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Interaction of Staphylococcal Enterotoxin B with Cell Cultures of Human Embryonic Intestine

W. I. SCHAEFFER, J. GABLIKS, AND R. CALITIS

Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts

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ABSTRACT

SCHAEFFER, W. I. (Massachusetts Institute of Technology, Cambridge), J. GABLIKS, AND R. CALITIS. Interaction of staphylococcal enterotoxin B with cell cultures of human embryonic intestine. J. Bacteriol. 91:21–26. 1966.—The cytotoxic effect of staphylococcal enterotoxin B upon human embryonic intestine cell cultures is characterized by retraction of cells from the monolayer. This is followed by clumping of the retracted cells to form clear areas in the monolayer and finally by sloughing of the clumps from the glass surface. The 50% effective dose of the toxin, determined by protein analysis of the cultures used in titration studies, was found to be between 40 and 60 μg/ml. The cytotoxic property of the enterotoxin was completely neutralized by 3.9×10^{-5} ml of specific antitoxin per μg of toxin. The cytotoxicity was found to be slightly enhanced by 2.2 g of bicarbonate per liter of Eagle's basal medium (Earle's salt solution level), the absence of serum, the absence of penicillin and streptomycin, and the presence of 2.8 mmoles of calcium in the medium. The cytotoxicity was profoundly influenced by the age of the culture. No cytotoxicity was evident until after 2 days of growth had taken place, when the cell number was approximately 4.0×10^5 cells per culture.

Staphylococcal enterotoxin is known to produce vomiting in monkeys and kittens and food poisoning in man. In addition, staphylococcal enterotoxin will produce enterocolitis in chinchillas (4) and enteritis in varying severity in cats and kittens (6) and in dogs (7).

Attempts at studying the effect of enterotoxin in in vitro systems have met with varying results. Guerin, Jackson, and Morgan (Abstr. Ann. Meeting Tissue Culture Assoc., 12th, p. 37, 1961) presented evidence of cytopathogenicity of staphylococcal α, β, and Δ hemolysins and purified staphylococcal enterotoxin when these substances were added in graded series to cultures of chick embryonic heart fibroblasts. Milone (3), in an attempt to establish an assay system for enterotoxin, used a variety of cell cultures and enterotoxin preparations varying in purity from crude to highly purified. He demonstrated that the more highly purified the toxic preparation, the less did it elicit cytopathogenic effects. In addition, cytopathogenicity could not be neutralized by specific antiserum. The author concluded that the agent causing the cytopathogenic effect was different from enterotoxin.

In the light of the discrepancy between the reports of Guerin et al. and Milone, we decided to investigate further the biological properties of enterotoxin as part of our continuing studies on the effect of microbial toxins in cell cultures.

The purpose of this report is to present evidence for the specific interaction of staphylococcal enterotoxin B with cell cultures of human embryonic intestine and to describe some of the factors which influence this interaction.

MATERIALS AND METHODS

Cell culture. The heteroploid cell culture used in this study was human embryonic intestine (Microbiological Associates, Inc., Bethesda, Md.) derived from ileum and jejunum tissue.

The stock cultures were grown in milk-dilution bottles in Eagle's basal medium containing Earle's salt solution and 10% human serum (Grand Island Biological Co., Grand Island, N.Y.). For maintenance of the stock cultures, the medium contained 100 units of penicillin and 100 μg of streptomycin per ml. For testing purposes, the penicillin and streptomycin were omitted. The cultures were incubated at 37 C at a *p*H of 7.2 to 7.4 maintained with a 5% CO_2 atmosphere.

Enterotoxin preparation. The enterotoxin preparation used in this study was identical with that recently described by Shantz et al. (5). These investigators prepared the enterotoxin from culture filtrates of *Staphylococcus aureus* strain S-6, and reported it to

be of a purity between 96 and 99%. They also reported the 50% effective dose (ED_{50}) for this toxin preparation to be 0.11 μg/kg when injected intravenously into Rhesus monkeys. The usual tests for α and β hemolysins, apyrase, and dermonecrotic substances were negative. All of the toxin used in this study was from the same batch, isolated and freeze-dried at one time.

For experimental purposes, one or more 2-mg vials of toxin were reconstituted with either medium or deionized distilled water. Dilutions were then made from these stock solutions. All freeze-dried toxin preparations and reconstituted stocks were stored at −43 C.

Antitoxin. The preparation of antitoxin was kindly provided by Earle W. Grogan, U.S. Army Veterinary Corps, Fort Detrick, Md. It was prepared by repeated administration of the purified toxin to ponies and by harvesting the serum. The titer of antitoxin was 0.006 ml of antitoxin per μg of the toxin, and was determined in the Rhesus monkey by protection against the lethality of 300 μg/kg of toxin.

Calcium chloride. A 0.1 N stock solution of calcium chloride was prepared in deionized distilled water. Dilutions to obtain the desired final concentration in the cultures were made at the time of testing.

Toxin test procedure. Test cultures were prepared in screw-capped culture tubes (16 by 150 mm). This was accomplished by trypsinizing stock cultures and by suspending the cells in medium at a level of 7.5×10^4 to 8.5×10^4 cells per milliliter, depending upon when the subsequent cultures were to be used. When Medium 199 was used, tube cultures were first planted and grown in Eagle's basal medium.

The growth medium was decanted 1 day prior to complete monolayer formation. The monolayers were washed with Hanks' balanced salt solution, and the medium was replaced with 1 ml of fresh medium containing the desired dilution of toxin. All toxin tests were set up in triplicate. The cultures were then reincubated at 37 C and were observed daily for cytopathogenic effects for an additional 48 hr.

Cytotoxicity was determined by microscopic examination and classified as follows: ±, less than 10% destruction of culture monolayers; +, 10 to 25% destruction; 2+, 25 to 50% destruction; 3+, 50 to 75% destruction; and 4+, 75 to 100% destruction.

For testing the effect of the age of cultures upon the response to the toxin, cultures were planted at a level of 8.5×10^4 cells per milliliter. Toxin was added to the tubes at a level of 100 μg/ml at the time of planting and after 1, 2, and 3 days of growth in the absence of toxin. After the addition of toxin, each set of cultures was incubated for an additional 48 hr, after which the medium was decanted and protein determinations were run.

To test the thermolability of the toxin preparation, 2 mg of freeze-dried enterotoxin was reconstituted with 2 ml of deionized distilled water. This solution was placed in a boiling-water bath for 10 min. After it was cooled, 8 ml of complete medium was added to the boiled preparation of enterotoxin, and the proper dilutions were made. As a control, a second 2-mg vial of freeze-dried enterotoxin was treated similarly except for the boiling step.

Toxin neutralization by specific antiserum. Serial twofold dilutions were made of the antiserum preparation in complete medium. An equal volume of medium containing 200 μg/ml of enterotoxin was added to each dilution. The resulting solution was then incubated for 4 hr at 37 C, after which an equal quantity of medium lacking toxin was added. A 1-ml amount of this solution was then added to each cell culture.

A control system treated as above, with normal horse serum in place of antiserum, was employed to exclude the possibility of nonspecific protection against enterotoxin by horse serum.

Protein determination. In all cases, these determinations were done after 48 hr of incubation with the toxin solution. The medium was decanted from the cultures and the remaining cells were washed twice with 3 ml of Hanks' balanced salt solution. The Lowry modification of the Folin Ciocalteau test (2) was then used to determine the protein content of each culture.

Culture tubes containing complete growth medium without cells were always included in the 48-hr incubation and were treated in the same manner as those containing cell cultures. This was done to correct for any remaining proteinaceous material as a result of the medium but not of the cells. In general, this amounted to approximately 25 μg of protein per culture tube after 48 hr of incubation.

Calculation of ED_{50}. The ED_{50} value, that level of toxin destroying 50% of the cell monolayer, for our tissue culture system was calculated from the protein determination data by the method of Litchfield and Wilcoxon (1). This value was calculated by use of at least six different concentrations of toxin.

RESULTS

Figure 1 illustrates the typical dose response obtained when various concentrations of staphylococcal enterotoxin B were incubated with human embryonic intestine cell cultures for 48 hr. The ED_{50} and the 95% confidence limits were found to be 51.5 (34.1 to 77.8) μg/ml. In a series of such titrations, the ED_{50} has been found to vary between 40 and 60 μg/ml.

Cytopathogenic effect. The cytopathogenic effect of staphylococcal enterotoxin B upon human intestine cell cultures is characterized first by retraction of individual cells from the glass surface, leading to formation of holes in the monolayer. As the cell retraction process progresses, these cells then clump as cell masses, thus forming larger holes with thin bridges of cell material extending across the cleared areas of the monolayer. In the final phase, these clumps of cells slough from the glass.

To determine whether the sloughed cells were dead, the floating cell masses were harvested by centrifugation of the medium from cultures containing various levels of toxin. The cells were then suspended in fresh medium and triturated in an attempt to break up the clumps. Only relatively

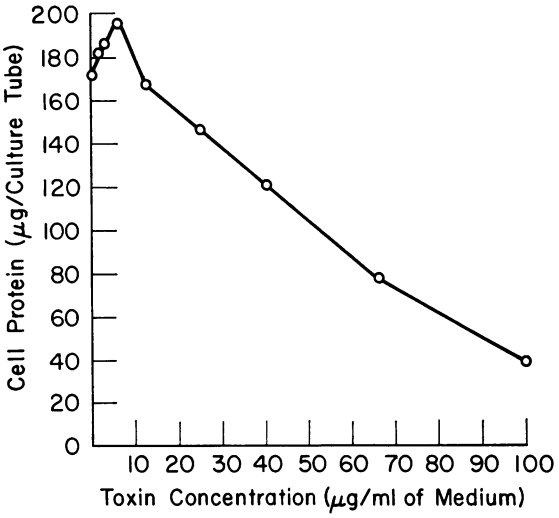

FIG. 1. *Titration of staphylococcal enterotoxin B preparation in cell cultures of human embryonic intestine as determined by protein analysis 48 hr after toxin addition.*

few cells were separated from the clumps, and, as a result, both clumps and free cells were replanted. Within the first 24 hr, some cells and clumps had attached to the glass surface. The cells appeared normal morphologically, although no multiplication took place. During the second 24-hr period of incubation, the entire culture had degenerated, and the cells once again detached from the glass surface.

One recurrent observation indicated that the retraction process begins in the areas of heaviest growth, that is, approximately 2 cm from the bottom of the tube, and then progresses from this area along the median line of the monolayer. In general, the cells at the periphery of the monolayer respond least to the toxin.

Factors influencing the response of the cell cultures to the toxin. Early in this study, it became apparent that the response of the cells to the toxin was influenced by one or more factors. As a result, we undertook a study of the effects of a number of parameters which could influence the toxic response.

Bicarbonate concentration. The bicarbonate concentration of the medium affected the toxic reaction only slightly. The cytotoxicity level was always higher with 0.22 g of bicarbonate per 100 ml of medium (the bicarbonate level in Earle's salt solution) than at 0.035 g of bicarbonate per 100 ml (the level in Hanks' salt solution). With the lower bicarbonate level, there was a morphological change in the cells from an epithelioid type to a fibroblastic form, and, concomitantly, a slight increase in the resistance of the culture to the toxin.

Penicillin and streptomycin. The effect of penicillin and streptomycin was also slight, with the absence of both antibiotics yielding a somewhat higher level of cytotoxicity. There was no dose response effect when the penicillin and streptomycin solution was added in a graded series, ranging from 100 units of penicillin and 100 μg of streptomycin (normal level) to 1,000 units of penicillin and 1,000 μg of streptomycin per ml of medium, to cultures containing a dose of 52 μg of toxin per ml.

Serum. The absence of serum, that is, with Medium 199, increased the ED_{50} level by approximately 40%. However, since the human intestine cells did not grow in Medium 199 without serum, and, in addition, since the cells appeared granular and somewhat rounded, this condition of the cultures may have accounted partly for the increased toxicity.

Age of culture. Figure 2 illustrates the effect of the age of the culture upon the response of the cell culture to the toxin. If toxin is added to the cultures at the time of planting or after 1 day of growth in the absence of toxin, there is no cytopathogenic effect upon the cultures 48 hr after the addition of toxin. Indeed, if the cultures to which

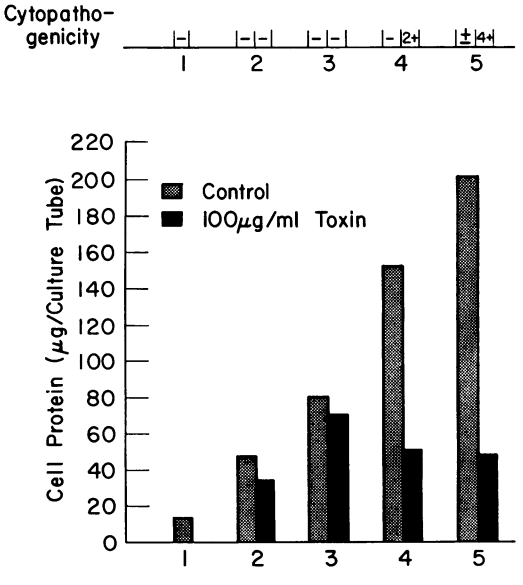

FIG. 2. *Determination of toxicity of staphylococcal enterotoxin B upon human embryonic cell cultures by microscopic observation of cytopathogenicity and cell protein analyses 48 hr after toxin addition. (1) Quantity of protein planted; (2) toxin added at the time of planting; (3) toxin added 1 day after planting; (4) toxin added 2 days after planting; and (5) toxin added 3 days after planting.*

toxin has been added at the time of planting and after 1 day's growth in the absence of toxin are maintained for up to 1 week in the presence of 100 μg/ml of toxin, no cytopathogenicity is evident. The lethal action of the toxin becomes evident only after 2 days of prior growth in the absence of toxin. At this point, there are approximately 4.0×10^5 cells per culture tube.

Calcium concentration. Figure 3 illustrates the effect of varying the concentration of calcium in the medium from the normal level of 1.4 mM, present in Eagle's basal medium, to a maximum of 11.7 mM. At each level of calcium, there was a culture treated with 50 μg/ml of toxin. The values shown on the graph represent the differences in protein content between the treated and the control cultures, expressed as percentages of the corresponding control culture.

The optimal level of calcium for a maximal reaction of the toxin was 2.8 mM calcium, whereas higher levels of calcium decreased the cytotoxicity. Growth stimulation was observed in the control cultures containing increased calcium concentrations above 1.4 mM.

Figure 4 shows the results obtained when a complete toxin titration was run with the normal level of calcium, 1.4 mM, and with 2.42 mM calcium. The ED_{50} values and the 95% confidence limits were found to be 58.5 (29.3 to 117.0) μg/ml for 1.4 mM calcium, and 44.0 (22 to 88) μg/ml for 2.42 mM calcium.

Thermostability and the effect of freezing and thawing. Figure 5 illustrates the results obtained when a titration of toxin was conducted with a boiled and an unboiled sample of toxin. Boiling the enterotoxin preparation for 10 min caused the toxin to coagulate and to precipitate from the solution. This procedure effected the loss of 100% of the biological activity demonstrable by our test system. Shantz et al. (5) reported that boiling for 5 min caused the enterotoxin B preparation to coagulate. However, only less than 50% of the biological activity detectable in their in vivo system was destroyed after this treatment.

When the toxin is reconstituted from the freeze-dried state with deionized distilled water, it may be frozen and thawed for up to two times without significant loss of activity. However, when the toxin is reconstituted in complete medium (minus penicillin and streptomycin), the toxin is completely inactivated after freezing and thawing once.

Neutralization of toxin by antitoxin. Figure 6 illustrates the results of a neutralization test run with staphylococcal enterotoxin B antitoxin.

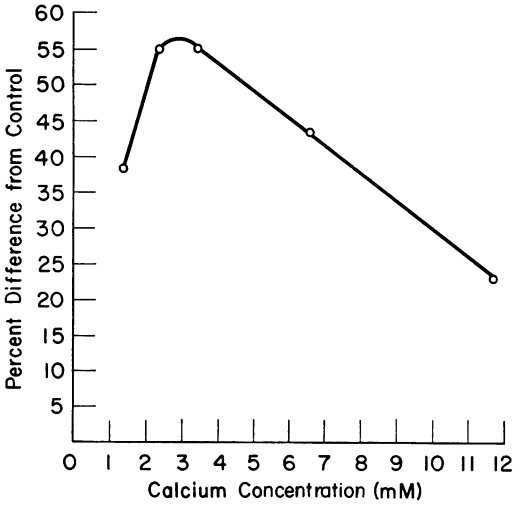

FIG. 3. *Effect of the concentration of calcium on the biological activity of staphylococcal enterotoxin B. The data plotted represent the differences in remaining protein of the control and treated cultures 48 hr after toxin addition, expressed as percentages of the control.*

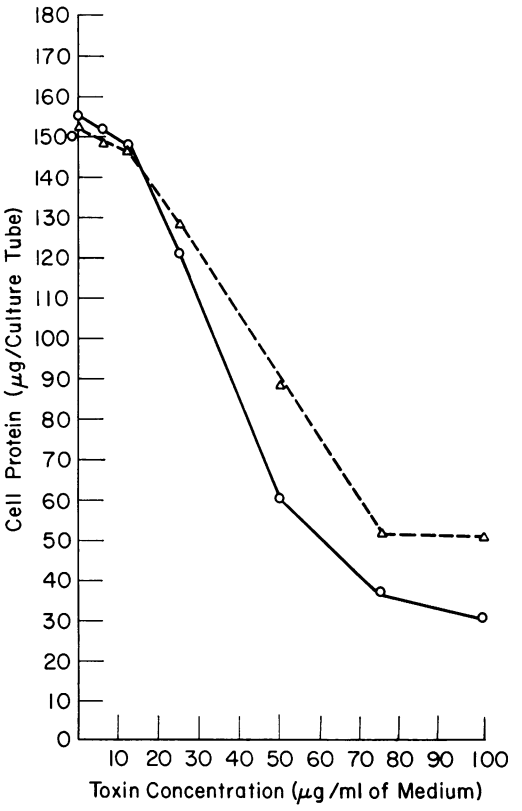

FIG. 4. *Toxin titration in the presence of 1.4 mM (normal) and 2.42 mM calcium. Symbols: ○ = 1.4 mM calcium; △ = 2.42 mM calcium.*

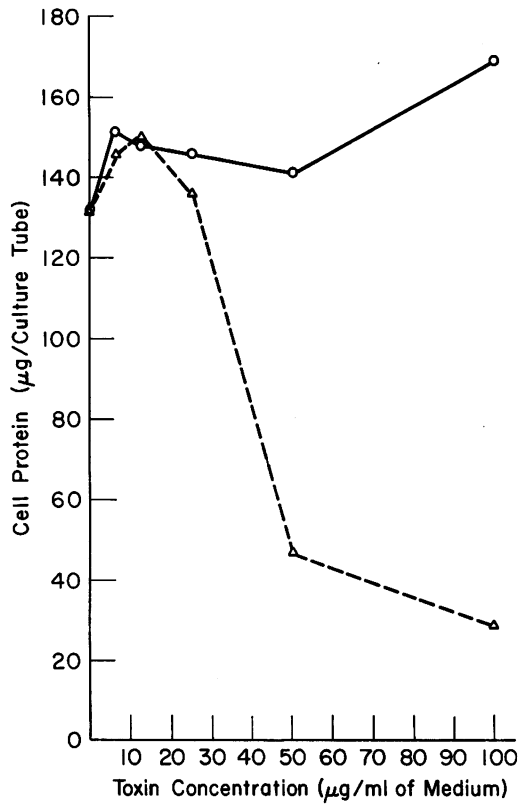

FIG. 5. *Toxin titration with boiled and unboiled staphylococcal enterotoxin B preparations. Symbols: ○ = boiled preparation; △ = unboiled preparation.*

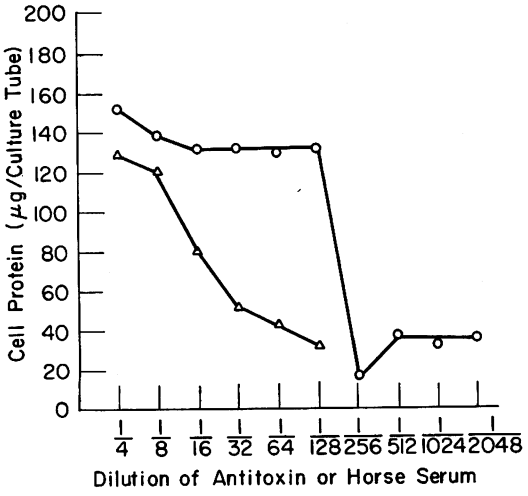

FIG. 6. *Neutralization of staphylococcal enterotoxin B by specific antitoxin. Symbols: ○ = specific antiserum; △ = normal horse serum.*

Complete neutralization of 200 μg of enterotoxin was effected by a 1:128 dilution of the antitoxin. This dilution represents 7.8×10^{-3} ml of antitoxin, and, therefore, in our system, 3.9×10^{-5} ml of antitoxin is required per microgram of enterotoxin for complete protection. The results obtained from the parallel test, with use of normal horse serum in place of the antitoxin, indicated no protection against the lethal reaction of the toxin.

DISCUSSION

It is apparent from this study that a number of factors influence the cytotoxic effect of staphylococcal enterotoxin B upon human embryonic intestine cell cultures. Such factors as the bicarbonate concentration, the presence of serum, and the presence of penicillin and streptomycin played a small role, but undoubtedly added to the variability between experiments which we experienced during the early phases of our study.

The most important single variable which has become evident is the effect of the age of the culture upon the magnitude of cytotoxicity. The cell number may also be a prime factor, since both the age of the culture and the cell number are related. We do not, as yet, know how the calcium concentration is involved in the toxic reaction. It may be related to the cell number-age of culture phenomenon, as calcium is known to be essential for attachment of the cells to glass, or it may act independently as, for example, in cell-membrane permeability. These factors are presently under examination.

The toxic reaction described is specific, since specific antitoxin neutralized the toxicity. The fact that the toxin preparation is 96 to 99% pure minimizes the possibility that cytotoxicity was a result of contaminating substances. The level of contamination would be exceedingly low after the toxin solution was carried through the dilution schedule.

We have no explanation for the discrepancy between our results and those of Milone (3), who used human embryonic intestine cell cultures in the early portion of his study. Possibly the discrepancy lies in the parameters which we have found essential for a toxic reaction to take place, especially the age of the culture.

From the results which we obtained, it appears as though cell-culture systems are not practical as a means of assaying for staphylococcal enterotoxins in food. Relatively long periods would have to be spent in isolating the toxin in purified form so as to render negligible the cytotoxicity of contaminating substances. Also, at least 30 μg of toxin would be required for a detectable reaction. There is little doubt, however,

concerning the use of cell cultures for mode of action studies.

Preliminary work in our laboratory concerning the biochemical effect of staphylococcal enterotoxin upon the human embryonic intestine cell line showed that there are definite alterations in the volume, protein, ribonucleic acid, and deoxyribonucleic acid on a per cell basis in treated cultures. Further investigations with the use of radioactive tracers are currently in progress.

ACKNOWLEDGMENT

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