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BINDING VERSUS BIOLOGICAL ACTIVITY OF CLOSTRIDIUM PERFRINGENS ENTEROTOXIN IN VERO CELLS

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SUMMARY

Cells resistant to Clostridium perfringens enterotoxin were selected from cultures of highly sensitive Vero (African green monkey kidney) cells. Studies were done with the sensitive and resistant cells to determine the relationship between binding and biological activity. Binding studies using ^{125}I-enterotoxin revealed the apparent existence of high and low affinity binding sites for the enterotoxin on both cell types. The binding site density on resistant cells was found to be 1/10 that of sensitive cells. It was found that, even with high doses of enterotoxin, only partial affect upon DNA synthesis, membrane permeability, and plating efficiency was noted in resistant cells. It is concluded that without specific binding there is little or no ability of the enterotoxin to effect biological activity in cells.

INTRODUCTION

Recent studies on the mechanism of action of Clostridium perfringens enterotoxin have led to the belief that transport alterations (1,2,3), metabolic disturbances (4,5), histological damage (1,6,7), and complete inhibition of macromolecular synthesis in cells within 30 minutes of exposure to enterotoxin (8) ultimately are due to membrane damage (7,9) induced by the enterotoxin. McClane and McDonel (8) have demonstrated that Vero (African green monkey kidney) cells respond quickly (within 15-20 minutes) and with high sensitivity to the enterotoxin. The fact that the cells respond by inhibition of macromolecular synthesis, loss of viability, and morphological alterations has established them as an excellent tool to study the mechanism of action of the enterotoxin.

In the studies reported here, resistant cells were selected from cultures of highly sensitive Vero cells for the sake of performing studies on binding

Abbreviations: TCA, trichloroacetic acid; Ca-Mg free HBSS, calcium and magnesium free Hank's balanced salt solution.

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capabilities and biological activity of the enterotoxin. It was intended that the studies undertaken would establish the resistant cells as an important tool for further studies on the mechanism of action of C. perfringens enterotoxin.

METHODS

Enterotoxin. Enterotoxin was prepared as described (10) and its biological activity determined in erythemal units, EU (11). The specific activity was 2500 EU/mg. Enterotoxin was iodinated by the chloramine T method. Free ^{125}I was separated from bound level by column chromatography (sephadex G-25) and dialysis. Ninety-five percent of counts in the final preparation were found to be associated with TCA precipitable material. The same percent was found to be associated with antigen for rabbit anti-enterotoxin serum. Analysis of the ^{125}I enterotoxin showed that the iodination procedure did not detectably alter erythemal or serological activity, or physical properties as determined by polyacrylamide disc gel electrophoresis.

Vero cells. Normal Vero cells were grown in roller bottles and 75 cm^2 (Corning) flask with Medium 199 (Flow) with 5% fetal calf serum (Flow) and 0.75% sodium bicarbonate. Resistant cells were selected by treating normal Vero cells with enterotoxin in Medium 199. After incubation overnight, the enterotoxin was removed and fresh medium added. The few surviving cells were allowed to grow to confluency (initially, 10-14 days). This process was repeated 9 times. No apparent loss of cells was noted upon addition of enterotoxin after the 7th treatment. Karyotypic analysis (12) verified that the resistant cells derived from the normal Vero cells.

Binding studies. Binding to cells was accomplished by mixing ^{125}I-enterotoxin with cells suspended in 1.5 ml polyethylene microcentrifuge tubes containing 0.5 ml of Ca-Mg free HBSS. Specific binding was determined by preincubating (45 minutes) a third tube of cells for each enterotoxin dilution with an excess of native enterotoxin. Each set of duplicates plus the corresponding third tube were then treated with equal amounts of ^{125}I-enterotoxin. The level of binding in the third tube (non-specific binding) was then subtracted from the levels in the duplicates. Non-specific binding averaged 3% of total counts added. All tubes were incubated with shaking at 37 C for 45 minutes. After incubation the cells were spun down (20 seconds) and washed 2 times with Ca-Mg free HBSS. The washed pellets were transfered to clean tubes. Pellets and supernatants (original plus washes) were counted in a Packard gamma counter. Cell protein was determined, after dissolving pellets in 1 N NaOH, by the method of Lowry, et al. (13).

DNA synthesis. The incorporation of ^{3}H thymidine (53 Ci/mmol, Amersham) into acid insoluble material was used as an index of DNA synthesis. After washing, 24 hour monolayers (seeded with 2.5×10^5 cells/dish) in 35 mm culture dishes were labeled by addition of 1.9 ml of Medium 199 without serum containing 1 µCi of ^{3}H thymidine. A 100 µl aliquot was added of Medium 199 with active or heat inactivated (60 C, 10 minutes) enterotoxin. At the time period specified the dishes were placed on ice. The culture supernatants were collected into cold centrifuge tubes followed by cell scrapings into 0.32 M sucrose buffer containing 3 mM $MgCl_2$ from each dish. The final TCA concentration was made to 5% prior to standing for 30 minutes in the cold. Precipitates were washed 3 times with 1 ml of 5% TCA and then solubilized with 1 ml of 0.1 N NaOH. One aliquot was used for scintillation counting and another for determination of cell protein by the method of Lowry, et al. (13).

^{3}H-uridine release. Release of ^{3}H uridine from cells treated with enterotoxin was used as an index of membrane damage and permeability (14). One hundred µl

(2.8×10^5 cells/ml) of cell suspension containing 0.67 μCi/ml ^{3}H uridine were added to each of a series of wells in a Microtest II plate (Falcon). After 24 hours of incubation, the medium was removed and the cells were washed with medium without serum. To each well was added 100 μl of medium without serum containing serial dilutions of enterotoxin. Controls received either heat inactivated, or, no enterotoxin. After 3 hours at 37 C the supernatants were collected and counted in a scintillation counter. The remaining cells were treated with cold 10% TCA and the supernatants (TCA-soluble material) were counted with a scintillation counter.

Plating efficiency. One hundred μl of cell suspension (2×10^3 cells/ml) and the desired concentration of enterotoxin (0.1 ηg to 10 μg/ml) were added to wells in Microtest II plates. After the plates were incubated for 18 hours, the medium was removed and the cells were washed with 0.85% saline solution. The cells were then stained with methylene blue and counted. Test well plating efficiencies were calculated as a percent of cells that plated in control wells.

RESULTS

Figure 1 shows levels of specific binding of ^{125}I-enterotoxin in normal and enterotoxin resistant Vero cells. It can be seen that binding increases with dose of enterotoxin added over a range of 0.039 to 20 EU. However, the level of binding in resistant cells is, on the average, only 13.8% (range: 6.9-23.3%) of levels observed in normal cells. Calculations from the specific activity of the ^{125}I-enterotoxin revealed that the maximum number of enterotoxin molecules that could be bound specifically to normal cells was 1.42×10^6 molecules/cell (range: $1.13\text{-}1.71 \times 10^6$) and for resistant cells was 1.15×10^5 molecules/cell (range: $0.95\text{-}1.35 \times 10^5$). This amounts to nearly a 12 fold reduction in binding capacity by resistant cells.

Scatchard analysis of binding in normal cells (Figure 2) showed that there apparently are 2 binding sites, one a high affinity, low capacity site (association constant, $K_a = 2.21 \times 10^8$ liter/mol) and the other a low affinity, high capacity site ($K_a = 1.57 \times 10^7$ liter/mol). The apparent density of the high affinity sites and the low affinity sites was 0.52×10^6 and 1.29×10^6 sites/cell, respectively. Scatchard analysis also was done (not shown) of the binding data for the resistant cells. This analysis was far more subject to error because the resistant cells bound a very small percent of the total labeled molecules added. However, a biphasic curve resulted which was similar in appearance to that shown in Figure 2. The calculated association constant

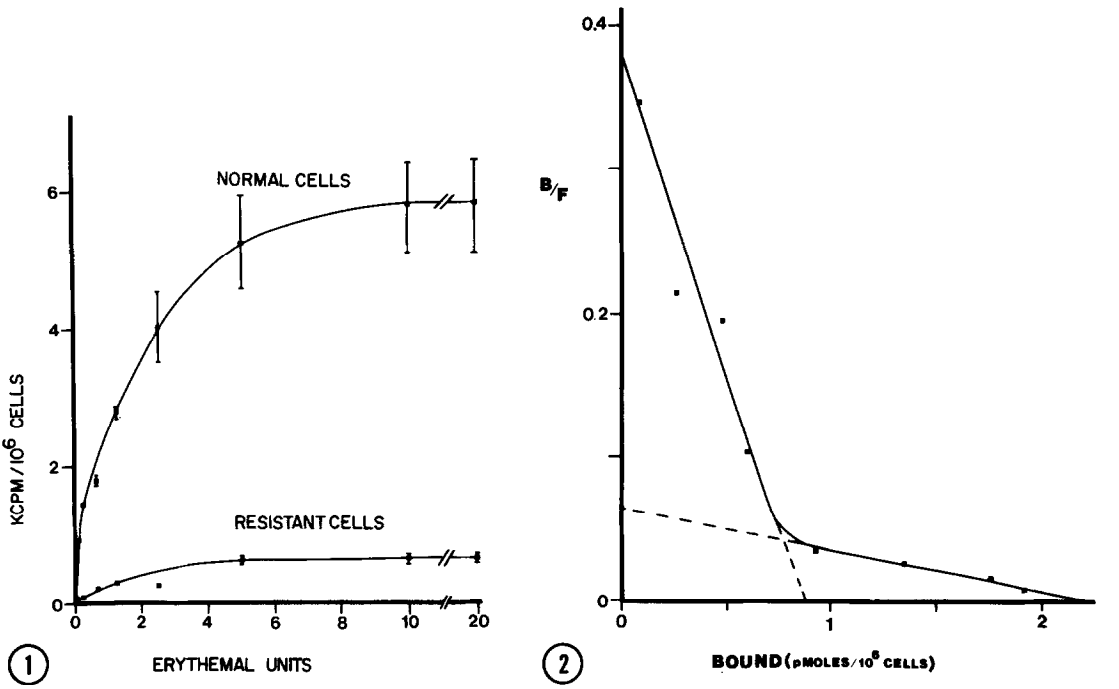

Figure 1. Effect of ^{125}I-enterotoxin concentrations upon enterotoxin-cell association. Cells were treated with ^{125}I-enterotoxin for 45 minutes at 37 C. Levels of binding represent "specific binding" (that binding which could be inhibited by pretreatment with native enterotoxin). Vertical bars represent standard error of the mean. Points without bars had standard errors too small to depict. Normal cell values are averages of 4 experiments, resistant cell values are averages of 2 experiments.

Figure 2. Plot of bound/free (B/F) ^{125}I-enterotoxin as a function of enterobound to Vero cells. These data were used to calculate apparent association constants (K_a) and binding site densities. The biphasic curve with upward concavity has been construed to imply the existance of two sites with different affinities (high and low). Points are derived from averages of 4 experiments.

for the high affinity, low capacity site was $K_a = 2.58 \times 10^8$ liter/mol and for the low affinity, high capacity site was $K_a = 2.46 \times 10^7$ liter/mol. The apparent density of the high affinity and low affinity sites on the resistant cells was 0.54×10^5 and 1.35×10^5 sites/cell, respectively.

Comparisons of biological activity of the enterotoxin in normal and resistant cells are given in Table I and Figures 3 and 4. Table I shows that enterotoxin causes a rapid (within 30 minutes) cessation of precursor incorp-

TABLE I

Effect of Enterotoxin on DNA Synthesis in Normal and Toxin Resistant Cells

INCUBATION TIME	TREATMENT[a]	(n)	INCORPORATION (CPM/μg PROTEIN)[b]
30 min.	Control	(6)	236 ± 8
	Normal	(6)	169 ± 11[c]
	Resistant	(4)	211 ± 13[d]
60 min.	Control	(8)	492 ± 5
	Normal	(6)	171 ± 12[c]
	Resistant	(5)	449 ± 10[e]

[a]Controls received either heat inactivated or no enterotoxin. All other cells were treated for the time given with 2.5 EU/culture dish. "n" represents the number of culture dishes treated.

[b]Values are means ± standard error of the mean. Statistical significance was determined by Student's "t" test.

[c]$p < 0.001$

[d]Not significant ($p > 0.1$)

[e]$p < 0.01$

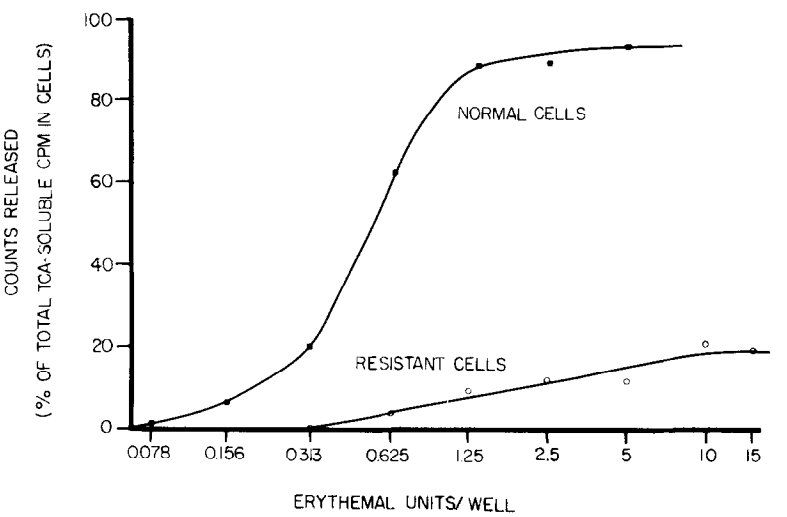

Figure 3. Effect of enterotoxin concentration upon release by cells of pre-loaded ^{3}H-uridine. Cells which were loaded for 24 hours were exposed to enterotoxin for 3 hours. Cells were pelleted and the supernatants and pellets counted. Background leakage was determined from control wells and subtracted from experimental values. Values given are the average from 2 experiments.

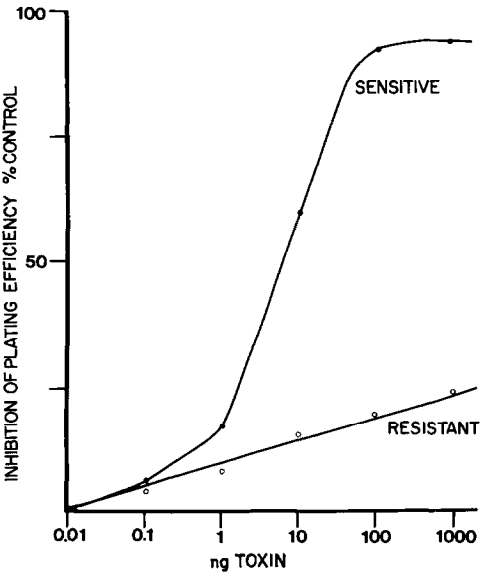

Figure 4. Effect of enterotoxin concentration upon plating efficiency of normal and resistant cells. Plating efficiency of enterotoxin treated cells has been expressed as a percent inhibition of control plating efficiency levels. Values are means of 5 experiments.

oration into DNA in normal cells. No statistically significant inhibition is noted in the resistant cells at 30 minutes. However, a significant ($p < 0.01$) depression of precursor incorporation is evident in the resistant cells at 60 minutes. It is important to note that the effect is mild in the resistant cells (precursor incorporation is 93% of control value), compared to essentially complete shutdown in normal cells (0.8% of control value).

Figure 3 shows the effect of increasing doses of enterotoxin on release by preloaded cells of ^{3}H-uridine. It can be seen that greater than 90% of TCA soluble counts in the normal cells are caused to leak out after addition of 1.25 EU or more whereas at doses as high as 15 EU less than 20% of TCA soluble counts leak out of resistant cells.

The effects of very small amounts of enterotoxin on plating efficiency of Vero cells is shown in Figure 4. Greater than 90% inhibition of plating efficiency is noted in normal cells with addition of 100 ηg (0.25 EU) or more of enterotoxin. Less than 25% inhibition is seen in the resistant cells with addition of 1000 ηg (2.5 EU).

DISCUSSION

Cells grown in tissue culture provide an excellent system for studying the mechanism of action of C. perfringens enterotoxin. McClane and McDonel (8) have demonstrated several important rapid responses by Vero cells to the enterotoxin including inhibition of macromolecular synthesis and plating efficiency, and morphological alterations to the cells. In this report we have established a resistant cell strain derived from the highly sensitive Vero cells. A further advantage of this system is that Vero cells have been used for studies dealing with binding and membrane interaction with diptheria toxin (15) which makes important comparison possible.

We have demonstrated that the enterotoxin has significantly reduced activity in these resistant cells. Cells resistant to diptheria toxin have been isolated from diptheria toxin sensitive lines (16,17). Moehring and Moerhing (16) selected resistant cells by the same basic method as we used to select enterotoxin resistant cells. The degree of resistance to diptheria toxin varied from 10 fold to 10,000 fold. In this report, our derived cells were approximately 10 fold less sensitive to enterotoxin than were the normal cells as determined by DNA synthesis, ^{3}H-uridine release and plating efficiency.

It is particularly interesting to note that the calculated binding site density on the resistant cells is approximately 1/10 that of normal cells, and that the observed maximum number of enterotoxin molecules binding to the resistant cells is nearly 1/10 the number in normal cells. Furthermore, the association constants of the high and low affinity sites are comparable in both cell types. We believe that this indicates that the primary difference between the two cell types is availability of binding sites. Our data demonstrate that, when the density of binding sites is reduced by a factor of ten, the number of enterotoxin molecules binding per cell is reduced by 10 fold, which is concurrent with approximately a 10 fold reduction in response by the cells to the enterotoxin. These observations strongly suggest that without binding the enterotoxin is capable of inducing little or no response.

Chang and Neville (18) demonstrated that receptor site density in several different cell types is not necessarily related to cell sensitivity or resistance to diptheria toxin. It is not possible to conclude from our data that enterotoxin that binds need necessarily be biologically active. The identification of a resistant cell type would be necessary that binds large amounts of enterotoxin but exhibits little or no biological response in order to make such a conclusion. The events in the mechanism of action that occur after binding to the membrane of these cells is unknown at this point. Studies currently underway are being done in an attempt to identify a receptor and to characterize in greater detail what is happening to the membranes of sensitive Vero cells treated with the enterotoxin.

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