sensitive assay be used to determine with great accuracy the absolute biological activity (unit activity/unit weight or volume of solution) of an enterotoxin preparation to be used for some experimental purpose or to determine the activity of enterotoxin in unknown samples. The assay can also be used to detect very small quantities of enterotoxin activity in preparations that have been diluted for one reason or another. Very slight changes in activity should also be detectable in preparations that have been treated with chemicals and enzymes or extremes in temperature or pH. The applicability of this assay to enterotoxin that is not in a highly purified form has yet to be determined. Enterotoxin from such sources as human stools or contaminated foods may not be assayable with this system because of the likely interference with plating of cells by innumerable other compounds from such sources.

The results of the lability studies are very interesting in that biological and serological activities have, for the first time, been clearly distinguished as separable entities. Furthermore, the importance has been enhanced for expressing data in terms of biological activity as well as serological activity. From these studies it can be seen that substantial amounts of serologically active enterotoxin can exist with no biological activity whatsoever. Further studies with the enterotoxin molecule will be necessary to characterize in greater detail the relationship between serological and biological activities.

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