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CELL CYCLE DEPENDENT RESISTANCE TO STAPHYLOCOCCAL DELTA TOXIN-INDUCED LYSIS OF CULTURED CELLS

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SUMMARY: Staphylococcal delta toxin is a protein capable of rapidly disrupting cell membranes. Synchronized populations of 3T3 mouse fibroblasts in mitosis and early G_1 phases of the cell cycle exhibit resistance to delta toxin at concentrations cytolytic to interphase cells. Similar results were obtained with HeLa cells grown attached or in suspension culture. Increased resistance appears to result from structural or biochemical features other than cell rounding or detachment. Delta toxin stimulated significantly less cellular phospholipase A_2 (a potentially lytic enzyme activity) in mitotic 3T3 cells than in interphase cells.

INTRODUCTION

A series of toxins have been identified which appear to induce self-destruction in cultured cells (1-3) by prolonged activation of high levels of endogenous phospholipase A_2 activity. Included in this series is staphylococcal delta toxin, an amphipathic, cytolytic polypeptide produced by pathogenic strains of the skin bacterium *Staphylococcus aureus* (4,5). Stimulation of high levels of phospholipase A_2 results in the hydrolysis of a substantial percentage of cell membrane phospholipids, producing as the principal products two natural detergent lipids, lysolecithin and free fatty acids. Cell lysis could then occur by one or more mechanisms including 1) loss of essential membrane-forming lipids, 2) dissolution of essential membrane proteins or lipids by detergent action, and 3) lysolecithin-induced selective alterations in membrane permeability leading to osmotic lysis (6). Partial characterization of the phospholipases stimulated by various toxins has provided evidence for multiple, independently activated enzymes (1,2) whose normal role in cells remains to be established. Possible roles include regulation of prostaglandin biosynthesis (7) and regulation of membrane-associated enzymes by alteration of membrane lipid composition (8).

We report here the observation that delta toxin exhibits cell cycle dependent cytotoxicity. Mitotic cells are resistant to toxin concentrations much higher than that required to effectively lyse interphase cells, despite the greater fragility reported for mitotic cell surface membranes (9). Mitotic cells have been observed to exhibit a variety of altered metabolic processes, including a virtual shutdown of transcription (10), reduced

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protein synthesis (11), enhanced tubulin phosphorylation (12), altered composition of the cell surface as indicated by enhanced electrophoretic mobility (13) and lectin agglutinability (14), reduced substratum adhesion (15) and glycoprotein glycosylation (16), and altered antigenic properties (17). We have observed that delta toxin induces significantly less phospholipase A_2 activity in mitotic cells than in interphase cells treated in a similar manner. This observation provides evidence for a novel mitosis-associated alteration in membrane lipid metabolism of a type potentially capable of playing an important regulatory role in the mitotic cell.

MATERIALS AND METHODS

Delta toxin was prepared and purified by the procedure of Kreger et al. (18). Amino acid analysis indicated that this material was a homogenous protein preparation. It gave results identical to those obtained with an authentic sample of delta toxin kindly provided by Dr. A. W. Bernheimer.

Cultures of Swiss mouse 3T3 fibroblasts (subclone of a line of 3T3-4a obtained from Dr. R. W. Holley, this Institute) were maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 10% calf serum. All incubations were conducted at 37°C in a humidified CO_2 incubator. Cultures were tested (19) and found to be free from mycoplasma. Additional methods and conditions are described in the legends to figures and tables.

RESULTS AND DISCUSSION

Mitotic cell resistance to the cytolytic effects of delta toxin

3T3 Cells were synchronized by release from a hydroxyurea (G_1/S) block (20) and the progression of the population through the cell cycle monitored by flow microfluorimetry (21). Concurrently, cell populations were tested for their ability to survive a 30 min exposure to cytolytic levels of delta toxin (50 µg/ml). The time of appearance of a toxin-resistant population was concurrent with mitosis (i.e. late G_2 + M) and appeared to persist into early G_1 (Table I). In order to study resistance in mitotic cells, growing 3T3 cells were treated with low levels of colcemid (0.02 µg/ml) so as to increase the ratio of mitotic to interphase cells in the culture. These cell populations were subsequently treated with delta toxin (50 µg/ml) in medium containing fluorescein diacetate (0.025 µg/ml). Fluorochromasia, the intracellular accumulation of fluorescein generated by the action of cellular enzymes on a fluorogenic substrate, has been shown to be dependent upon the structural integrity of the cell membrane (22). When colcemid-treated cells were exposed to fluorescein diacetate, they rapidly (< 1 min) accumulated

Table I. Delta toxin resistance as a function of cell cycle location of a synchronized 3T3 fibroblast population

	% cell population in			% resistant cells
Time (h)	G_1	S	G_2 + M	± SEM
0	84.7	12.8	2.4	5.7 ± 1.6
3	7.6	92.2	<1	5.3 ± 1.7
5	<1	90.4	9.6	5.7 ± 1.4
7	<1	56.6	43.4	8.8 ± 2.2
9	23.0	29.5	47.5	21.1 ± 2.1
11	59.3	21.8	18.9	30.2 ± 0.24
13	49.9	29.2	20.9	8.6 ± 0.64

Cultures of 5 X 10^4 Swiss mouse fibroblasts, in 5 ml of Dulbecco's modified Eagle's medium (DME) supplemented with 10% calf serum, were cultured for 36 h in 6-cm diameter Falcon plastic dishes containing 15-mm coverslips. The medium was changed to 5 ml fresh medium containing 1% calf serum for 70 h. Cell populations were synchronized with hydroxyurea according to the procedure of Dooley and Ozer (20). Following removal of the blocking agent, coverslips were taken at various times, placed into 3.4-cm diameter Falcon plastic dishes, and incubated in 0.70 ml of DME containing delta toxin (50 µg/ml) for 30 min at 37°C. The percentage of cells surviving was determined in triplicate at each time point by exclusion of trypan blue dye. After removal of a coverslip, the cells remaining on the dish were suspended with 0.05% trypsin and the DNA distribution of the cell population determined by flow-microfluorometric analysis (21) using mithramycin and a Los Alamos design microfluorimeter with an argon laser at 457-nm. The photograph obtained from the storage oscilloscope was analyzed graphically as described (34).

fluorescence and retained it at high levels for at least 30 min (Fig. 1b). However, about 10 min after the addition of delta toxin to the system, the well-spread morphology of interphase cells altered rapidly. Large protrusions (blebs) of the outer membrane developed (5), the cells became turgid and by 30 min cellular fragmentation was observed (Fig. 1c). Concomitant with the appearance of blebs, a gradual diminution of accumulated cellular fluorescence was detected and by 30 min, total loss of fluorescence from interphase cells was observed (Fig. 1d). Mitotic cells, in contrast, retained both their characteristic morphology and high levels of accumulated fluorescence in the presence of delta toxin.

In order to corroborate these findings, delta toxin was labeled using either fluorescein isothiocyanate (23) or [^{3}H]acetic anhydride (24). Prior to the onset of bleb formation, there was no detectable binding or internalization of either probe. However, in cells treated with fluorescein-labeled toxin, a rapid cellular influx of labeled toxin was noted concomitant with the appearance of blebs. This material appeared to associate predominantly with the cell surface and perinuclear membranes. Since delta toxin exists in solution in a highly aggregated state (25), the initial lag observed may reflect slow dissociation of toxin into active subunits upon exposure to cellular components.

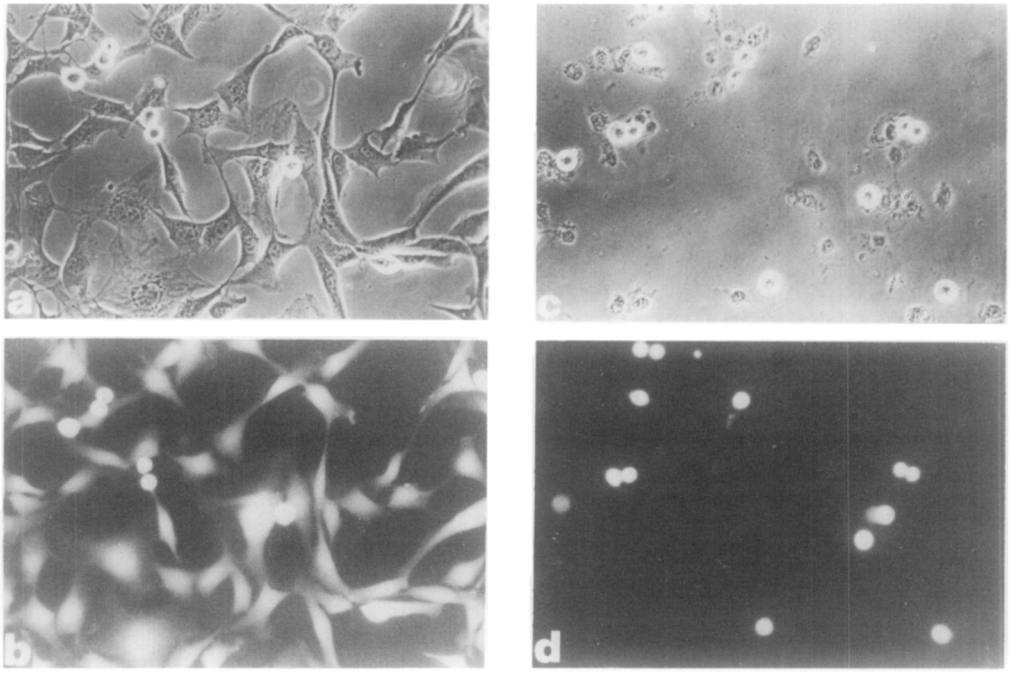


Fig. 1. Effects of delta toxin on a growing 3T3 fibroblast population. 3T3 Fibroblasts were cultured on 15-mm coverslips (1.5×10^3 cell/cm^2) in DME containing 10% calf serum for 24 h at 37°C at which time colcemid was added (0.02 µg/ml). Following a 4 h incubation at 37°C, coverslips were removed, gently rinsed in DME, inverted onto 20 µl of the following solutions on clean microscope slides and sealed with melted paraffin. Photographs represent: a field of cells incubated 30 min at 37°C in DME containing fluorescein diacetate (0.025 µg/ml) and observed by *(a)* phase microscopy and *(b)* fluorescence microscopy; and a field of cells incubated 30 min at 37°C in DME containing fluorescein diacetate (0.025 µg/ml) and delta toxin (50 µg/ml) observed by *(c)* phase microscopy and *(d)* fluorescence microscopy. Cells presumably in early G_1 (i.e. partially spread and existing as doublets) are resistant to toxin concentrations (e.g. 20 µg/ml) lytic to other interphase cells. Magnification x87.5.

Effects of cell rounding and detachment on toxin induced cytolysis

Specific changes occur in morphology and cell surface chemistry as cells enter mitosis (13-17). We investigated the possibility that surface changes associated with the process of cell rounding are responsible for the failure of delta toxin to complete a crutial step in its cytolytic mechanism. The delta toxin sensitivity of mitotic and suspended, trypsin-treated interphase 3T3 cells was compared. Removal of attached and spread interphase 3T3 fibroblasts from the substratum by agents such as trypsin induces co-ordinate alterations in cell-shape, membrane structure and cytoskeleton (26). Mitotic and suspended interphase 3T3 fibroblasts share several morphological and surface biochemical features in common (26-29), the most apparent of which is

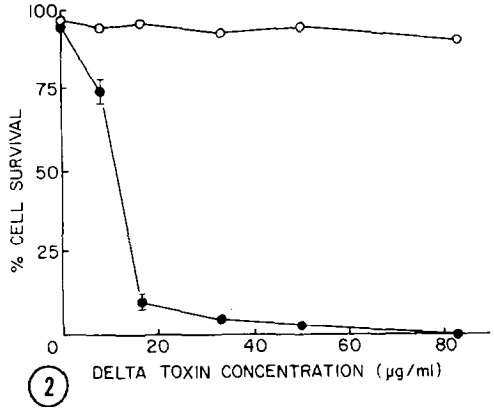
spherical shape. As shown in Fig.2, mitotic cell viability was unaffected throughout the concentration range of toxin tested, while interphase 3T3 cells in suspension exhibited a sensitivity similar to that observed for attached G_1 cells. In addition, treatment of mitotic cells with trypsin failed to significantly alter their sensitivity to delta toxin. Thus, neither cell rounding nor cellular features associated with the rounding process (e.g. changes in intrinsic membrane structure [26] or cytokinetic element redistribution [27]) appear responsible for the observed resistance of mitotic fibroblasts to the cytolytic effects of delta toxin. This conclusion was supported by results from experiments utilizing HeLa cells. Certain lines of HeLa cells which readily attach and spread on plastic dishes will grow as spheroids in suspension culture in calcium-free medium. HeLa cells (line S-3) were found to possess similar sensitivities to delta toxin whether attached and spread or growing in suspension (LD_{50} = 12 and 13 µg/ml, respectively). HeLa cells in mitosis, collected (30) from growing populations of spread cells, were resistant to approximately three times more delta toxin (LD_{50} = 12 µg/ml) than suspended interphase cells under the same experimental conditions.

Delta toxin effects on the virally transformed subline, SV3T3

Numerous surface alterations which occur during mitosis in untransformed cells have been shown to be expressed during interphase of transformed cells (15). In order to determine if delta toxin resistance was also expressed in the interphase portion of the transformed cell cycle, the toxin sensitivities of 3T3 and SV3T3 fibroblasts were compared at various points in their respective cell cycles. No oncogenic transformation-associated differences in toxin sensitivity were observed; comparable toxin sensitivity in interphase and resistance in mitosis was observed in the two cell lines. The results reported above using HeLa cells are consistent with this conclusion.

Possible mechanisms of mitotic cell resistance to delta toxin

The results indicate that a structural or biochemical feature unique to mitosis and early G_1 is responsible for enhanced resistance to lysis by delta toxin. The observation that delta toxin retains partial lytic activity even after being rendered incapable of entering a cell by covalent attachment to large Sepharose polymers (31) argues against mechanisms dependent on internalization. Similarly, we have failed to observe cellular internalization of labeled delta toxin prior to the onset of bleb formation. Therefore, alterations in endocytotic activity within the cell cycle (32) are unlikely to constitute the mechanism for increased resistance to delta toxin in mitosis.



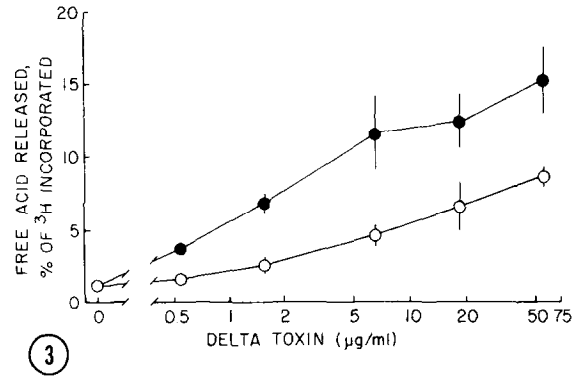


Fig. 2. Effect of delta toxin on the viability of mitotic and suspended interphase 3T3 fibroblasts. The medium on cultures of 3T3 fibroblasts in DME containing 10% calf serum was changed to 10 ml of DME containing 10% calf serum plus colcemid (0.02 µg/ml) and the cells cultured for an additional 4 h. Mitotic cells were then gathered by shake-off (29) washed and resuspended in DME (200 µl) at a density of 5 X 10^5 cells/ml. In a microfuge tube (0.75 ml capacity), 30 µl of the cell suspension was added to an equal volume of DME containing twice the indicated concentration of delta toxin. The mixture was incubated for 30 min at 37°C in a CO_2 incubator, then centrifuged, and the cells gently resuspended in a small volume of DME containing 0.002% trypan blue. Cells were immediately observed under phase microscopy and percent survivors determined. The interphase cells remaining after removal of mitotic cells were suspended after incubation with 10 ml of 0.05% trypsin in DME for 10 min at 37°C. The cells were collected by centrifugation and resuspended in DME at 5 X 10^5 cells/ml. Aliquots of this cell suspension (30 µl) were treated as described for mitotic cells. Mitotic cells which had been treated for 30 min with high concentrations of toxin (∿ 75 µg/ml) were found capable of continued growth when cultured in DME containing 10% calf serum. Mitotic cells (—O—); trypsin-suspended interphase cells (—●—).

Fig. 3. Effects of a range of concentrations of delta toxin on the release of phospholipase A_2 products in mitotic (O) and interphase (●) 3T3 fibroblasts. Triplicate cultures of 8.3 X 10^5 cells plated in 15 ml of Dulbecco's modified Eagle's medium (DME) containing 0.2% calf serum in 8.5-cm plastic dishes were cultured for 24 h, then the medium changed to 15 ml of fresh growth medium containing 10% calf serum and 7 µCi of [^{3}H]arachidonic acid (1). After 24 h, colcemid was added to a final concentration of 0.02 µg/ml and the cells incubated an additional 4 h. Mitotic cells were gathered by shake-off (29), washed three times in 5 ml of DME and resuspended in DME at a density of 4 X 10^5 cells/ml. Equal volumes of this cell suspension and delta toxin containing medium were mixed (final volume, 300 µl) and allowed to incubate 30 min at 37°C. The lipids released were analyzed as described (1). Phospholipase A_2 activity is expressed as the percentage of the total incorporated [^{3}H]arachidonic acid released into the medium as chromatographically identifiable free [^{3}H]arachidonic acid and conversion products. The interphase cells remaining on the dish after removal of the mitotic cell population were suspended after incubation with 10 ml of 0.05% trypsin in DME for 10 min at 37°C. The suspended interphase cells were treated in a manner identical to that described for mitotic cells. All washes and incubations were carried out in the presence of 0.02 µg/ml colcemid.

It has been suggested that similarities exist between the membrane disruptive mechanisms of delta toxin and detergents such as lysolecithin and Triton X-100 (5,33). The observed cell cycle dependence of delta toxin cytolysis is inconsistent with a lytic mechanism based solely upon detergent action. This view is substantiated by our observation that both melittin (a potent cytolytic peptide from bee venom possessing a variety of properties in common with delta toxin) and lysolecithin lyse mitotic and interphase 3T3 cells with equal efficiency.

We have shown that delta toxin stimulates high levels of cellular phospholipase A_2 in 3T3 fibroblasts, and we have partially characterized the enzyme activity (2; Durkin and Shier, in preparation). These studies included a demonstration that, across a range of delta toxin concentrations, cytolysis correlates closely with the degree of activation of phospholipase A_2 above a threshold level which can be tolerated without significant cell lysis. This threshold level corresponds to hydrolysis (in 30 min) of about 10% of the lipids labeled by biosynthetic incorporation of [^{3}H]arachidonic acid. In this respect delta toxin is similar to other toxins (1,2), which appear to induce self-destruction of cultured cells through prolonged activation of high levels of cellular phospholipase A_2. In colcemid-treated cultures of 3T3 cells biosynthetically labeled with [^{3}H]arachidonic acid, mitotic cells exhibited substantially less toxin-induced activation of cellular phospholipase A_2 than did attached or trypsin-suspended interphase cells from the same culture (see Fig.3). At concentrations of delta toxin cytolytic to interphase cells in suspension (10-20 μg/ml), the extent of hydrolysis of labeled cell lipids exceeded the cytolytic threshold level, whereas in mitotic cells insufficient phospholipase A_2 was activated at any toxin concentration tested to achieve the cytolytic threshold level of lipid hydrolysis. This difference in the degree of activation of phospholipase A_2 may account for the increased resistance of mitotic cells to the cytolytic effects of delta toxin.

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REFERENCES

1. Shier, W. T. (1979) Proc. Natl. Acad. Sci. USA 76, 195-199.
2. Shier, W. T. (1980) in Natural Toxins (Eaker, D., and Wadström, T., eds). Pergamon Press, London, in press.
3. Durkin, J. D., and Shier, W. T. (1980) Fed. Proc. Fed. Am. Sco. Exp. Biol. 39, 645.
4. Bernheimer, A. A. (1974) Biochim. Biophys. Acta 344, 27-50.

5. Gladstone, G. P., and Yoshida, A. (1967) Brit. J. Exp. Pathol. 48, 11-19.
6. Bierbaum, T. J., Bouma, S. R., and Hestis, W. H. (1979) Biochim. Biophys. Acta 555, 102-110.
7. Kunze, H., and Vogt, W. (1971) Ann. N. Y. Acad. Sci. 180, 123-125.
8. Shier, W. T., Baldwin, J. H., Nilsen-Hamilton, M., Hamilton, R., and Thanassi, N. M. (1976) Proc. Natl. Acad. Sci. USA 73, 1586-1590.
9. Graham, J. M. Sumner, M. C. B., Curtis, D. H., and Pasternak, C. A. Nature 246, 291-295.
10. Taylor, J. H. (1960) Ann. N. Y. Acad. Sci. 90, 409-421.
11. Baserga, R. (1962) Biochim. Biophys. Acta 61, 445-450.
12. Piras, R., and Piras, M. (1975) Proc. Natl. Acad. Sci. USA 72, 1161-1165.
13. Kraemer, P. M. (1967) J. Cell Biol. 33, 197-200.
14. Smets, L. A., and Deley, L. (1974) J. Cell. Phsyiol. 84, 343-348.
15. Mannino, R. F., and Burger, M. M. (1975) Nature 256, 19-22.
16. Glick, M. C., and Buck, C. A. (1973) Biochemistry 12, 85-90.
17. Thomas, D. B. (1971) Nature 233, 317-321.
18. Kreger, A. S., Kim, K. S., Zaboretzky, F., and Bernheimer, A. W. (1971) Infec. Immunity 3, 449-465.
19. Chen, T. R. (1977) Exp. Cell Res. 104, 255-262.
20. Dooley, D. C., and Ozer, H. L. (1977) J. Cell. Physiol. 90, 337-350.
21. Crissman, H., and Tobey, R. A. (1974) Science 184, 1297-1298.
22. Celada, F., and Rotman, B. (1967) Proc. Natl. Acad. Sci. USA 57, 630-636.
23. Rinderknecht, H. (1962) Nature 193, 167-168.
24. Hille, M. B., Barret, A. J., Dingle, J. T., and Fell, H. B. (1970) Exp. Cell Res. 61, 470-472.
25. Kantor, H. S., Temples, B., and Shaw, W. V. (1972) Arch. Biochem. Biophys. 151, 142-156.
26. Furcht, L. T., and Wendelschafer-Crabb, G. (1978) Exp. Cell Res. 114,1-4
27. Wells, V., and Mallucci, L. (1978) Exp. Cell Res. 116, 301-312.
28. Burger, M. M. (1969) Proc. Natl. Acad. Sci. USA 62, 994-1001.
29. Burger, M. M. (1978) in ICN-UCLA Symposia on Molecular and Cellular Biology, Vol. 12 (Dirksen, E. R., Prescott, D. M., and Fox, C. F., eds) pp. 15-32, Academic Press, New York.
30. Terasima, T., and Tolmach, L. J. (1963) Exp. Cell Res. 30, 344-362.
31. Lee, S. H., and Haque, R. (1976) Biochem. Biophys. Res. Commun. 68, 1116-1118.
32. Berlin, R. D., Oliver, J. M., and Walter, R. J. (1978) Cell 15, 327-341.
33. Rahal, J. J. (1972) J. Infect. Diseases 126, 96-103.
34. Holley, R. W., and Kiernan, J. A. (1974) Proc. Natl. Acad. Sci. USA 71, 2942-2945.