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Differential Chemical Protection of Mammalian Cells from the Exotoxins of *Corynebacterium diphtheriae* and *Pseudomonas aeruginosa*

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Many drugs or chemicals had markedly different effects on the cytotoxicity induced by *Pseudomonas aeruginosa* exotoxin A (PE) or *Corynebacterium diphtheriae* exotoxin (DE). The glycolytic inhibitor NaF protected cells from DE but potentiated the cytotoxicity of PE. Another energy inhibitor, salicylic acid, also protected cells from DE but had no effect with PE. Colchicine and colcemid did not affect the cytotoxicity of either toxin. Cytochalasin B exhibited a modest protection from DE but no effect with PE. Ouabain, a specific inhibitor of the Na^+,K^+-dependent adenosine 5'-triphosphatase (ATPase), did not affect the cytotoxicity of either toxin. Ruthenium red, a specific inhibitor of the Ca^{2+},Mg^{2+}-dependent ATPase, conferred marked protection from DE-induced cytotoxicity but did not affect PE-induced cytotoxicity. A number of local anesthetics were tested, and they too presented differential results with PE and DE. Most chemicals that affected toxin-induced cytotoxicity had little or no influence on the in vitro adenosine 5'-diphosphate-ribosylation catalyzed by either toxin. This work presents further evidence that PE and DE have different mechanisms of intoxication and suggests that these differences lie in the attachment or internalization stages of intoxication.

The cytotoxic response of cultured mammalian cells to diphtheria toxin (DE) has been the subject of numerous studies (4, 7, 16). It is generally believed that the toxin causes cell death by inhibiting protein synthesis. DE has been shown to inactivate an enzyme necessary for peptide synthesis, elongation factor 2 (EF-2), by catalyzing its adenosine 5'-diphosphate-ribosylation using nicotinamide adenine dinucleotide (NAD) as substrate (4).

Although much is known about the enzymatic activity of DE, little is known about its initial interaction with the cell and entry into the cytoplasm. Kim and Groman (11, 12) found that NH_4Cl protected cells from DE, possibly by blocking its specific (but not nonspecific, i.e., pinocytotic [19]) uptake. Duncan and Groman (6) extended that work to include a study of the ionic conditions necessary for toxin adsorption to the cell membrane. These workers also found that the glycolytic inhibitor NaF prevented expression of toxicity. Ivins et al. (10) recently tested the ability of a number of chemicals to protect cells from DE. Their results, however, did not lead to any firm conclusions concerning the attachment and internalization process. Finally, Saelinger et al. (19) have presented evidence that a significant fraction of DE internalized by cells gains entry by a nonspecific (pinocytotic) mechanism.

Iglewski and Kabat (9) recently demonstrated that *Pseudomonas aeruginosa* exotoxin A (PE) has an enzymatic ribosylating activity similar to that of DE. Prompted by that report, we compared the sensitivities of 21 mammalian cell lines to *Pseudomonas* and diphtheria toxins (J. L. Middlebrook and R. B. Dorland, Fed. Proc. 35:1394, 1976). Every cell line examined exhibited a different sensitivity to each toxin. Since the differences in sensitivities could not be attributed to different in vitro ribosylating activities, it was concluded that the two toxins do not have strictly analogous mechanisms of cellular intoxication.

We have extended our comparative study of *Pseudomonas* and diphtheria exotoxins by testing the ability of various chemicals to protect cells from their cytotoxic actions. It was found that although several of the chemicals examined protected cells from DE, none protected from PE. Indeed, some of these chemicals potentiated the cytotoxic effects of the latter. We believe these findings present further evidence that *Pseudomonas* and diphtheria exotoxins

have significantly different mechanisms of intoxication on intact cells.

MATERIALS AND METHODS

Cells and cell culture. Seed stock for all cell lines was obtained from the American Type Culture Collection, Rockville, Md. Each line was maintained in 75-cm² T-flasks (Costar no. 3075) with the media and serum supplements recommended by the American Type Culture Collection.

Media and sera. All media, vitamins, antibiotics, and amino acids were obtained from Grand Island Biological Co., Grand Island, N.Y. Fetal calf serum was purchased from Reheis Chemical Co., Phoenix, Ariz. The serum was heat-inactivated for 30 min at 56°C before use in cell culture.

Toxins. *P. aeruginosa*, strain PA103, was obtained from P. V. Liu. PE was produced and purified by Stephen Leppla of this institute (13). The final product behaves as a single polypeptide of 66,000 daltons, contains no measurable carbohydrate, and is ≥95% pure as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Heating the purified toxin at 70°C for 1 h completely destroys its cytotoxic activity. The 50% lethal dose of PE purified in this manner was 0.1 μg per 20-g CD-1 mouse (13). Purified *Corynebacterium diphtheriae* exotoxin (23 minimum lethal doses/μg) was the generous gift of A. M. Pappenheimer, Jr., Harvard University. Toxin concentrations were determined using extinction coefficients ($E_{1cm}^{1\%}$) at 280 nm of 10.5 and 11.9 for PE and DE, respectively.

Chemicals. Butacaine hemi-sulfate, colchicine, colcemid, 2-deoxyglucose, 2,4-dinitrophenol (2,4-DNP), *p*-chloromercuribenzoic acid, 1-nonanol, ouabain, procaine hydrochloride, ruthenium red, sodium fluoride, and poly-L-α-ornithine HBr were purchased from Sigma Chemical Co., St. Louis, Mo. Cytochalasin B and Tuftsin were obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis., and Calbiochem, La Jolla, Calif., respectively. Lidocaine hydrochloride and chlorpromazine hydrochloride were the kind gifts of Astra Pharmaceuticals, Worcester, Mass., and Smith, Kline & French, Philadelphia, Pa., respectively. [^{14}C]NAD was obtained from Amersham/Searle, Arlington Heights, Ill. The calcium ionophore A23187 was the generous gift of Robert Hamill, Eli Lilly & Co., Indianapolis, Ind. All other chemicals were reagent grade and were used without further purification.

Cytotoxicity assay. Details of our cytotoxicity assay for PE and DE have been recently described (J. L. Middlebrook and R. B. Dorland, Can. J. Microbiol., in press). We used a slight variation of the method for this work. Cells in multiwell tissue culture plates were incubated at 37°C with the drug or chemical under investigation for 1 h before toxin addition. Toxin was then added (replicates of three), and incubation of cells with drug plus toxin continued for 2 to 3 h. The incubation was terminated by washing each monolayer three times with serum-free medium (37°C) and adding complete medium to continue cell culture. After 48 h the monolayers were washed with Hanks balanced salt solution (37°C), and the remaining cells were dissolved in 0.1 M NaOH for protein assay. When solvents other than water or saline were required for drug solubility (dimethyl sulfoxide for cytochalasin B and ethanol for nonanol), controls were run to ascertain that the solvents themselves did not influence cytotoxicity.

Ribosylation assay. Wheat germ, a convenient source of EF-2, has been found to yield results similar to rabbit riticulocyte EF-2 in ribosylation assays with DE and PE (Stephen Leppla, unpublished observations). Partially purified wheat germ EF-2 was prepared by incubating a 33 to 50% $(NH_4)_2SO_4$ cut of ground wheat germ with 4 mM iodoacetamide for 15 min. The alkylated material was adsorbed on diethylaminoethyl-cellulose, washed with 0.05 M NaCl, eluted (batchwise) with 0.15 M NaCl, and concentrated using an Amicon ultrafiltration cell with an XM-50 filter. The ribosylation assay (100 μl, total volume) contained 10 μl of toxin, 10 μl of [^{14}C]NAD (0.1 μCi), 40 μl of a 0.001 M ethylenediaminetetraacetic acid–0.5 M tris(hydroxymethyl)aminomethane (pH 8.2) buffer, 10 μl of 0.4 M dithiothreitol, 20 μl of EF-2, and 10 μl of the chemical to be tested. Assays were routinely performed in triplicate, the standard errors of which were usually less than 5%. After 1 h at room temperature, 90 μl was spotted on paper disks and processed as previously described (14) to obtain the acid-precipitable radioactivity. The radioactivity on each disk was measured with a Nuclear-Chicago Mark II liquid scintillation spectrometer using Liquifluor/toluene (New England Nuclear Corp., Boston, Mass.).

RESULTS

Effect of energy inhibitors. It has been demonstrated that at least one inhibitor of energy metabolism (NaF) can protect both HeLa (6) and HEp-2 (10) cells from DE-induced cytotoxicity. We tested this and several other inhibitors to determine whether PE-induced cytotoxicity could also be prevented. As with all the chemicals studied in this work, the effects of each inhibitor were assessed in at least two separate experiments using both the HeLa and HEp-2 cell lines. Similar results were obtained with both cell lines, although data are presented from only one.

The effect of NaF on PE- and DE-induced cytotoxicity is depicted in Fig. 1. It is apparent that though NaF was a very effective protective agent against DE, it markedly potentiated the cytotoxicity induced by PE. Maximal potentiation occurred in the range of 3 to 6 mM, followed by a slight but reproducible rebound effect up to the point where the drug itself became toxic (>10 mM). Similarly, salicylic acid was found to protect cells from DE but to slightly potentiate PE-induced cytotoxicity (Fig. 2).

The pattern observed with NaF and salicylic acid was not common to all energy inhibitors.

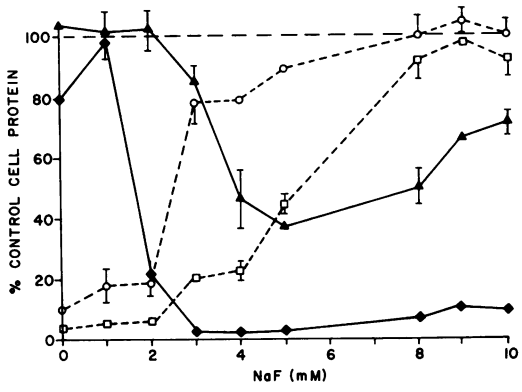

FIG. 1. *Effect of NaF on PE- and DE-induced cytotoxicity for HEp-2 cells. Cells were incubated at 37°C with drug at the concentration indicated on the abscissa for 1 h. Toxin was then added to the various concentrations indicated below, and incubation was continued for 3 h at 37°C. Cells were then washed and cytotoxicity was assayed as described in Materials and Methods. Drug toxicity was observed at >50 mM. Error bars indicate standard error of the mean, which, if not shown, was smaller than the symbol. Symbols: (▲) PE, 10 ng/ml; (◆) PE, 100 ng/ml; (○) DE, 30 ng/ml; (□) DE, 300 ng/ml.*

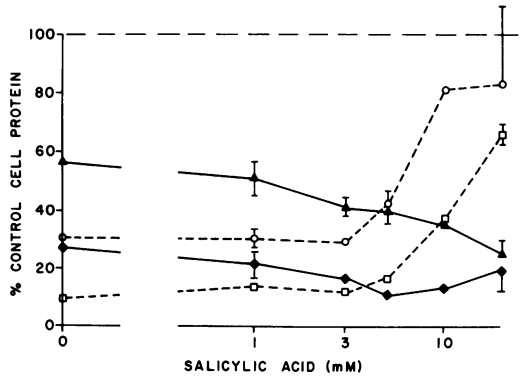

FIG. 2. *Effect of salicylic acid on PE- and DE-induced cytotoxicity for HeLa cells. Protocol as in Fig. 1. Error bars indicate standard error of the mean, which, if not shown, was smaller than the symbol. Symbols: (▲) PE, 100 ng/ml; (◆) PE, 500 ng/ml; (○) DE, 1 ng/ml; (□) DE, 5 ng/ml.*

No measurable protection from either toxin was observed with 2,4-DNP (1×10^{-5} to 5×10^{-4} M), potassium cyanide (1×10^{-4} to 5×10^{-3} M), 2-deoxyglucose (1×10^{-3} to 1×10^{-1} M), or sodium azide (1×10^{-6} to 1×10^{-2} M) over concentration ranges known to effectively inhibit energy production (1, 2). To eliminate the possibility that high concentrations of inhibitors (or any other tested drug) would protect cells, our studies were always extended to the point of obvious toxicity by the inhibitor itself. However, this approach did not uncover any additional information, and no data presented in this work are in the drug toxicity range.

Effect of cytoskeletal perturbants. Colchicine and colcemid affect the assembly and function of microtubules (21). These drugs were tested at concentration ranges of 1×10^{-8} to 1×10^{-5} M and 1×10^{-8} to 1×10^{-4} M, respectively, and no effect on the cytotoxicity of either toxin was observed (data not shown). Cytochalasin B, which disrupts microfilaments (19), appeared to partially protect cells from DE, but had no effect on the cytotoxicity induced by PE (Fig. 3). We found the threshold dose for protection to be $\simeq 0.1$ μg/ml; concentrations >5 μg/ml were toxic to the cells.

Effect of ATPase inhibitors. There are two generally recognized classes of membrane-bound adenosine 5'-triphosphatases (ATPases), a Na^+,K^+-dependent type and a Ca^{2+},Mg^{2+}-dependent type. Ouabain specifically inhibits the Na^+,K^+-dependent ATPase (22). In our system, ouabain had no detectable effect on either PE- or DE-induced cytotoxicity (data not shown). In contrast, ruthenium red, a specific inhibitor of the Ca^{2+},Mg^{2+}-dependent ATPase (20, 23), protected cells from DE but had no measurable effect on PE-induced cytotoxicity (Fig. 4). Protection was first observed at 1 μM and reached a maximum at $\simeq 10$ μM. Although we did not directly measure the effect of ruthenium red on ATPase activity, this concentration range is close to that shown by others to be inhibitory to enzyme function (20, 23). Since ATPase inhibition by ruthenium red should block calcium transport and lead to its intracellular accumulation, we tested another drug that promotes intracellular accumulation of calcium, the iono-

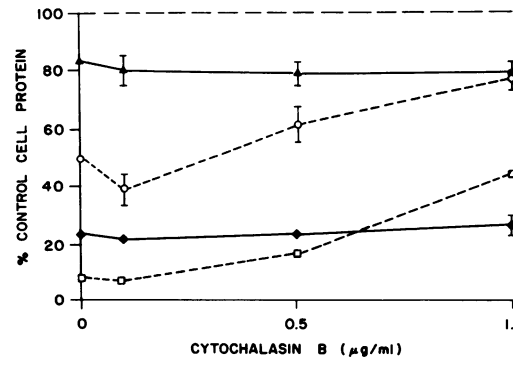

FIG. 3. *Effect of cytochalasin B on PE- and DE-induced cytotoxicity for HeLa cells. Protocol as in Fig. 1. Error bars indicate standard error of the mean, which, if not shown, was smaller than the symbol. Symbols: (▲) PE, 100 ng/ml; (◆) PE, 500 ng/ml; (○) DE, 1 ng/ml; (□) DE, 5 ng/ml.*

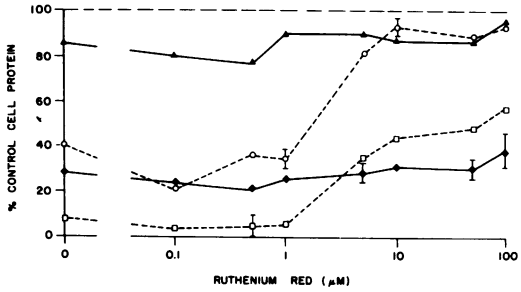

FIG. 4. *Effect of ruthenium red on PE- and DE-induced cytotoxicity for HEp-2 cells. Protocol as in Fig. 1. Error bars indicate standard error of the mean, which, if not shown, was smaller than the symbol. Symbols:* (▲) *PE, 100 ng/ml;* (◆) *PE, 500 ng/ml;* (○) *DE, 1 ng/ml;* (□) *DE, 5 ng/ml.*

phore A23187 (18). This drug, examined over a 0.001 to 10 mM concentration range, did not affect the cytotoxicity of either PE or DE.

Effect of sulfhydryl agents and arsenite. The sulfhydryl reagents *p*-chloromercuribenzoic acid and *N*-ethylmaleimide were found to have no effect on either PE- or DE-induced cytotoxicity (data not shown). However, arsenite, which also affects sulfhydryl enzymes, was effective in protecting cells from DE challenge while markedly potentiating the cytotoxicity of PE (Fig. 5). Potentiation of PE-induced cytotoxicity appeared at a lower concentration than protection from DE. Maximum potentiation occurred at $\simeq 0.6 \times 10^{-5}$ M, whereas DE protection was still increasing at concentrations of arsenite that approached drug-induced toxicity. Since arsenite concentrations higher than 10^{-4} M were toxic, it was not possible to determine whether there was a rebound effect such as that seen with NaF potentiation (Fig. 1).

Effect of ammonium chloride. Duncan and Groman (6) demonstrated that NH_4Cl effectively protected cells from DE, probably by blocking toxin internalization. The effects of NH_4Cl on PE- and DE-induced cytotoxicities are shown in Fig. 6. In agreement with other workers (6, 10), we found that NH_4Cl prevented expression of DE-induced cytotoxicity. However, no such effect was observed when cells were challenged with PE (the apparent slight protection at 0.5 ng of PE per ml was not a reproducible result). Unlike most of the drugs investigated, it was possible to incubate NH_4Cl with cells for periods of time greater than 2 to 3 h without toxic effect. Protection from DE was still observed when cells were treated with NH_4Cl and toxin for as long as 48 h, whereas PE cytotoxicity remained unaffected (data not shown).

Effect of membrane perturbants. Poly-L-ornithine is believed to stimulate cells to a higher rate of pinocytosis (15). The effects of this compound on the cytotoxicity of both toxins are depicted in Fig. 7. In agreement with previous workers (10), we found that poly-L-ornithine protected cells from DE. However, we did not detect any significant effect on the cytotoxicity induced by PE. These results are probably not due simply to stimulation of pinocytosis, since another agent believed to increase pinocytosis, Tuftsin (10), had no effect on either PE- or DE-induced cytotoxicity (data not shown).

Effect of local anesthetics. Local anesthetics have been shown to affect the mobility and distribution of cell surface receptors (17). We tested the local anesthetics lidocaine, procaine, butacaine, nonanol, and chlorpromazine in our system. Neither butacaine nor nonanol significantly affected the cytotoxicity of either PE or DE (data not shown). Procaine provided partial protection from DE-induced cytotoxicity, but had no effect on that due to PE (Fig. 8).

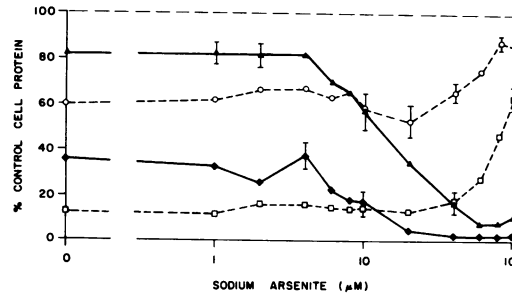

FIG. 5. *Effect of sodium arsenite on PE- and DE-induced cytotoxicity for HEp-2 cells. Protocol as in Fig. 1. Drug toxicity was observed at >300 μM. Error bars indicate standard error of the mean, which, if not shown, was smaller than the symbol. Symbols:* (▲) *PE, 100 ng/ml;* (◆) *PE, 500 ng/ml;* (○) *DE, 1 ng/ml;* (□) *DE, 5 ng/ml.*

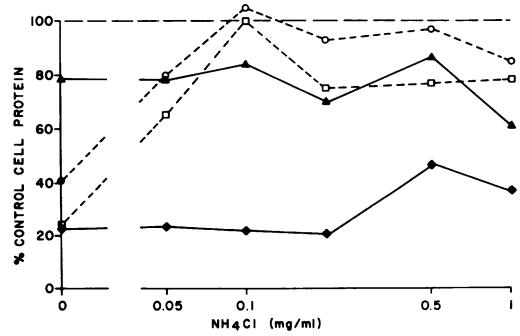

FIG. 6. *Effect of NH_4Cl on PE- and DE-induced cytotoxicity for HEp-2 cells. Protocol as in Fig. 1. Symbols:* (▲) *PE, 100 ng/ml;* (◆) *PE, 500 ng/ml;* (○) *DE, 1 ng/ml;* (□) *DE, 5 ng/ml.*

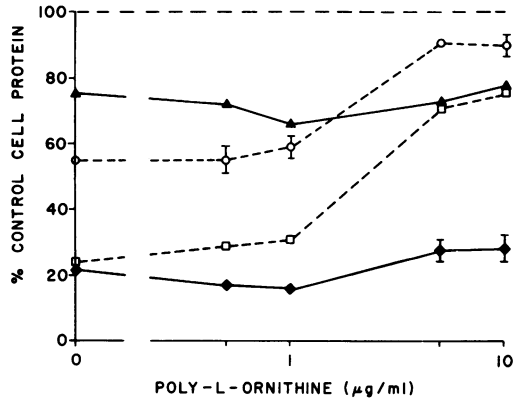

FIG. 7. *Effect of poly-L-ornithine on PE- and DE-induced cytotoxicity for HeLa cells. Protocol as in Fig. 1. Drug toxicity was observed at >20 μg/ml. Error bars indicate standard error of the mean, which, if not shown, was smaller than the symbol. Symbols: (▲) PE, 100 ng/ml; (◆) PE, 500 ng/ml; (○) DE, 1 ng/ml; (□) DE, 5 ng/ml.*

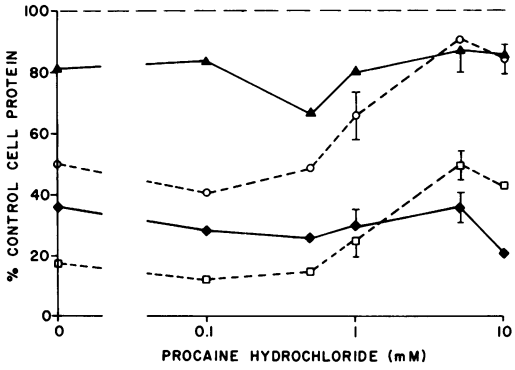

FIG. 8. *Effect of procaine on PE- and DE-induced cytotoxicity for HeLa cells. Protocol as in Fig. 1. Drug toxicity was observed at ≥20 mM. Error bars indicate standard error of the mean, which, if not shown, was smaller than the symbol. Symbols: (▲) PE, 100 ng/ml; (◆) PE, 500 ng/ml; (○) DE, 1 ng/ml; (□) DE, 5 ng/ml.*

Lidocaine provided partial protection from DE but potentiated PE-induced cytotoxicity (Fig. 9). Chlorpromazine also potentiated PE-induced cytotoxicity, but did not appear to affect DE (Fig. 10).

Effect of active agents on ribosylation activity. One possible explanation for the various agents' protective or potentiating effects on cytotoxicity is that they influence the enzymatic reaction with EF-2. To test this possibility, we examined the effects of all active agents on the in vitro ribosylation activity of both toxins. The agents tested were used at concentrations corresponding to their maximal effect in cell culture. The results (Table 1) were somewhat variable, but certain patterns emerged. Adenine, a known inhibitor of DE-catalyzed ribosylation (8), markedly reduced incorporation of the label, indicating that our assay can detect inhibitors when they are present. Chlorpromazine, lidocaine, NH_4Cl, poly-L-ornithine, and NaF did not affect the ribosylation activity of either toxin. Cytochalasin B reduced the activity of both toxins, but this effect was most likely due to action of the solvent (dimethyl sulfoxide). The ribosylation activities of both toxins were consistently reduced by ruthenium red and increased by arsenite. Since this is not the situation observed with intact cells (see Fig. 4 and 5), we do not believe that the effects of these drugs on ribosylation can explain their effects in cell culture. On the other hand, both procaine and salicylic acid reduced the ribosyla-

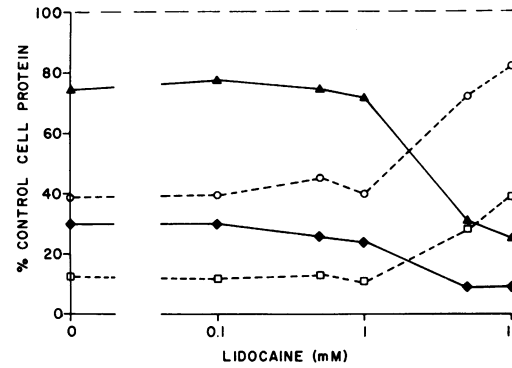

FIG. 9. *Effect of lidocaine on PE- and DE-induced cytotoxicity for HeLa cells. Protocol as in Fig. 1. Drug toxicity was observed at ≥20 mM. Symbols: (▲) PE, 100 ng/ml; (◆) PE, 500 ng/ml; (○) DE, 1 ng/ml; (□) DE, 5 ng/ml.*

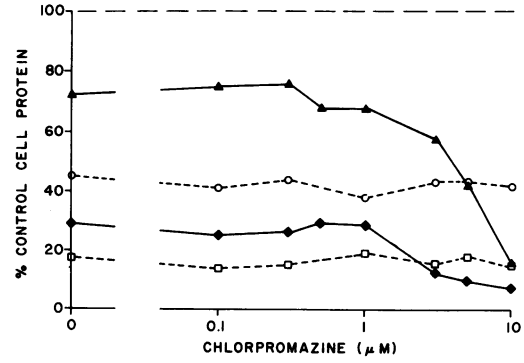

FIG. 10. *Effect of chlorpromazine on PE- and DE-induced toxicity for HeLa cells. Protocol as in Fig. 1. Drug toxicity was observed at ≥30 μM. Symbols: (▲) PE, 100 ng/ml; (◆) PE, 500 ng/ml; (○) DE, 1 ng/ml; (□) DE, 5 ng/ml.*

TABLE 1. *Effect of active agents on PE- and DE-catalyzed adenosine 5'-diphosphate-ribosylation*

Agent	Concn	Percent of control[a]	
		DE	PE
NaF	10 mM	94 ± 9	100 ± 5
Salicylic acid	10 mM	51 ± 10	93 ± 6
Cytochalasin B	1 μg/ml	46 ± 4	46 ± 5
Dimethyl sulfoxide	10% (vol/vol)[b]	34 ± 7	43 ± 5
Ruthenium red	0.05 mM	55 ± 2	42 ± 6
Sodium arsenite	0.1 mM[c]	133 ± 6	122 ± 10
NH_4Cl	0.1 mg/ml	107 ± 12	90 ± 7
Poly-L-ornithine	10 μg/ml	89 ± 22	259 ± 97[d]
Procaine	10 mM	54 ± 3	87 ± 8
Lidocaine	10 mM	89 ± 6	102 ± 8
Chlorpromazine	0.01 mM	108 ± 3	100 ± 5
$CaCl_2$	2 mM	86 ± 10	149 ± 16
Adenine	1 mM	3 ± 1	6 ± 1

[a] Ribosylation assays were carried out as explained in Materials and Methods. Values are the percentage of control incubations with water and are expressed as the mean of three separate experiments ± the standard error of the mean. Controls for the three experiments were in the range of 1,620 to 2,360 cpm for DE (0.5 μg/ml) and 1,240 to 2,650 cpm for PE (25 μg/ml).

[b] Although 1% (vol/vol) dimethyl sulfoxide was used in cell culture experiments, volume considerations required us to use 10% in our ribosylation assay.

[c] Precipitation was noted.

[d] Results were highly variable and precipitation was noted.

tion activity of DE but had no effect on that of PE. These results are analogous to those obtained in cell culture (Fig. 2 and 8) and may well explain the effects of procaine and salicylic acid on cytotoxicity.

DISCUSSION

The use of chemicals or drugs to protect cells from DE has been described in the studies of Ivins et al. (10) and Duncan and Groman (6), which served as models for much of this work. We have extended these earlier studies to include an investigation of drug effects on PE-induced cytotoxicity and a number of previously unexamined drugs. Although our data do not provide insights sufficient to propose detailed mechanisms of intoxication for either toxin, they do indicate which subcellular systems or organelles may be involved in toxin action.

NaF was capable of protecting cells from very high concentrations of DE (note the concentrations of DE used in Fig. 1 as compared with other experiments), yet it markedly potentiated PE at concentrations normally having little or no cytotoxicity. The DE-protective effect of NaF has been previously described by Duncan and Groman (6) and Ivins et al. (10). The latter group determined that NaF has no direct effect on the in vitro toxin-catalyzed inactivation of EF-2. Our data confirm their finding and extend it to PE (Table 1). We believe these results indicate that the NaF effects are probably due to perturbation of the attachment or internalization stages of intoxication. If, as Duncan and Groman (6) suggest, DE requires glycolytically derived energy at some step of intoxication, it follows that intoxication by PE either requires little or no glycolytic energy or utilizes some other energy pool. Recent experiments with quinidine, another inhibitor of glycolysis (1), provide some support for this theory. Quinidine, like NaF, was found to protect cells from DE and to potentiate cytotoxicity induced by PE (J. L. Middlebrook and R. B. Dorland, unpublished observations). However, since the pharmacology of these drugs is complex (1), the results must be interpreted with caution. Further investigations will determine whether or not all glycolytic inhibitors follow the pattern of NaF and quinidine.

Salicylates are believed to uncouple oxidative phosphorylation in a manner similar to that of 2,4-DNP (2), yet 2,4-DNP itself had no measurable effect on either PE- or DE-induced cytotoxicity. There are at least two explanations for this apparent paradox. First, it is possible that the DE-protective effect of salicylic acid results from action on a cellular process other than oxidative phosphorylation. Protection may be due to the apparent ability of salicylic acid to partially block DE-induced ribosylation of EF-2 (Table 1). Second, it is possible that our assay system is not sufficiently sensitive to measure weak protection. Duncan and Groman (6) found that neither cyanide nor 2,4-DNP affected DE cytotoxicity, whereas Ivins et al. (10) detected modest levels of protection. The latter group suggested that this contradiction may have resulted from the greater sensitivity of their assay system (radioactive amino acid incorporation). Since our methodology is somewhat similar to that of Duncan and Groman, this may also explain our negative findings with 2,4-DNP.

The results obtained with the ATPase inhibitors are intriguing in light of the proposed interconnection between glycolysis and the Ca^{2+},Mg^{2+}-dependent ATPase (5). Whereas inhibition of the Na^+,K^+-dependent ATPase had no apparent effect on the cytotoxicity of either toxin, inhibition of the Ca^{2+},Mg^{2+}-dependent ATPase protected cells from DE. This ruthenium red-induced protection is probably not due simply to intracellular accumulation of calcium since the calcium ionophore A23187 was found to have no effect on the cytoxicity of either toxin.

Although many sulfhydryl enzymes (and systems of which they are a part) are probably

inhibited by arsenicals, one system known to be quite sensitive to these compounds is glycolysis (1). This is interesting in view of the pronounced effects observed with arsenite and their similarity to the effects of NaF. Arsenite exhibits a protection-potentiation pattern similar to that of NaF, although arsenite protection from DE is not as effective as that provided by NaF. The protection-potentiation pattern should not, however, be interpreted as resulting only from glycolytic inhibition since the local anesthetic lidocaine also presents this pattern. The effects of arsenite on PE cytotoxicity began at a lower concentration than effects on DE, and this may indicate that inhibition of different systems is responsible for the opposing results with PE and DE.

NH_4Cl was quite potent in protecting cells from DE but had no effect on PE cytotoxicity (Fig. 6). Kim and Groman (12) have presented evidence that NH_4Cl probably exerts its protective effect by preventing (specific) DE internalization without affecting toxin adsorption to the cell membrane. Ivins et al. (10) demonstrated that NH_4Cl does not inhibit DE-catalyzed ribosylation. Our data (Table 1) confirm this observation and indicate that PE-catalyzed ribosylation is also unaffected by NH_4Cl. It therefore seems likely that our results with NH_4Cl in cell culture reflect some difference(s) in the mechanisms of internalization of PE and DE.

Previous studies have shown that local anesthetics can affect the mobility and distribution of cell surface receptors (17). In our system, the anesthetics procaine, lidocaine, and chloropromazine all affected cytotoxicity, but not in the same manner (Fig. 8–10). The absence of a general pattern of anesthetic effects may indicate that the putative cell surface receptors for these toxins are not the only cellular components affected by the drugs. For example, the effects of procaine in cell culture may result from its ability to inhibit DE- but not PE-catalyzed ribosylation (Table 1). Poste et al. (17) found that lidocaine, procaine, and cytochalasin B all inhibit capping of immunoglobulin receptors on spleen cells, whereas colchicine does not. Similarly, we found that lidocaine, procaine, and cytochalasin B all protected cells from DE whereas colchicine did not. This is quite unlike the pattern of effects on PE-induced cytotoxicity. The use of radiolabeled toxins will best determine whether or not these anesthetics are affecting membrane receptors for PE and DE. Unfortunately, despite success with DE, we have been unable as yet to obtain labeled PE of unaltered cytotoxicity.

Only salicylic acid and procaine affected DE- and PE-catalyzed ribosylation in a manner that might explain their protective or potentiative effects in cell culture. Since DE is a proenzyme (4, 7, 16), it is possible that these chemicals act at the level of either "activation" or enzymatic (transferase) activity. The latter appears to be the case inasmuch as all tested chemicals affected fragment A- and whole-toxin-catalyzed ribosylation identically (data not shown). Whether or not PE also undergoes an "activation" step has not yet been determined. There was a recent report that simultaneous treatment of PE with denaturant and reductant leads to a 20- to 50-fold increase in its adenosine 5'-diphosphate-ribosylation activity (13). However, the molecular events responsible for this type of "activation" and their relevance to cell or animal intoxication are unclear.

A complete explanation for the results obtained with individual drugs is difficult since their effects on cytotoxicity may be either primary or secondary. Moreover, despite our similar results with both HeLa and HEp-2 cells, it is possible that cell lines more sensitive to either toxin might not exhibit the same pattern with a given chemical (15). However, the absence of a drug effect can probably be taken as evidence that the subcellular system or organelle perturbed is not directly involved in the toxin's mode of action. Thus, according to our results, it is unlikely that oxidative phosphorylation, pinocytosis, cell surface sulfhydryls, or the Na^+,K^+-dependent ATPase are involved in the mechanisms of intoxication of either toxin. Our data also indicate that mitochondrial electron transport, the microfilaments, and the Ca^{2+},Mg^{2+}-dependent ATPase are not directly associated with the mechanism of intoxication of PE.

In toto, our results present an interesting and consistent pattern. Although many of the drugs examined protected cells from DE, none protected from PE. On the other hand, although some of these same drugs potentiated the cytotoxicity of PE, potentiation was never observed with DE. We believe that this pattern provides additional support for our previous proposal (J. L. Middlebrook and R. B. Dorland, Fed. Proc. 35:1394, 1976) that DE and PE have different mechanisms of intoxication on intact cells. Since the majority of drugs tested had no effect on the enzymatic activity of either toxin, it seems most likely that these differences lie in the attachment or internalization stages of intoxication.

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