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INTERACTION BETWEEN *CLOSTRIDIUM DIFFICILE* TOXIN A AND MAMMALIAN CELLS

Te-Wen Chang (张德汶), MD (*Infectious Disease Service, New England Medical Center & Dept of Medicine, Tufts University School of Medicine, Boston MA 02111, USA*)

ABSTRACT The interaction between *Clostridium difficile* toxin A and human cells was studied using both WI-38 cells and red cell ghosts. The adsorption to cultured cells was rapid during the first 10 min of exposure. There was a late increase in adsorption at higher temp. Toxin binding to the RBC cell ghost was temperature-dependent; higher at 37° than at 22° or 0°C. The adsorption to both WI-38 cells and to RBC ghost was not influenced by pH changes ranging from 6.4 to 7.8. The release of bound toxin from WI-38 cells was minimal from intact cells, but greatly accelerated after the cells had been broken by repeated freezing and thawing. The release of toxin from the RBC cell ghost was not detected until 28 h after incubation, regardless of the incubation temp. Only ghosts of human RBC were shown to bind toxin. RBC ghosts were inactivated in toxin binding in: rhesus monkey, dog, rabbit, sheep, rat, hamster, guinea pig and chicken. Treatment with either 2-mercaptoethanol or *N*-ethyl maleimide caused inactivation of toxin which was not reversible after its removal, suggesting that disulfide and sulfhydryl bonds are essential for toxicity.

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C. difficile toxin induced cytotoxicity in a variety of cell cultures.[1-3] Examination by electron microscopy indicated that the toxin caused pronounced changes in cell membranes which began several h before cytopathic changes were detected by light microscopy. These alterations persisted without further progression during the subsequent 48 h [4]. In this report, we describe interaction between toxin and mammalian cells. We tried to examine the kinetics of toxin attachment and the release of bound toxin; factors which influence toxin attachment; and various agents that interfere with toxin binding. Because of unavailability of radioactive toxin, the methods used in this study are very different from the usual approach.

MATERIALS AND METHODS

Toxin A *C. difficile* toxin was obtained from the cecum of a hamster with diarrhea and colitis, 2 d after 2 mg of clindamycin had been given by a gastric tube. The cecal contents were harvested, spun at 20,000 × *g* for 20 min,

and passed through a 45 nm millipore filter. The crude toxin contained a titer of $10^{6.8}$ TCD-50/ml (50% tissue culture dose), estimated by method of Reed and Muench[5] and it could be neutralized by either monovalent *C. difficile* antitoxin or by polyvalent gas gangrene antitoxin containing a *C. sordelli* component. Partially purified toxin, obtained from a culture supernatant, had a potency of producing cytotoxicity at a concentration of 1.4 ng/ml protein. The purification procedure included ultrafiltration, precipitation with $(NH_4)_2SO_4$, gel infiltration, and ion-exchange chromatography [6]. Both toxins were stored at -70℃ in small quantities in a number of tubes. For each experiment, a fresh tube was used. All studies were done with both crude toxin from the cecal contents and partially purified toxin from a culture supernatant.

Preparation of cell culture and red cell ghost WI-38 cell cultures from the Flow Laboratories were maintained in minimal Eagle medium + 5% fetal calf serum. RBC ghost was prepared: Blood in a heparinized tube was spun at 2500 rpm for 30 min. The plasma and buffy coat were removed. For each 5 ml blood, 40 ml of distilled water were added. The container was vigorously shaken on a voltax machine for 3 min, and spun at 2500 rpm for 20 min. The supernatant was discarded, and the sediment was washed with distilled water 3 times. After final washing, the packed RBC ghost was resuspended in phosphate buffered saline (PBS) at pH 7.2 to make a 10% suspension, and was stored at −70℃ until used.

Toxin adsorption to and elution from WI-38 cells A serial 10-fold dilution (10^{-1} to 10^{-6}) of the toxin preparation was made in PBS. 0.1 ml of each dilution was added to each of 8 cell cultures. At the desired interval, one set of cultures, covering a series of dilutions, was washed with PBS 8 times for each tube, using a Cornwall repeating syringe to deliver PBS. After washing, fresh culture medium was added and the cultures were incubated at 37℃ for 24 h, at which time microscopical examination for cytopathic changes was carried out.

For elution experiments, flask cultures (25 cm^2 growth area) of WI-38 cells were inoculated with 10^5 TCD-50 of toxin. After incubation at 37℃ for 1 h, the flask cultures were washed 8 times with PBS, and fresh medium was added. At the desired intervals, aliquots were removed for toxin titration. Another set of flask cultures of WI-38 were inoculated with 10^5 TCD-50 of toxin and incubated at 37℃ for 1 h. Following incubation, the cultures were washed 8 times with PBS, once with gas gangrene antitoxin at 1:5 dilution, and 8 times again with PBS. After washing, fresh medium was added and the cultures were incubated at 37℃ for 2 h. Following incubation, the medium was tested for toxin content and the cells were washed 3 times with PBS, and subjected to quick freezing and thawing, also 3 times in 0.2 ml of PBS. This procedure resulted in complete detachment of cells from the glass surface and breakage of individual cells. After centrifugation, the supernatant was saved for toxin determination. The sediment was further washed twice in PBS and was resuspended in 0.2 ml PBS for toxin determination.

Toxin binding to and elution from RBC ghost 10% suspensions of human RBC ghost were prewarmed to the desired temp (0, 22 and 37℃). To each suspension, 1000 TCD-50/ml of toxin was added. At the desired time intervals (3, 6, 10, 20, 30, 40, 50 and 60 min), aliquots were taken for toxin titration. For the elution experiment, 10% RBC membrane suspensions were mixed with 100 TCD-50 of toxin and incubated at 37℃ for 1 h. The mixtures were then washed 3 times with PBS. After washing, the membrane fragments were resuspended in PBS to the original volume, and were incubated at 0, 22, and 37℃. Aliquots were titrated for toxin at 12, 24, 36 and 48 h.

Effect of pH on toxin binding Ten-fold serial dilutions of toxin were prepared in PBS at pH 6.4, 7.0, 7.4, and 7.8. A triplicate of WI-38 cell cultures was inoculated with each toxin dilution, after the cells had been washed with

PBS at different pH's. Following incubation for 1 h at 37℃, all cultures were washed with PBS 8 times, and fresh medium was added. Cytopathic changes were recorded the next day, and the TCD-50 was calculated.

10% human RBC ghost was made in PBS at different pH's. 1000 TCE-50 toxin in 0.1 ml amounts were added to ghost suspensions at a ratio of 1:10. After incubation at 37℃ for 1 h, titration for residual toxin was carried in WI-38 cell cultures by 2-fold serial dilutions.

RESULTS

Toxin adsorption to and elution from WI-38 cells The effect of temperature on the rate of toxin adsorption to WI-38 cells is shown in Fig 1. At 3 min, approximately 0.1% of maximum toxin binding was detected at all temp of incubation. At 10 min, more than 1% binding capacity had been reached. No further increase in binding was detected until 40 min, when more binding took place at 24 and 37℃, but not at 6℃. Although the speed of adsorption was the same with both crude and partially purified toxin, the efficiency of attachment was 30 times better with purified material (Table 1).

The amount of toxin released from intact cells was small, reaching the maximal titer of 1:27 at 6-10 min. No further release was observed in spite of continued incubation. However, after the cells were broken up and centrifuged, the sediments contained much larger amounts of toxin (Table 2).

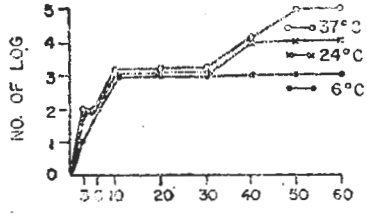

Fig 1. Toxin binding to WI-38 cells. The abscissa represents minutes of exposure, the ordinate, degree of toxin adsorption. The cells were exposed to serial 10-fold dilutions of toxin, washed × 8 with PBS at varying time intervals, and fed with fresh medium. Cytotoxicity was read after overnight incubation; its presence indicates toxin adsorption before repeated washings. Initial adsorption was fast. As toxin exposure continued, more adsorption occurred at higher temp.

Tab 1. Toxin adsorption to WI-38 cells: crude vs partially purified log toxin titer (TCD-50)

Treatment	Crude Toxin	Partially Purified Toxin
No washing	6.5	5.2
Repeated washings after 50 min exposure	5.0	5.0
Difference in toxin titers	30:1	1.1:1

Tab 2. Toxin release from WI-38 cells

Temperature of Incubation(℃)	0	6	22	37
Toxin in eluate	<3*	<3	<3	<3
Cell-bound toxin				
Supernate	81	81	27	27
Sediment†	1,000	——	1,000	1,000

* Reciprocal of dilutions.

† Much less toxin was recovered when partially purified toxin was used, where both supernate and sediment contained about the same titers.

Toxin-binding to and elution from RBC ghost 10% suspensions of human RBC ghost were mixed with 1000 TCD-50 toxin and were incubated at different temp. The reduction of toxin titers plotted against time is shown in Fig 2. At 0℃, no binding was detected until 30 min; continued exposure did not produce

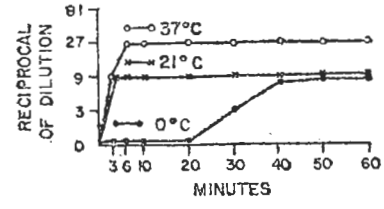

Fig 2. Toxin binding to human erythrocyte lysate. The ordinate represents degree of binding measured in terms of log of toxin disappearance from the medium. The abscissa represents the time of toxin exposure. The rate of binding is temperature dependent.

increased binding. At higher temp, rapid toxin binding was observed, more at 37℃ than at 22℃. A late increase in toxin binding was detected at 50 min.

The extent of toxin binding also depended on the concentration of RBC ghost suspension. A 10% suspension was enough to remove 100 TCD-50 toxin, and at a 2% level, 10 TCD-50 toxin was removed; 0.4% ghost suspension was insufficient to produce detectable toxin reduction.

Elution of bound toxin from ghost cell did not take place until 48 h. This was the case at 0, 22 or 37℃. At 0℃, 90% of bound toxin was released at 48 h.

Effect of pH on toxin binding The degree of toxin binding to WI-38 cells were expressed as the TCD-50. The titers (number of logs) at pH 6.4, 7.0, 7.4 and 7.8 were 5.5, 6.2, 5.7 and 5.5, respectively. The differences were not statistically significant.

A more direct measurement of toxin binding to RBC ghost showed that within pH 6.4–7.8, all ghost suspensions were found to bind toxin equally well. There was no reduction of binding capacity at either the high or low pH tested.

Effect of reducing agents on toxin activity The effect of 2-mercaptoethanol on toxin activity was studied by mixing it with toxin for 10 min before inoculation to WI-38 cell cultures. After incubation at 37℃ for 1 h, all cultures were washed repeatedly with PBS and fed with fresh medium. A complete inhibition of toxicity was observed when the concentration of 2-ME was 5% or higher. After removal of 2-ME from the mixture by dialysis against PBS, toxic activity could not be restored. Treatment of toxin with 1.2 m*M* *N*-acetyl maleimide caused 2 log reduction of toxin titer.

RBC ghosts from different animal species and toxin binding RBC ghosts were prepared from 9 animal species and were tested for toxin binding capacity. Only human RBC ghost was active. None from other species was found to have toxin-binding capacity at the 10% level. The negative results were obtained with the following species: rhesus monkey, dog, guinea pig, rabbit, rat, chicken, sheep, and hamster.

DISCUSSION

A number of bacterial toxins have been known to cause biochemical and morphologic alterations of membranes of susceptible cells[8]. The toxins for which there is best definition of the toxin: membrane interaction include staphylococcal β-toxin, *Clostridium perfringens* α-toxin, staphylococcal δ-toxin, subtilysin, and oxygen-labile hemolysins. Other toxins which cause membrane alterations, but for which the mechanisms are unclear include staphylococcal leucocidin, α-lysin and τ-lysin, streptolysin-S, and hemolysins produced by gram-negative organisms [7,8]. One feature is held in common by all of these toxins: all cause damage to cell membrane. In addition to these cytolytic toxins, diphtheria and cholera toxins bind to the membranes of susceptible cells [9,10]. It appears that binding of toxin to the cell membrane plays an important role in the eventual physiologic or cytopathic activity of the toxin.

The interaction between toxin and WI-38 cells is a complex phenomenon. Toxin binding to RBC ghost showed that there was an initial, rapid binding, then a slowdown, and then a late increase in binding. Within a narrow range of pH studied, no significant differences in binding were observed. RBC ghost was used because the intact cells were found to be inactive. Ghost cell preparations other than that described here were also inactive, suggesting that the inner membranes and other components were essential for binding.

By using antitoxin in the washing process, we were able to remove traces of toxin attached to the outer membrane of WI-38 cells. When these cells were broken up by repeated freezing and thawing, large amounts of toxin could be recovered in the membrane fraction of the broken cells. It appears that the toxin had high affinity for the membranes inside the cells.

The inactivation of toxin by 2-mercapto-ethanol and *N*-ethyl maleimide raises the possibility that both disulfide and sulfhydryl bonds are essential for toxicity. The presence of disulfide bonds has been demonstrated in diphtheria toxin[11] and cholera toxin[12]. These toxins consist of two peptides bound together by a disulfide bond.

The species difference in susceptibility of RBC ghosts for toxin binding is somewhat surprising. When tissue cultures were used to detect *C. difficile* toxin, the species from which the cell cultures are derived did not seem to make any difference; indeed, toxin susceptibility has been demonstrated in cells derived from human, monkey, rabbit, mouse and hamster. With the RBC membrane system, however, only human cells show consistently active binding to toxin.

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