THE RESPONSE OF CULTURED MAMMALIAN CELLS TO DIPHTHERIA TOXIN

II. The Resistant Cell: Enhancement of Toxin Action by Poly-L-Ornithine*,‡

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Plate 69

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Inhibition of protein synthesis is the lethal effect resulting from the action of diphtheria toxin upon cultured mammalian cells. The component of the cell's synthetic apparatus which is sensitive to toxin has been localized in the 100,000 g supernatant fraction of extracts prepared from sensitive cells[1] (6). In the reticulocyte cell-free system, it has been shown that a transfer factor, which may be the peptide synthetase enzyme, is inactivated by toxin plus cofactor NAD[2] (6). These represent significant advances toward an understanding of the mode of action of toxin in cultured mammalian cells and cell-free systems. Conversely, little attention has been given to describing the mechanism of resistance which is a property of certain mammalian cells, enabling them to survive an excess of 100,000 times the lethal dose for sensitive cells.

Our present study was undertaken to determine whether resistance to toxin of the L cell, derived from the toxin-resistant mouse, was due (*a*) to actual differences in subcellular components directly involved in protein synthesis, or (*b*) to other cellular structures or functions. This toxin-resistant cell syn-

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[1] Using the sensitive KB cell, we have found the site of toxin action to be localized in the 100,000 g supernatant fraction of intoxicated whole cells. Toxin added to cell-free systems from normal KB cells inhibited protein synthesis to approximately the same extent as similar concentrations of toxin in HeLa cell and reticulocyte systems (6, 7). These data will be reported elsewhere.

[2] Abbreviations used in this paper: NAD, nicotinamide adenine dinucleotide; MEM, Eagle's minimum essential medium; HBSS, Hanks balanced salts solution; BME, Eagle's basal medium; TCA, trichloroacetic acid; PCA, perchloric acid; ATP, adenosine triphosphate; GTP, guanosine triphosphate.

thesizes protein in the same manner as sensitive cells do; in cell-free systems, we have combined 100,000 *g* supernatant fractions from L cells with washed ribosomes from sensitive KB cells, and vice versa, and determined that incorporation of labeled amino acids into protein was as efficient as in homologous systems (Moehring and Moehring, unpublished data). From this it was predicted that the latter hypothesis was correct. Our studies on the mode of action of toxin in the sensitive KB and HeLa cells, both intact cells (11) and cell-free systems, were used as a basis for comparison of the toxin-cell interactions and provided some of the techniques for testing the above hypotheses.

We have investigated the sensitivity of both intact L cells and cell-free systems, and have determined that resistance is linked to the cell membrane and the process of macromolecular uptake, rather than to any intrinsic property of the polyribosomes or associated protein synthesizing factors.

Materials and Methods

Cell Cultures.—L-929, mouse fibroblast cells, were obtained from Grand Island Biological Co., Grand Island, N. Y. They were maintained in antibiotic-free MEM containing 10% calf or 5% fetal calf serum, in milk dilution bottles.

Diphtheria Toxin.—Purified, 5 × recrystallized, diphtheria toxin, lot RX 7328, previously described (11) and crude toxin, lot 1051 (Wyeth Laboratories, Marietta, Pa.), dialyzed and concentrated to contain 1187 Lf (51, 041 guinea pig MLD) per ml were used. Crude toxin was used in some preliminary experiments. The results were verified using the purified toxin.

Reagents.—ATP (dipotassium salt), GTP, creatine phosphate, creatine phosphokinase, and bovine pancreatic ribonuclease (5 × crystallized) were obtained from Calbiochem, Los Angeles, Calif. Puromycin-dihydrochloride, 2-mercaptoethanol, and naphthol AS-BI phosphoric acid (sodium salt) were supplied by Sigma Chemical Co., St. Louis, Mo. Poly-L-ornithine-HBr, molecular weight 175,000, was obtained from Pilot Chemicals, Inc., Watertown, Mass.

Radioactive Compounds.—The following labeled compounds were used: purified ^{14}C-amino acid mixture (15 amino acids, 100 μc/ml) and ^{14}C-L-valine (195 mc per mmole). Both were from New England Nuclear Corp., Boston, Mass.

Assay Procedure in Intact Cells.—Replicate cultures of L cells were distributed to 25 ml Erlenmeyer flasks and were used after 48 hr of growth. Cell sheets were washed once with warm HBSS and 3 ml of test medium (MEM or BME), containing radioactive amino acids and the appropriate test compounds, were added. Cultures were incubated in a water bath shaker at 36°C, and incubation was terminated by addition of TCA to 5%. After chilling at least 1 hr, samples were heated at 90°C for 15 min and scraped free of the flask with a rubber scraper. Samples were collected on filter discs with three washes of 5% TCA, placed in scintillation vials, and dried. 15 ml of a solution of 0.5% 2,5-diphenyloxazole (PPO) and 0.03% 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (dimethyl POPOP) in toluene was added and the samples were counted in a Packard Tri-Carb liquid scintillation counter.

Preparation of Cell Extracts.—48 hr cell sheets were chilled, and the cells suspended in cold HBSS. The cells were collected by centrifugation and washed twice in cold HBSS. They were then suspended in a hypotonic buffer (HB), containing 0.01 M Tris-HCl, pH 7.4, 0.01 M KCl, 0.0015 M magnesium acetate, and 0.006 M 2-mercaptoethanol, to a concentration of approximately 62 million cells per ml. Cells were allowed to swell for 10 min, then were disrupted with 5 to 7 strokes of a tight-fitting, all glass, Dounce homogenizer. Enough 1.25 M sucrose in

HB to give a final concentration of 0.25 M sucrose was then added. This homogenate was centrifuged at 800 *g* for 10 min. The 800 *g* supernatant was centrifuged at 13,000 *g* for 15 min. 13,000 *g* supernatant was used in the studies of in vitro amino acid incorporation.

Amino Acid Incorporation by Cell-Free Extracts.—Amino acid–incorporating systems contained 0.4 ml of L cell extract per ml. The complete reaction mixture also contained 50 μmoles of Tris-HCl buffer, pH 7.5, 5 μmoles of magnesium acetate, 50 μmoles of KCl, 6 μmoles of 2-mercaptoethanol, 1 μmole of ATP, 0.25 μmole of GTP, 10 μmoles of creatine phosphate, 120 μg of creatine phosphokinase, and 2 μc ^{14}C-L-valine, supplemented with 0.05 μmoles per ml of each of 19 other amino acids. All components of the system except the extracts were combined in the cold and the reaction was initiated by addition of the extract. Reaction mixtures were incubated at 36°C and 0.3 ml samples were collected in 7.0% PCA containing 20 mg per ml casamino acids. After 1 hr at 4°C, samples were heated at 90°C for 15 min. They were then centrifuged, the pellet dissolved in 1 N NaOH for exactly 2 min, and reprecipitated with 10% TCA. After chilling for 1 hr, samples were collected on filters and washed 3 times with 5% TCA. The precipitates were extracted with 1:1 ether-ethanol, followed by ether, then dried and counted as described above.

TABLE I

Relative Sensitivity to Diphtheria Toxin of KB, HeLa, and L Cells

Parameter measured	KB*	HeLa*	L
Concentration of toxin which completely inhibits protein synthesis in 12 hr	0.0005 MLD/ml	—	>1000 MLD/ml
Saturating level of toxin	15 MLD/ml	15 MLD/ml	>1000 MLD/ml
Time of first detectable toxic effect	15 min	30 min	8–9 hr

* Data from reference 11.

Acid Phosphatase Staining.—Cells to be stained were grown on cover slips in Leighton tubes and used after 48 hr of incubation at 36°C. Before staining, cells were treated with various test substances (i.e., toxin, serum, poly-L-ornithine). Cover slip cultures were washed twice with warmed HBSS and the appropriate test medium (MEM or MEM containing test substances) was applied. Cultures were incubated at 36°C for various periods. The cultures were then washed twice with HBSS, once with normal saline, and fixed for 1 hr in Baker's formol-calcium solution. After fixation, cover slips were washed in three changes of normal saline, over a 30-min period. Cells were then stained by the method of Barka and Anderson (2), utilizing naphthol AS-BI phosphate and pararosaniline dye. Best results were obtained using a 1-hr staining period. Sites of acid phosphatase activity were stained red.

RESULTS

Table I shows the relative sensitivities to toxin of KB, HeLa, and L cells. Protein synthesis is completely inhibited in 12 hr by five ten-thousandths of a guinea pig MLD per ml in KB cells. In L cells tested under the same conditions, 1000 MLD per ml (the highest level tested) fails to inhibit protein syn-

thesis completely. In L cells, the saturating level[3] is greater than 1000 MLD per ml and has not been precisely determined. The first evidence of toxin action in KB cells is noted in only 15 min; in HeLa cells, in 30 min. In L cells, even with the highest concentrations of toxin tested, the first inhibition is detected only after 8 to 9 hr exposure to toxin.

To eliminate the possibility that contact with the L cell or its products inactivated toxin, samples of toxin of 2 MLD per ml were incubated with L cells for 24 hr, then tested on sensitive cells. Toxin so incubated lost none of its activity against KB cells (Table II).

TABLE II

The Effect of Incubation with L Cells upon Toxicity of Diphtheria Toxin

Sample	2 hr		3 hr	
	CPM	Per cent inhibition	CPM	Per cent inhibition
Control	14,762	—	21,522	—
Toxin	8,900	40	8,803	59
"L cell" toxin	8,763	41	10,126	53

10 ml of MEM containing 2 MLD/ml of toxin was incubated for 24 hr at 36°C on a full cell sheet of L cells ("L cell" toxin). A similar sample was incubated with no cells. Toxin was tested at 1 MLD/ml on flask cultures of 48 hr KB cells, containing 0.11 μc/ml of ^{14}C-valine. Samples were taken at 2 and 3 hr.

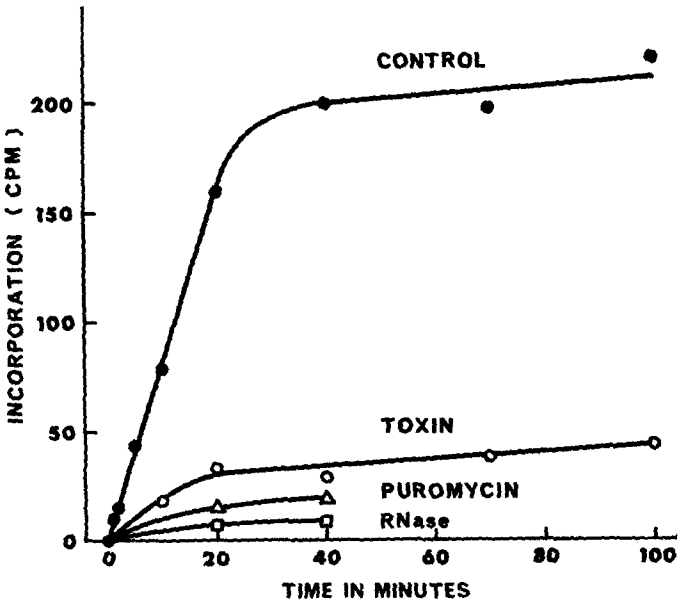
To investigate whether the actual protein-synthesizing components of the L cell differed from those of sensitive cells, cell-free amino acid–incorporating systems were prepared, using postmitochondrial extracts from disrupted cells. Text-fig. 1 shows incorporation of ^{14}C-valine into hot acid-insoluble material in an L cell cell-free system. Incorporation is linear for approximately 20 min and complete after 40 min. 100 MLD per ml of toxin caused 85% inhibition of protein synthesis in 40 min.

Table III shows the effect on protein synthesis of levels of toxin ranging from 0.1 MLD to 500 MLD per ml. After a 40-min reaction time, the lowest concentration of toxin caused 11% inhibition, while 100 and 500 MLD per ml caused 85 and 88% inhibition respectively. Complete inhibition was not achieved, even when higher levels of toxin were used.

Comparison of these figures with results from HeLa cell and rabbit reticulocyte systems (6, 7) and with our results from the sensitive KB cell-free system (Moehring and Moehring, unpublished data) revealed that the amino acid–

[3] A saturating level of toxin in cell cultures is defined as the lowest concentration of toxin that will produce a detectable effect in the minimum time, where higher concentrations cannot shorten this time.

incorporating system from L cells was equally sensitive to diphtheria toxin. The cell-free system did not reflect the resistance of the intact cell. It therefore appeared that in the intact cells the toxin did not gain access to the cytoplasmic sites of protein synthesis; thus, we turned our attention to uptake of the toxin molecule by the intact L cell.



TEXT-FIG. 1. The effect of toxin on incorporation of ^{14}C-valine by cytoplasmic extracts from L cells. Crude extracts of L cells were prepared and reacted in the cell-free system, as described in the text. Purified toxin was included at a concentration of 100 MLD per ml. Puromycin was included at 1×10^{-4} M and ribonuclease at 10 μg per ml. Incorporation of ^{14}C-valine is plotted as CPM per 0.3 ml sample.

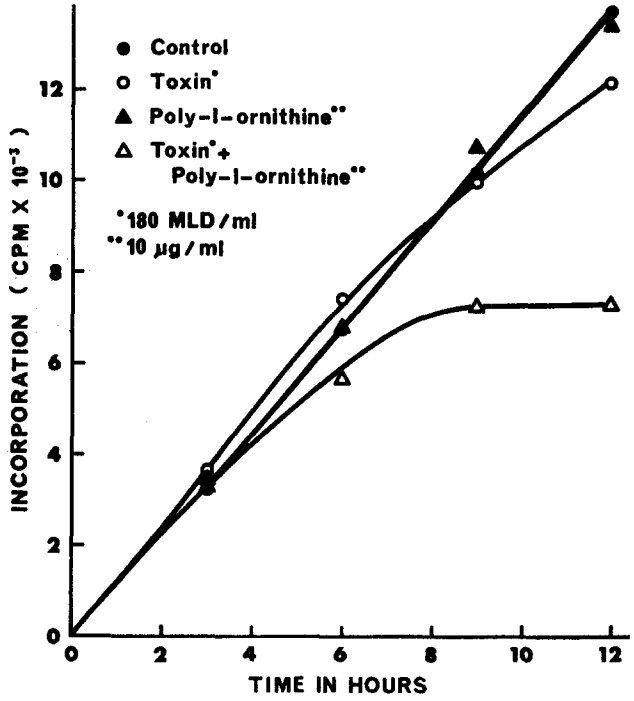
It has been reported that high molecular weight poly-L-ornithine is a potent stimulator of protein uptake in sarcoma 180 cells (12). Using levels similar to those reported, we exposed L cells to poly-L-ornithine and toxin, in serum containing medium. Text-fig. 2 shows the results of such a test. The presence of polyornithine enhanced the ability of the toxin to inhibit protein synthesis. The time of earliest detectable effect was shortened by approximately 6 hr, and synthesis was completely stopped after 9 hr of exposure. From this type of experiment, we also had our attention drawn to the effects of serum on toxin action in the L cell. Previously, we had carried out most of our tests in serum-free medium, having determined that the rate of incorporation of ^{14}C-amino acids was little influenced by its presence or absence over the time of our experiments. We had tested levels of toxin ranging from 10 to 1000 MLD per

TABLE III

The Effect of Diphtheria Toxin on the Incorporation of ^{14}C-Valine by Extracts of L Cells

Sample	CPM/ml	Per cent inhibition
Control	663	—
Toxin		
0.1 MLD/ml	593	11
1.0 "	380	43
10.0 "	153	77
100.0 "	97	85
500.0 "	80	88
Puromycin		
10^{-4} M	63	90
RNase		
10 μg/ml	27	96
100 " "	30	95

Assay conditions were as described in the text. Samples were taken after 40 min of incubation.



TEXT-FIG. 2. The effect of toxin and poly-L-ornithine on incorporation of ^{14}C-amino acids into intact L cells. Assay conditions were as described in the text. The test medium was MEM, containing 5% calf serum and 0.1 μc of ^{14}C-amino acid mixture per ml. The test substances were included as indicated in the figure.

ml in serum-free medium, against our L cell line, and had observed no significant inhibition of protein synthesis in a 12-hr period (see Table IV). Because the more active concentrations of polyornithine tend to be inhibitory to the cells in serum-free medium, we included serum in these tests, and observed that the presence of calf serum alone caused the toxin to have some effect by 12 hr. Fetal calf serum was slightly more effective in promoting toxin activity than was calf serum.

TABLE IV

Response of L-929 Cells in Serum-Free MEM to Increasing Levels of Diphtheria Toxin

Concn. of toxin	CPM per culture*
MLD/ml	
0	5489
10	5733
100	5923
250	5728
500	5722
750	5320
1000	5407

* The reported values are the average of duplicate cultures. Standard deviation of duplicates was no greater than 4%.

TABLE V

The Response of L Cells to Diphtheria Toxin in Several Media

Eagle's MEM* containing:	Inhibition of amino acid incorporation
	%
No serum	0
5% calf serum	10
5% fetal calf serum	14
5% calf serum plus 10 μg/ml poly-L-ornithine	46

100 MLD/ml of toxin measured after 12 hr exposure.

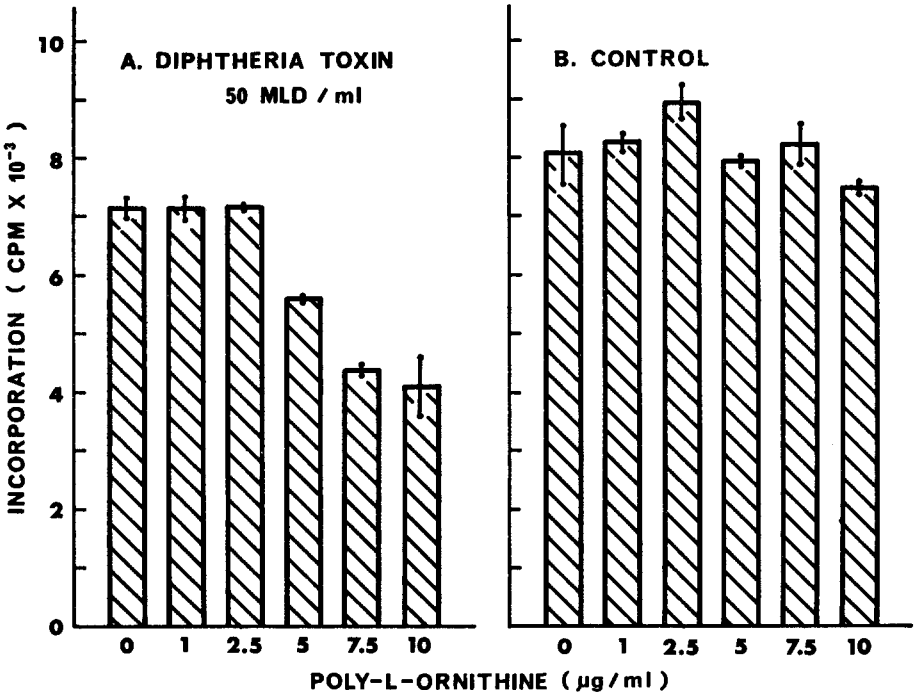
* Minimum essential medium.

Table V shows the per cent inhibition of amino acid incorporation caused by 100 MLD per ml of toxin in four different media. In medium with no serum, no inhibition is detected. With 5% calf serum, 10% inhibition is seen; with 5% fetal calf serum, 14% inhibition; and with 5% calf serum plus 10 μg per ml of polyornithine, 46% inhibition is seen.

Text-fig. 3 shows the results of a titration of polyornithine on L cells, measured at 12 hr. On the right (B), concentrations of polyornithine ranging from 1 to 10 μg per ml have little effect on incorporation of ^{14}C-amino acids into cell protein. On the left (A), 50 MLD per ml of toxin was incor-

porated into the polyornithine-containing medium. Concentrations of polyornithine below 5 μg per ml did not stimulate toxin action; 5 μg and above did.

Diphtheria toxin was titrated in the presence and absence of 10 μg per ml of polyornithine. The results are shown in Text-fig. 4. In medium without polyornithine, concentrations of toxin of 50 MLD per ml and higher have a de-



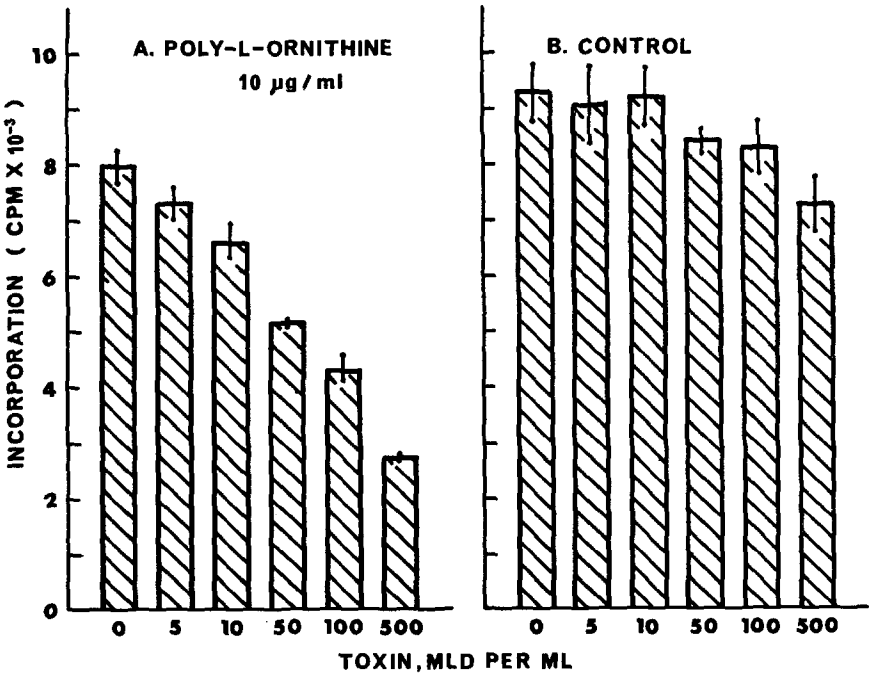
TEXT-FIG. 3. The effect of increasing concentrations of poly-L-ornithine on incorporation of ^{14}C-amino acids in normal and toxin-treated L cells. Assay conditions were as described in the text. The test medium was BME, containing 5% calf serum and 0.06 μc of ^{14}C-amino acid mixture per ml. Toxin and poly-L-ornithine were included as indicated in the figure.

tectable toxic effect, not exceeding 22% inhibition seen with 500 MLD per ml. In medium containing polyornithine, all levels tested, from 5 to 500 MLD per ml have detectable toxic effects, ranging from 9% inhibition with 5 MLD per ml to 66% for 500 MLD per ml.

We have tested polyornithine in cell-free systems prepared from L cells, both alone and in combination with dialyzed calf serum, and have determined that polyornithine itself is not inhibitory (Table VI).

It has been reported that structures derived from pinocytotic processes may be demonstrated by acid phosphatase staining and that the acid phosphatase

activity of various cells is related to the development and actual activity of the pinocytotic apparatus (1, 3, 14). We undertook acid phosphatase staining of sensitive and resistant cells, in the presence and absence of serum and polyornithine, to determine whether differences in pinocytotic activity could be detected.



TEXT-FIG. 4. The effect of increasing concentrations of toxin on incorporation of ^{14}C-amino acids in polyornithine-treated and control cells. Assay conditions were as described in the text. The test medium was BME, containing 5% calf serum and 0.06 μc of ^{14}C-amino acid mixture per ml. Poly-L-ornithine and toxin were included as indicated in the figure.

Fig. 1 shows the results of such staining. The sensitive KB cell (Fig. 1 *a*) has a high level of acid phosphatase activity, as shown by the presence of red-staining granules. This high level is detected in the presence or absence of polyornithine or serum. The L cell, in serum-free medium (Fig. 1 *b*), has no detectable acid phosphatase but when incubated in medium containing serum and polyornithine, in this case for 8 hr (Fig. 1 *c*), red-staining granules form, indicating increased lysosomes and increased pinocytotic activity. In Fig. 1 *d* we have included a picture of fibroblasts cultured from guinea pig peritoneal exudate, nonphagocytic cells which stain negatively for acid phosphatase. They are shown in comparison with intensely positive macrophages. These

fibroblasts, although derived from a sensitive animal, were found, in preliminary experiments, to be resistant to diphtheria toxin, further supporting our belief that cellular resistance to toxin is based on the ability of the membrane to exclude the toxin molecule.

TABLE VI

The Effect of Poly-L-Ornithine and Serum on the Incorporation of ^{14}C-Valine by Extracts of L Cells

Sample	CPM/ml
Control	663
Poly-L-ornithine, 1.0 μg/ml	636
Serum (dialyzed calf), 0.5%	546
Poly-L-ornithine plus 0.5% serum	
0.01 μg/ml	560
0.1 " "	610
1.0 " "	576

Assay conditions as described in the text. Samples taken after 40 min incubation.

DISCUSSION

It has been suggested that cells in culture reflect the sensitivity or resistance to diphtheria toxin of the animal species from which they were derived (8, 13). Numerous examples of the former have been cited in the literature and recently were reviewed by Solotorovsky (13). Examples of the latter, while less numerous, include cells cultured from both the resistant mouse and rat. There are also reports of sublines from normally sensitive cell lines which developed toxin resistance (8, 9). To study this general phenomenon of resistance to toxin in cultured mammalian cells, we selected as our model the L cell, both for its resistance to the lethal action of high levels of diphtheria toxin and for its derivation from the resistant mouse (4).

When studied under the same specified conditions, the L cell is more than 100,000 times as resistant to diphtheria toxin as the KB and HeLa human carcinoma cells (see Table I) (13). This resistance does not result from neutralization or inactivation of the toxin during interaction with either the cells or cell products. Further, it was shown that the protein-synthesizing components of the L cell were sensitive to toxin. Comparison of data from the cell-free system of the L cell with data from similar toxin-treated systems from the sensitive KB cell (Moehring and Moehring, unpublished data), HeLa cell, and rabbit reticulocyte (6, 7) showed that the protein-synthesizing components of the L cell were equally sensitive.

Since it appeared that the toxin molecule did not gain access to the cytoplasmic sites of protein synthesis in the intact L cell, we investigated the uptake of toxin by this cell. It has been shown that poly-L-ornithine can stimulate the uptake of protein molecules in cultured cells (12). We found that when toxin was applied to L cells in the presence of serum and poly-L-ornithine, the toxin was able to inhibit protein synthesis to a marked degree; not, however, as efficiently as in the normally sensitive cells. We can increase the sensitivity of L cells to toxin, but we cannot render them as sensitive as KB or HeLa cells. Neither the polyornithine nor the serum was significantly inhibitory in cell-free systems.

It was apparent that the resistance of the L cell to diphtheria toxin was related to the failure of the toxin to be taken into the cell, at least in sufficient amount to express its effect. Polyornithine has been considered a possible stimulator of pinocytosis (12). Fetal calf serum, especially the fetuin constituent, has been shown, by Cohn and Parks (5), to stimulate the formation of pinocytotic vesicles in mouse macrophages. Since structures derived from pinocytotic processes may be demonstrated by acid phosphatase staining (1, 3, 14), this technique was applied to sensitive and resistant cells, in the presence and absence of polyornithine, to determine whether differences in pinocytotic activity could be detected. Sensitive cells had a high level of acid phosphatase activity, whereas the resistant cells had a very low level, unless incubated in medium containing serum and polyornithine. The acid phosphatase–positive lysosomes appeared as sensitivity to toxin increased, suggesting that toxin enters the cells by pinocytosis and that cellular resistance to toxin in the L cell is based on the ability of the membrane to exclude the toxin molecule.

Determining the effects upon the cell of an interaction between polyornithine and diphtheria toxin also served to clarify a point regarding the stimulatory nature of polyornithine. The inhibition of protein synthesis by diphtheria toxin is a result of its unique action upon cytoplasmic components (6). It does not inhibit protein synthesis by any surface or membrane associated mechanism (11). Thus, the ability of polyornithine to enhance toxin action, while at the same time stimulating acid phosphatase activity, is good evidence for concluding that the uptake of protein stimulated by poly-L-ornithine represents absorption into the cytoplasm, or cytoplasmic bodies, and not merely adsorption to the cell surface.

SUMMARY

We have investigated the response of the resistant mouse L cell to diphtheria toxin. Intact cells and cell-free systems were studied. It was determined that the cell-free system is as sensitive to toxin as those from sensitive reticulocyte,

HeLa, and KB cells previously studied. Poly-L-ornithine, reported to stimulate macromolecular uptake, enhanced toxin activity under conditions which also stimulated acid phosphatase activity. Resistance, in the L cell, appears to be linked to the cell membrane and the process of macromolecular uptake, and not to any intrinsic property of either the polyribosomes or associated protein synthesizing factors. Because of the unique way in which diphtheria toxin inhibits protein synthesis, the ability of poly-L-ornithine to enhance this action provides convincing evidence that poly-L-ornithine stimulates a true absorption of macromolecules and not just increased surface adsorption.

We thank Dr. H. Eagle for suggesting the use of poly-L-ornithine in these studies and Dr. M. Goihman-Yahr for providing the guinea pig fibroblast cultures. We also thank Dr. C. Schwerdt for reading the manuscript and Dr. S. Raffel for encouragement, helpful suggestions, and review of the manuscript.

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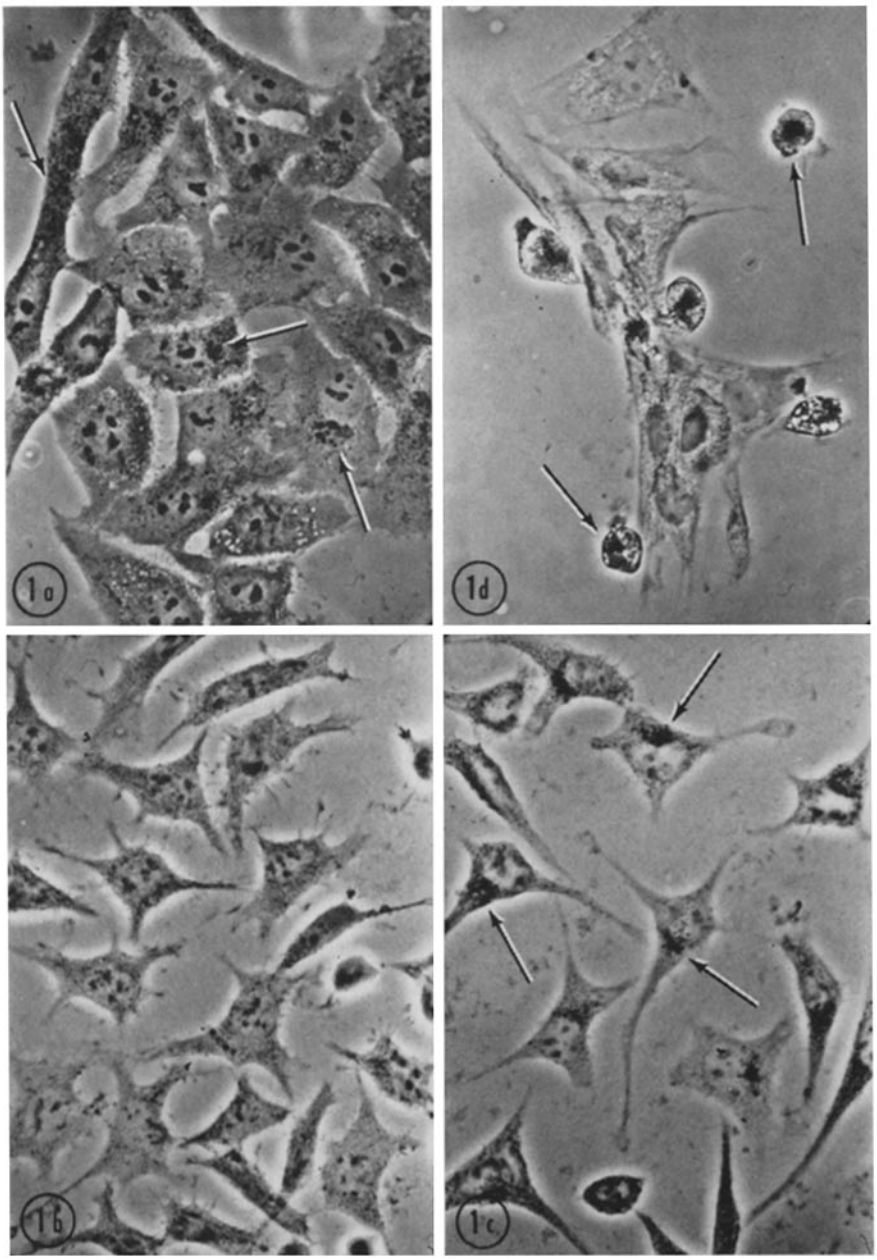
EXPLANATION OF PLATE 69

FIG. 1. Phase-contrast micrographs showing acid phosphatase staining of cells sensitive and resistant to diphtheria toxin. Red-staining granules are indicated by arrows. × 1500.

Fig. 1 *a*. KB cells, incubated 90 min in MEM, no serum. Fig. 1 *b*. L cells incubated 90 min in MEM, no serum. Fig. 1 *c*. L cells, incubated 8 hr in MEM, 5% calf serum and 10 μg per ml poly-L-ornithine. Fig. 1 *d*. Guinea pig fibroblasts and macrophages. Macrophages containing red-staining granules are indicated by arrows.



(Moehring and Moehring: Mammalian cell response to diphtheria toxin)