INTERACTION OF DIPHTHERIA TOXIN WITH CELL CULTURES FROM SUSCEPTIBLE AND RESISTANT ANIMALS*, ‡

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Studies on the biological action of diphtheria toxin on tissue and cell cultures have been reviewed by Solotorovsky and Gabliks (1) and by Pappenheimer, Jr., et al. (2). Numerous investigations on differences in cell susceptibility have shown that cell cultures derived from diphtheria toxin–sensitive animals (guinea pig, rabbit, dog, monkey, and man) and those derived from resistant animals (rat and mouse) reflect the characteristic susceptibility or resistance of the donor animal. The previous report by Gabliks and Solotorovsky (3) shows that the cultures derived from diphtheria toxin–resistant animals, the rat and mouse, tolerate about 100,000 times more toxin than the cultures derived from sensitive animals, such as the guinea pig, rabbit, and man. Explanation for these significant differences in cell susceptibility to diphtheria toxin is unknown; certain possibilities are explored in the present study which describes some of the initial effects of toxin on susceptible cells and indicates an alteration of the toxin during exposure to susceptible cell culture.

Methods and Materials

Cell Cultures.—Three human cell lines were used as diphtheria toxin–susceptible cells: Chang liver, derived from normal liver tissue; HeLa, derived from cervical carcinoma; and KB, derived from laryngeal carcinoma. The human cultures were grown in Eagle's basal medium (4) supplemented with 10% calf serum. The toxin-resistant mouse lines, liver NCTC 1469, derived from normal liver, and L-929, derived from subcutaneous tissue, were grown in medium 199 (5) supplemented with 10 to 20% horse serum. All listed stock cultures were obtained from Microbiological Associates, Inc., Bethesda, Maryland.

For experimental work, cells were suspended in the appropriate growth medium containing 100 units per ml penicillin and 100 μg per ml streptomycin. 1 ml volumes of cell suspensions at a concentration of 7×10^4 cells per ml were planted in screw-capped culture tubes. The cultures were incubated in a stationary position at 37°C for 2 days to develop cell monolayers.

Diphtheria Toxin and Antitoxin.—Most experiments were conducted with a crude diphtheria toxin preparation to assess the effects of all components which might be associated with

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the pathogenesis of diphtheria toxin. The crude toxin preparation assayed 3000 guinea pig MLD's[1] (90 Lf units)[2] per ml. For the toxin alteration studies we also included a highly purified toxin preparation which contained 1.35 mg nitrogen per ml and assayed 50,000 guinea pig MLD's (2000 Lf units) per ml (from Massachusetts State Biological Laboratory, Boston, Massachusetts). The corresponding diphtheria antitoxin preparations (also from Massachusetts State Biological Laboratory) assayed 275 units and 1000 units per ml of horse serum. For testing, stock toxins and antitoxins were diluted in culture medium immediately before use.

Test Procedures for Cytotoxicity.—When cell monolayers had developed, the growth medium was decanted and replaced with 1 ml of fresh medium containing the desired quantity of diphtheria toxin. The cultures were then reincubated and observed by microscope examination daily. The degree of cytotoxicity was evaluated by changes in cell morphology and by growth inhibition. The morphological changes were compared by using a scale based on the percentage of destroyed and damaged cells. Destruction or morphological damage of 75 to 100% of the cells was classified as 4+; 50 to 75% as 3+; 25 to 50% as 2+; and less than 25% as a 1+ reaction. The lowest concentration of diphtheria toxin causing a 2+ reaction during the incubation period was designated as the toxic dose (TD).

The growth was expressed as an increase of cell protein per culture. To measure protein, cell monolayers were washed twice with balanced salt solution to remove traces of protein present in the test medium. The washed cells were then dissolved in Lowry's solution (6) and the protein content was determined with the Folin-Ciocalteau reagent according to the method of Oyama and Eagle (7).

To prove that the lethal effects upon cells were due to the specific action of toxin, toxin neutralization tests were performed with antitoxin. Antitoxin was diluted in medium or in balanced salt solution, and 0.1 ml from each of the various dilutions of antitoxin was added directly to the cultures before the addition of the specified amount of toxin. It was established previously that complete neutralization of diphtheria toxin could be achieved by adding an excess of antitoxin to the cultures as late as 60 min after addition of toxin. Controls with antitoxin alone were included. All tests were done in triplicate.

Alteration of Toxin.—To study the biological activity of diphtheria toxin after its interaction with susceptible cells, toxin was diluted in the medium (Eagle's$_{90}$, calf serum$_{10}$) up to 100 MLD per ml, and 10 ml volumes of this toxin solution were added to Chang liver cell monolayers in milk dilution bottles. Control systems consisted of incubation of the same toxin solution without cells and of several identical Chang liver cultures without toxin, the latter serving as the spent medium-cell substance control. After 24 hr of incubation at 37°C, the cells in the toxin-containing cultures were in suspension, and a large number of them were destroyed as indicated by microscope examination of the cells. Immediately, all cultures (including the controls) were frozen and stored at −40°C. To test toxin activity, the cultures were thawed and the media were centrifuged at 1500 RPM for 15 min to remove cellular debris. The resulting supernatants were removed and tested for cytotoxicity in human and mouse liver cultures.

Parabiotic Culture Chambers.—The biological activity of diphtheria toxin after its previous interaction with the susceptible human liver cells was also investigated in parabiotic culture chambers (Bellco Glass Co., Vineland, New Jersey). Two chambers were connected with Millipore filter pads, pore size 220 mμ (Millipore Filter Corp., Bedford, Mass.) or with dialysis membranes, pore size 2.4 mμ (Union Carbide Corp., Chicago, Illinois). Cells were planted on one or both sides of the chambers, as indicated in the experimental results. After 2 or 3 days, when cell monolayers had developed, medium was decanted and all chambers received medium 199 without serum. Crude toxin was added to one side of the parabiotic culture chamber and then allowed to diffuse through the membranes so that its reactivity with the cells growing on the opposite side of the chamber could be studied.

[1] MLD, minimum lethal dose.

[2] Lf, Ramon flocculation unit.

Biochemical Testing Procedures.—Cell monolayers in 125 ml milk dilution bottles containing 10 ml of medium were used for biochemical analysis of the toxin-treated cells. At the termination of the experiment, the cells were washed twice with Hanks' balanced salt solution, and the growth of cells was measured by determination of the increases in cell number, packed cell volume, protein, RNA, and DNA per culture. Intact cells or nuclei were counted with a haemocytometer. When nuclei were counted, the cells were suspended in a citric acid and crystal violet solution (8). An increase in the number of cells per culture during a specified incubation period indicated growth of the culture.

The amount of protein was determined by the method of Lowry et al. (6) as modified by Oyama and Eagle for tissue cultures (7).

The total amount of DNA and RNA per culture was determined by the Schneider method (9, 10), with modifications for small tube cultures. The nucleic acids were extracted with 2.5 ml of 0.5 M trichloroacetic acid for 25 min at 90°C. The extracted cell residues were removed by centrifugation at 2500 RPM. Aliquots of the remaining supernatant were used for determination of DNA with diphenylamine reagent and of RNA with orcinol reagent.

TABLE I

Cytotoxicity of Crude and Purified Diphtheria Toxin in Human Chang Liver Cells

Toxin	Antitoxin	Degree of cytotoxicity at 48 hr*	
		Crude toxin	Purified toxin
MLD‡/ml per culture	*units/ml*		
1.0		++++	++++
0.5		+++	++++
0.1		++	+++
0.05		±	++
0.01		0	±
2.5	0.1	0	0
2.5	0.01	++++	++++

* Destruction or morphological damage of 75 to 100% of the cells was classified as 4+; 50 to 75% as 3+; 25 to 50% as 2+; and less than 25% as a 1+ reaction.

‡ Minimum lethal dose for guinea pigs.

RESULTS

Effects of Diphtheria Toxin upon Cells.—At cytotoxic levels, diphtheria toxin inhibited cell growth and induced progressive degenerative changes which led to destruction of the cells. The magnitude of cytotoxicity induced by the crude and the highly purified toxin preparations was similar and was neutralizable by diphtheria antitoxin. The results of toxin titrations are compared in Table I.

The susceptibility of the cell cultures was compared by using the toxic dose levels (TD) obtained by titration of crude toxin. Human KB cells were most susceptible with TD at 0.001 MLD per ml; followed by HeLa, 0.01 MLD per ml; Chang liver, 0.1 MLD per ml; mouse L-929, 100 MLD per ml; and mouse liver NCTC 1469, 500 MLD per ml. Accordingly, the mouse liver cells were

5000 times more resistant than the human liver cells, and the cell culture toxicity correlated well with the relative susceptibility of both man and mouse.

The initial effects of diphtheria toxin on susceptible cells were studied in human Chang liver cells using 0.05 MLD of toxin per ml of medium 199 (without serum). At this low toxin concentration, which is half of the TD level, there was no detectable cytopathogenicity during the first 48 hr, but it became evident on continued incubation. At the end of 48 hr of incubation, the cultures were analyzed for the number of cells, packed cell volume, protein, RNA, and

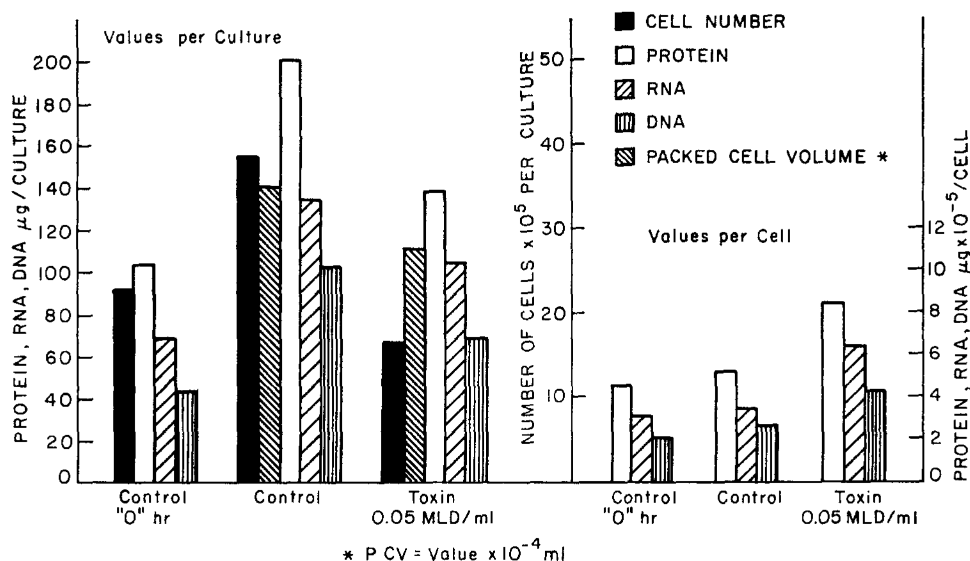


FIG. 1. Effects of crude diphtheria toxin on cell number, protein, RNA, DNA, and packed cell volume in human Chang liver cultures. Cells were exposed to 0.05 guinea pig MLD of toxin per ml of medium for 48 hr. The values per culture are illustrated on the left side of the graph and the values per cell on the right side.

DNA. The results are illustrated in Fig. 1. The number of cells in the toxin-treated cultures was 30% less than at the "0" hr levels and significantly less than in the controls. Protein values were 69% of the corresponding control values, RNA was 77%, and DNA, 67%. When the values of total protein, RNA, and DNA are expressed per cell, it is evident, as indicated on the right side of Fig. 1, that the individual toxin-treated cells contained 66% more protein, 84% more RNA, and 60% more DNA than the control cells. The cell volume was also increased 1.9 times.

In a similar test, when mouse L-929 cells were exposed to 50 MLD of toxin per ml (half of the TD), no toxic effects were detected by examination of cell morphology, number of cells, or cell protein content.

Alteration of Toxin by Susceptible Liver Cells.—The biological activity of diphtheria toxin after its previous interaction with human Chang liver cells was measured by its toxicity in both toxin-susceptible and toxin-resistant cells.

The toxin removed from Chang liver cultures and the control preparations (normal stock toxin, toxin incubated without cells, and the spent medium-cell substance control from the

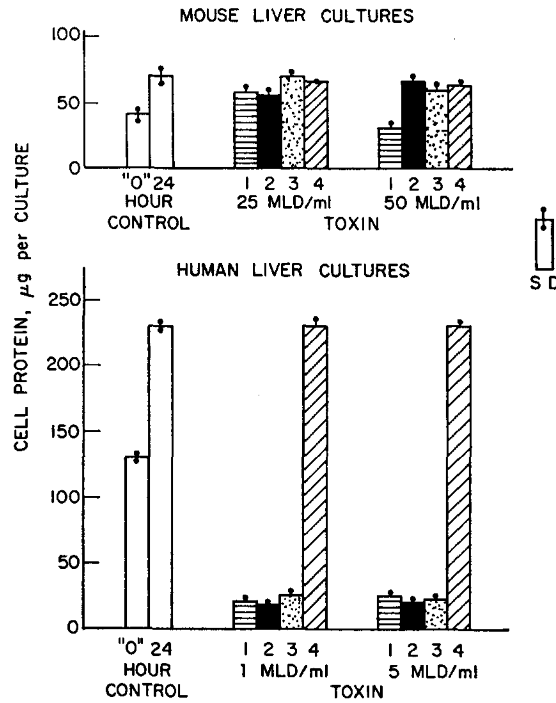


FIG. 2. Cytotoxicity of purified diphtheria toxin after its previous incubation with human Chang liver cells. The cytotoxicity in human Chang and mouse liver NCTC 1469 cultures at 24 hr is illustrated as growth inhibition—reduction of cell protein content per culture. Cultures: No. 1 contained toxin incubated with human liver cells; No. 2, toxin incubated without cells; No. 3, stock toxin; No. 4, spent medium from normal Chang liver culture.

normal Chang liver culture) were diluted equally in the corresponding culture medium and then added to the human and mouse liver cultures. The cytotoxicity was determined after 24 hr of incubation by measuring growth inhibition and cell destruction, i.e., reduction of cell protein per culture. The results are illustrated in Fig. 2.

In human liver cultures, all toxin preparations at 1 and 5 MLD of toxin per ml destroyed cells and accordingly reduced cell protein content up to 91% of the control values. In the toxin-resistant mouse liver cultures, only the toxin preparation removed from Chang liver cultures was cytotoxic. At 50 MLD per ml toxin level, the cell growth was completely inhibited, and the cells exhibited

abnormal morphology. Neither the normal stock toxin, the incubated toxin preparation, nor the spent medium-cell substance control induced any detectable cytotoxicity. When the cytotoxic effects caused by 500 MLD per ml of normal toxin are compared to those induced by 50 MLD per ml of "altered toxin," it is evident that the altered preparation is 10 times more toxic to the mouse liver culture.

To prove that the cytotoxicity induced by the altered toxin in resistant

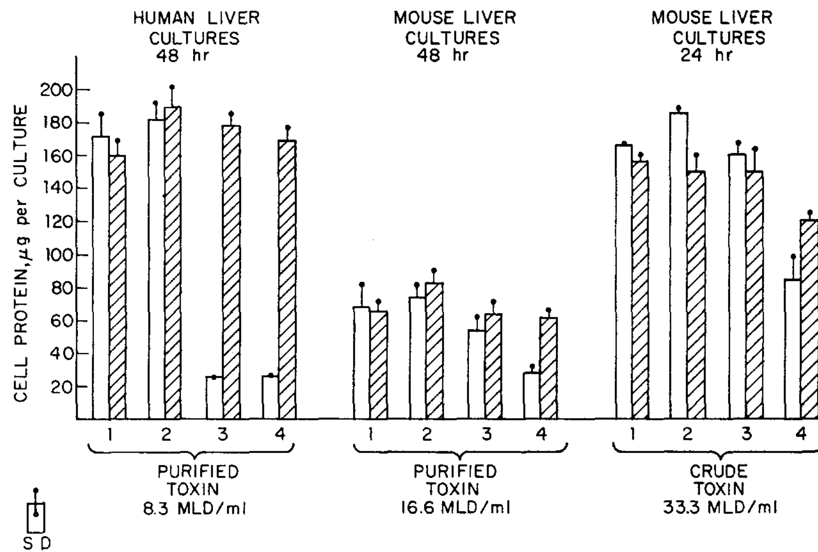


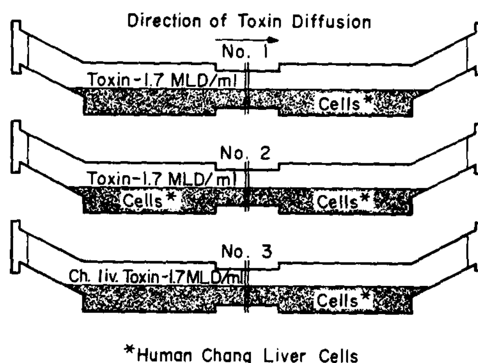
FIG. 3. Cytotoxicity of "altered toxin" (toxin incubated with Chang liver cells) and its neutralization with antitoxin. The cytotoxicity in human Chang and mouse liver NCTC 1469 cultures at 48 hr is illustrated as growth inhibition-reduction of cell protein content per culture. Cultures: No. 1, control after specified length of incubation; No. 2, spent medium from Chang liver culture; No. 3, toxin incubated in medium without cells; No. 4, toxin incubated with Chang liver cell cultures. The striped bars represent the same cultures containing antitoxin.

mouse liver cells is specific to the action of diphtheria toxin, we performed two additional experiments aimed at neutralizing the cytotoxicity with diphtheria antitoxin. Fig. 3 shows cytotoxicity results of both purified and crude toxin preparations. At 48 hr the general magnitude of cytotoxicity in human and mouse liver cultures was similar to that obtained in the first experiment. In the presence of antitoxin, the cytotoxicity induced with purified toxin preparations in human and mouse liver cells was neutralized completely, whereas the cytotoxicity induced with the altered crude toxin preparation was only partly neutralized as shown by reduced cell protein content per mouse liver culture.

In the above experiments, the spent medium-cell substance control from the

normal Chang liver cultures was not toxic to mouse liver cells at the concentration levels tested (dilutions 1/2 and 1/4). However, additional investigations showed that, with longer incubation, undiluted preparations were toxic for mouse liver cells.

An increased activity of diphtheria toxin after its interaction with Chang liver cells was also evidenced by testing the toxin diffusion rate in parabiotic



CHAMBER	CYTOTOXICITY SCORE	
	24 Hours	48 Hours
No. 1	±	2+
No. 2	2+	4+
No. 3	1+	4+

FIG. 4. Diffusion of diphtheria toxin in parabiotic culture chambers connected with filter of dialysis membranes. The toxin was added to the left hand chambers, and after it diffused through the membranes its activity was scored according to the magnitude of the cytotoxicity on human Chang liver cells growing in the right hand chambers. Destruction or morphological damage of 75 to 100% of the cells was classified as 4+; 50 to 75% as 3+; 25 to 50% as 2+; and less than 25% as a 1+ reaction.

culture chambers as indicated in Fig. 4. Toxin diluted in medium 199 without serum was added to the left hand chambers at concentrations of 1.7 MLD per ml. The activity of toxin after diffusion through the membranes was scored according to the magnitude of the cytotoxicity in the right hand chambers. Three experiments showed that the toxin which had interacted with human liver cells induced more pronounced reactions more rapidly than did the control toxin.

DISCUSSION

The susceptibility of the cell cultures used in this study ranges from 0.001 to 500 guinea pig MLD of diphtheria toxin per ml of medium. There is up to a

500,000-fold difference in susceptibility between cultures derived from the toxin-susceptible species such as man and the cultures derived from the mouse, one of the mammals relatively resistant to diphtheria toxin.

The initial effects of toxin upon susceptible cells were studied, using human Chang liver cells with a low toxin concentration which did not induce cell destruction in the first 48 hr. Under these conditions, the protein, RNA, and DNA content of the cultures remained fairly constant, whereas cell division was inhibited. This situation led to the formation of cells containing significantly more protein, RNA, and DNA, as illustrated in Fig. 1. The enlargement of toxin-treated cells is also evidenced by a 1.9-fold increase of cell volume. In agreement with our results are studies by Penso and Vicari (11) who reported the disappearance of mitotic figures and cessation of multiplication in human liver cells with low toxin concentrations.

Results of the biological activity of diphtheria toxin after its interaction with the toxin-susceptible human Chang liver cells show a significantly increased toxicity for the toxin-resistant mouse liver cells. The altered toxin, at concentrations ranging from 16 to 50 guinea pig MLD per ml, inhibited growth and induced progressive degenerative changes leading to cell destruction. In the same test, the normal stock toxin and the toxin incubated in medium (Eagle's$_{90}$, calf serum$_{10}$) without cells did not induce any detectable cytotoxicity. The cytotoxicity was specifically induced by the action of diphtheria toxin as shown by the neutralization of toxicity with specific diphtheria antitoxin. However, the possibility that some components of spent medium-cell substance from Chang liver cultures may affect the magnitude of cytotoxicity cannot be excluded with the present testing system.

When the same toxin preparations were titrated in the toxin-susceptible human liver cultures, they were all equally toxic and their toxicity was neutralizable with antitoxin. Since the toxin incubated in culture medium without cells was not inactivated as indicated by its cytotoxicity, the results suggest an alteration of diphtheria toxin during interaction with Chang liver cells.

The hypothesis of toxin alteration is also supported by the results of toxin diffusion through membranes in parabiotic culture chambers. After interaction with Chang liver cells, the toxin induced a more rapid and pronounced reaction upon the same cells growing on the other side of the membranes. These results indicate a possibility that the relatively large toxin molecule with a molecular weight of approximately 72,000 may be transformed by sensitive cells to smaller units which diffuse more rapidly and may more easily penetrate the cell membranes. The process of penetration of toxin through cell membranes has not yet been satisfactorily explained considering the large molecular weight of diphtheria toxin.

This study indicates that the normally resistant mouse liver cells are sus-

ceptible to diphtheria toxin after its interaction with susceptible human liver cells. The evidence presented suggests that the toxin is transformed to a more active derivative by enzyme systems present in susceptible cells. Such a transformation reaction may be essential as a preliminary step for the penetration of the cell membranes by toxin and may be one of the factors which determine a cell susceptibility or resistance to diphtheria toxin.

SUMMARY

The cytotoxicity (TD) level of diphtheria toxin for human Chang liver strain was 0.1 guinea pig MLD per ml; for mouse liver NCTC 1469 strain, the TD was 500 MLD per ml. The results of cell culture toxicity correlated well with relative susceptibility of both man and mouse. The initial effects of toxin on the susceptible Chang liver cells were studied at one half the TD level (0.05 MLD per ml). At this low concentration of toxin, the number of cells per culture was reduced slightly below the "0" hr values, whereas the amounts of cell protein, RNA, and DNA were increased. Analysis of the toxin-treated cells indicated an enlargement of the cells. The individual cells contained significantly more protein, RNA, and DNA than the control cells and the cell volume was increased 1.9 times.

When purified diphtheria toxin was incubated with the susceptible Chang liver cells and then tested for its biological activity, the results showed an increased diffusion rate in parabiotic culture chambers and definite cytotoxicity to the normally resistant mouse liver cells. The cytotoxicity was neutralizable with antitoxin. The results suggest that the toxin-susceptible cells transform the toxin molecule to a more active derivative which affects the highly resistant mouse liver cell.

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