Vol. 121, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1984 Pages 69-76

PRIMARY AMINES AND CHLOROQUINE INHIBIT CYTOTOXIC RESPONSES TO SHIGELLA TOXIN AND PERMIT LATE ANTIBODY RESCUE OF TOXIN TREATED CELLS

Gerald T. Keusch and Mary Jacewicz

Division of Geographic Medicine, Tufts University School of Medicine
136 Harrison Avenue, Boston, MA 02111

Received March 30, 1984

SUMMARY: The effect of lysosomotropic agents and primary amine inhibitors of transglutaminase on Shigella toxin activity in HeLa cells was determined by measuring cytotoxicity and late antibody rescue. All agents tested resulted in significant antibody rescue, but only ammonium chloride, dansylcadaverine, putrescine, bacitracin, serine/borate buffer, and chloroquine were inhibitory in the absence of added antibody. These compounds appear to be acting within the endocytic vacuole and/or inhibiting translocation of toxin from the cell surface to the cytosol. These data are consistent with a mechanism of translocation of ST from the cell surface to the cytosol by the process of receptor mediated endocytosis.

Some macromolecules presented to the surface of cells, including hormones and toxins, exert biochemical effects at intracellular targets (1,2). The mechanism of cellular uptake is, in most instances, not well defined but for many molecules may involve the process of receptor mediated endocytosis (RME)*. Shigella toxin binds to a cell surface receptor by means of a carbohydrate containing receptor (3), and is inhibited by a diverse group of metabolic inhibitors and by agents that interfere with endocytosis (4,5). However, we have not found a direct correlation between effects of metabolic inhibitors on ST action and endocytic uptake of horseradish peroxidase (6), a process involving nonspecific fluid phase endocytosis. These data suggest that RME may be involved in the cellular uptake of ST. The present study, therefore, examined the effects of amine inhibitors of RME on ST activity.

METHODS

ST was extracted from broth grown S. dysenteriae type 1 by previously reported methods (6). The same lot of toxin used in our previous experiments

* Abbreviations: ST, Shigella toxin; EGF, epidermal growth factor; α_2M, alpha$_2$ macroglobulin; RME, receptor mediated endocytosis; Me_2SO, dimethylsulfoxide; DT, diphtheria toxin; MM, McCoys modified medium; HBSS, Hanks balanced salt solution; TC_{50}, 50% tissue culture lethal dose.

0006-291X/84 $1.50

(3,4) was employed in the present investigation. Assay for cytotoxicity was by the method of Keusch et al (7). The percent mortality was calculated by comparison of results with sham intoxicated cells.

Primary amines were neutralized with 1 N HCl and diluted in McCoy's medium (MM). HeLa cells were preincubated with putrescine in 1% Me_2SO. All other inhibitors were in MM. Monolayers were incubated with noncytotoxic concentrations of inhibitors for 30 minutes and washed out just prior to the addition of ST. Toxin was then added simultaneously with the inhibitors for an additional 30-120 minutes. In all instances monolayers were then washed and the medium replaced with toxin-free MM with or without antibody. The ability of antiserum to rescue cells following at least 30 minutes of incubation with ST plus the amines was taken as evidence of the presence of toxin at the cell surface in a location accessible to antibody, and that this was due to an inhibitory effect upon ST internalization.

MATERIALS

Ammonium chloride was from Mallinckrodt (Paris, KY); methylamine, ethylamine, propylamine, t-butylamine, dansylcadaverine, bacitracin, putrescine, chloroquine, and serine/borate were from Sigma (St. Louis, MO). Antibody (Equine International Standard Anti-Dysentery Serum) was a gift of the Statens Seruminstitute, Copenhagen, Denmark.

RESULTS AND DISCUSSION

Effect of NH_4Cl on ST Cytototoxicity and Antibody Rescue

NH_4Cl protected HeLa cells in a dose related fashion from the effects of ST during an exposure period of 2 hours (Fig. 1), whereas in previous studies, 10-15 minutes was sufficient to express essentially all of the cytotoxic action of ST (8). When antibody was added at the end of the 2 hour incubation, protection due to the amine and antibody were additive when high concentrations of ST were used. Indeed, at the lowest concentration of NH_4Cl, the protection with antibody rescue was equivalent to that afforded by a 100 fold increase in quantity of amine alone. Preincubation of cells with NH_4Cl, however, failed to protect (data not shown), and the amine and ST had to be together to observe protection. These experiments indicate that NH_4Cl maintains toxin in a position on the cell surface from which it may be removed by washing, or neutralized by the late addition of antibody.

Effect of Primary Amines on ST Cytotoxicity and Antibody Rescue

In this experiment, cells were incubated with ST in the presence or absence of the amines for 30 minutes (Fig. 2). Addition of antibody was without effect

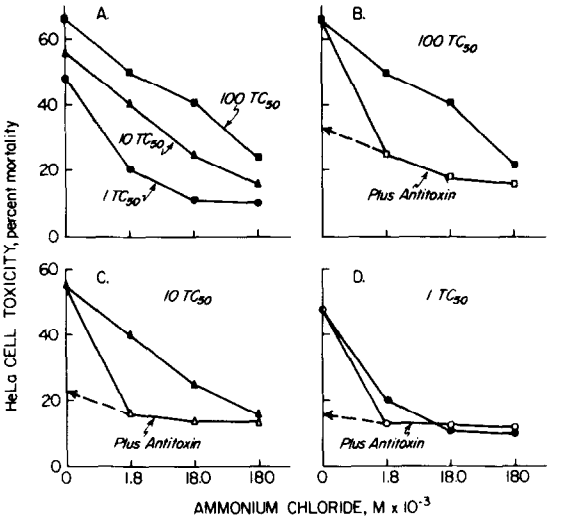

Fig 1. Duplicate HeLa cell monolayers were preexposed to 0.2 ml of MM, or 1.8, 18, or 180 mM NH_4Cl in MM for 30 min., then aspirated and treated with: A) 1, 10, or 100 TC_{50} doses of ST (1 TC_{50} = 0.5 ng)in 0.2 ml of MM for 2 hours. In B), C) and D), respectively, monolayers were incubated with 100, 10 or 1 TC_{50} doses of ST in MM and MM plus 1.8, 18, or 180 mM NH_4Cl for 2 hours, followed by a 1:20 dilution of antibody in 0.2 ml of MM for 1 hour. All incubations were performed at 37°C. After this, monolayers were washed twice with MM and then incubated overnight in 0.2ml of fresh MM. On the following day, percent mortality was determined. The dashed arrow head indicates the toxicity of preneutralized toxin following overnight incubation in HeLa cells.

in the absence of amines. NH_4Cl, dansylcadaverine, bacitracin and putrescine inhibited cytotoxicity to some degree whether or not antibody was added at the end of the incubation. All amines permitted a significant degree of antibody rescue. Prior addition of putrescine in the absence of Me_2SO failed to protect (data not shown), consistent with its intracellular locus of action and inability to penetrate cells without the permeabilizing agent (9,10).

Effect of Serine/borate and Chloroquine on ST Activity and Antibody Rescue

Serine (1mM), borate (20mM), or serine/borate had no direct effects on toxicity of ST (Table 1), however a small but significant degree of antibody rescue was observed when the serine/borate combination was employed.

Chloroquine was protective, as previously shown (4), however the highest concentration tested (500 µg/ml) was less effective than concentrations of 10-100 µg/ml (Table 2). Antibody rescue was also possible; in this case only the higher concentrations of chloroquine were effective. Indeed, lower concentrations able to inhibit ST action in the absence of antibody did not

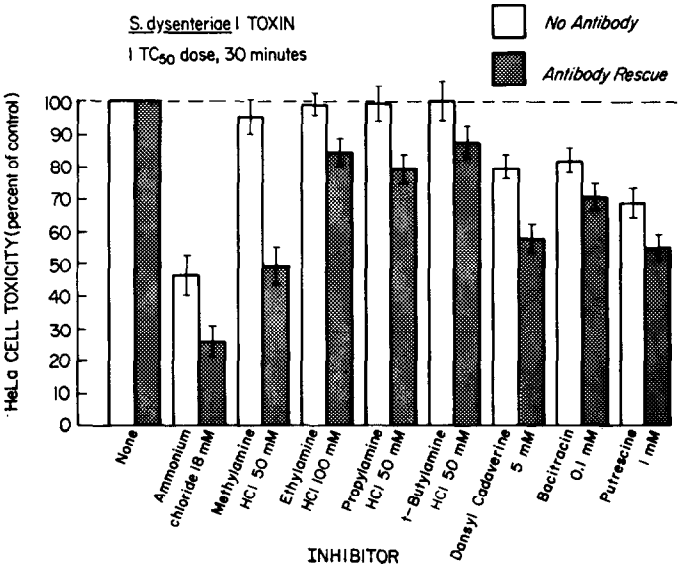

Fig. 2. Duplicate HeLa cell monolayers were preexposed to 18mM NH_4Cl, 50mM methylamine HCl, 100mM ethylamine HCl, 100mM propylamine HCl, 50mM t-butylamine HCl, 5mM dansylcadaverine, 0.1mM bacitracin in MM or 1mM putrescine in MM containing 1% Me_2SO. ST (1 TC_{50} dose) was then added in 0.2 ml of MM containing the above concentrations of the inhibitors. Duplicate monolayers treated in the same fashion were exposed to a 1:20 dilution of antibody. All monolayers were incubated for 1 hour, washed twice with MM, and incubated overnight in fresh MM. Cytotoxicity was measured the following day. The data were compared to the toxicity of ST in the absence of inhibitor (43% mortality), or in the case of putrescine, to the toxicity of ST in 1% Me_2SO (58% mortality).

result in an increment of protection following late addition of antibody. This suggests two loci of action of the drug.

Table 1. Effect of serine and borate on ST cytotoxicity and antibody rescue

Conditions	% HeLa Cell Mortality	
	Without antibody rescue	With antibody rescue
ST alone	44.7±3.0	44.0±2.2
ST + serine	44.7±3.6	45.0±0.9
ST + borate	44.7±2.9	44.7±2.3
ST + serine + borate	43.3±1.6	37.4±1.8

For cytotoxicity assay without antibody rescue, HeLa cells were exposed to ST for 30 minutes, followed by overnight incubation in fresh MM. For antibody rescue, ST exposure was followed by incubation for 30 minutes with antibody.

Table 2. Effect of chloroquine on ST cytotoxicity and antibody rescue

Chloroquine concentration (mg/ml)	% HeLa Cell Mortality	
	Without antibody rescue	With antibody rescue
0	45.9±1.7	45.5±3.2
500	37.9±1.2	17.1±1.6
100	29.9±1.2	10.0±0.9
50	27.1±2.4	23.5±2.4
10	28.3±2.3	27.6±3.3
5	39.7±2.0	36.8±3.3
1	45.0±3.4	44.3±3.7
0.5	45.7±2.0	44.8±3.0

Cells were incubated with ST plus chloroquine for 30 minutes (without antibody rescue) or with ST plus chloroquine for 30 minutes, then washed out, and incubated with antibody for 30 minutes (with antibody rescue). Cytotoxicity was determined following overnight incubation in fresh MM.

Effect of Permeabilization With Dimethylsulfoxide

Permeabilization of HeLa cells with Me_2SO markedly potentiates the action of toxin and reverses the inhibitory effects of metabolic inhibitors on ST activity (4), suggesting that the permeabilizer permits direct access of the toxin to the susceptible intracellular target. If primary amines protect HeLa cells by inhibiting RME of ST, then simultaneous addition of amine and ST together with Me_2SO should nullify this protection. Cells were preincubated with 1% Me_2SO in the presence or absence of putrescine, the monolayers washed three times with HBSS, and then exposed to ST with or without the permeabilizer. In other monolayers, NH_4Cl, ST and 1% Me_2SO were all added together. Addition of ST to permeabilized cells clearly reversed the protection afforded by either NH_4Cl or putrescine (Figure 3).

RME may be a common mechanism whereby eukaryotic cells import certain biologically important macromolecules from their external environment (1,2,11). Primary amines prevent clustering of EGF in coated pits, reduce EGF uptake in vesicles and potentiate its stimulatory action on DNA synthesis

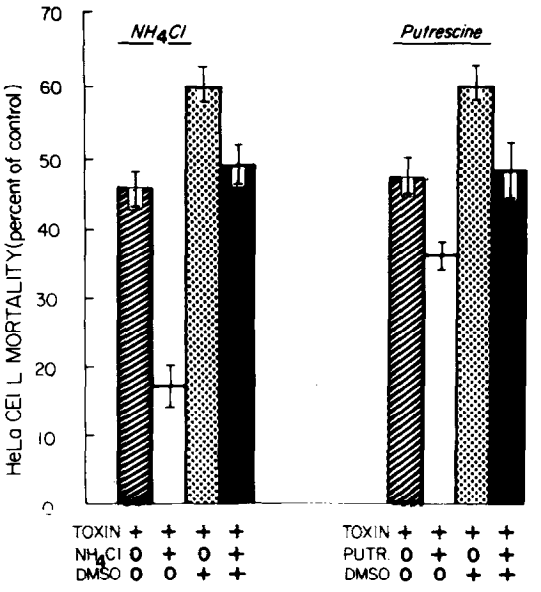

Fig. 3. Duplicate monolayers were preexposed to 0.2 ml of 18mM NH_4Cl in MM or 1mM putrescine in 1% Me_2SO for 30 minutes at 37°C, then aspirated, followed by treatment for 30 minutes with ST (1 TC_{50} dose), ST plus inhibitor, ST plus Me_2SO, or ST plus inhibitor plus Me_2SO. The monolayers were washed with MM, incubated overnight with fresh MM, and % mortality was determined the following day.

(12,13), suggesting that in this instance RME may be a mechanism to limit EGF hormone effects by rapidly removing it from the cell surface where it is active (12).

In this study we have shown that primary amines inhibit the cytotoxic action of shigella toxin and/or permit antibody rescue of intoxicated cells long after this becomes impossible in the absence of the amines. The latter effect demonstrates that the agents diminish translocation of ST from the cell surface to the cytosol. Since ST rapidly becomes inaccessible to removal by washing or antibody neutralization (8), is markedly potentiated by the membrane permeabilizer Me_2SO (4), and appears to act by inhibiting ribosomal protein synthesis (14), a rapid delivery system from the surface of the cell to the cytoplasm must be operative. The present experiments suggest that RME is the likely mechanism involved since the various amines we studied have been shown to prevent clustering at the cell surface of other ligands such as EGF and α_2-M with subsequent internalization by endocytosis (10). This conclusion is also consistent with the observed reversal of the

inhibition of ST activity by NH_4Cl or putrescine when Me_2SO is present in the medium to permeabilize the cells, providing a direct route for toxin into the cytosol.

It has been suggested that these amines alter RME by inhibition of the enzyme transglutaminase (15,16) which appears to function in cross-linking reactions needed for the translocation of receptor bound ligands (9,17). The effect of the amines on antibody rescue of ST cytotoxicity is consistent with this hypothesis, as is the effect of serine-borate, another inhibitor of cellular transglutaminase (18). In addition, the amines may be exerting other effects intracellularly, since as permeant weak bases they are known to accumulate within lysosomes and raise intralysosomal pH (19,20), and thereby alter intralysosomal processing of macromolecules and directly or indirectly inhibit their access to or action on cytoplasmic targets (21).

Chloroquine is a known lysosomotropic drug, and has been reported to inhibit degradation of DT following internalization by RME (22). The inhibitory action of relatively low concentrations of chloroquine on ST cytotoxicity may be explainable on this basis. At the same time, the antibody rescue observed at high concentrations of chloroquine is evidence of another action of the drug at the cell surface to limit the endocytic uptake of ST. Thus, chloroquine, like the amines, may also be acting at two loci, within the endocytic vesicle and at the cell surface as well.

ACKNOWLEDGMENT

This work was funded by grant RO1-AI-16242 from the National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD, grant 82008 from the Programme for Control of Diarrhoeal Diseases, WHO, Geneva, and a grant in Geographic Medicine from The Rockefeller Foundation, New York.

REFERENCES

1. Steinman, R.M., Mellman, I.S., Muller, W.A., and Cohn, Z.A. (1983) J. Cell Biol. 96, 1-27.
2. Neville, D.M.Jr., and Chang, T.-M. (1978) Curr. Top. Membr. Transp. 10, 65-150.
3. Keusch, G.T., and Jacewicz, M. (1977) J. Exp. Med. 146, 535-546.
4. Jacewicz, M., and Keusch, G.T. (1983) J. Infect. Dis 148, 844-854.
5. Eiklid, K., and Olsnes, S. (1983) Infect. Immun. 42, 771-777.
6. van Heyningen, W.E., and Gladstone, G.P. (1953) Br. J. Exp. Pathol. 34, 202-216.

7. Keusch, G.T., Jacewicz, M., and Hirschman, S.Z. (1972) J. Infect. Dis. 125, 539-541.
8. Keusch, G.T. (1973) Trans. N.Y. Acad. Sci. 37, 51-58.
9. Davies, P.J.A., Davies, D.R., Levitzki, A., Maxfield, F.R., Milhaud, P., Willingham, M.C., and Pastan, I.H. (1980) Nature 283, 162-167.
10. Maxfield, F.R., Willingham, M.C., Davies, P.J.A., and Pastan, I. (1979) Nature 277, 661-663.
11. Brown, M.S., and Goldstein, J.L. (1979) Science 76, 3330-3337.
12. Maxfield, F.R., Davies, P.J.A., Klempner, L., Willingham, M.C., and Pastan, I. (1979) Proc. Natl. Acad. Sci USA 76, 5731-5735.
13. Haigler, H.T., Maxfield, F.R., Willingham, M.C., and Pastan, I. (1980) J. Biol. Chem. 255, 1239-1241.
14. Reisbig, R.S., Olsnes, S., and Eiklid, K. (1981) J. Biol. Chem. 256, 8739-8744.
15. Lorand, L., Parameswaran, K.N., Steinberg, P., Tong, Y.S., Velasco, P. T., Jonsson, N.A., and Mikiver, L. (1979) Biochemistry 18, 1756-1765.
16. Folk, J.E., Park, M.H., Chung, S.I., Schrode, J., Lester, E.P., and Cooper, H.L. (1980) J. Biol. Chem. 255, 3695-3700.
17. Levitzki, A., Willingham, M., and Pastan, I. (1980) Proc. Natl. Acad. Sci. USA 77, 2706-2710.
18. Tate, S.S., and Meister, A. (1978) Proc. Natl. Acad. Sci. USA 75, 4806-4809.
19. Poole, B., and Okhuma, S. (1981) J. Cell Biol. 90, 665-669.
20. Maxfield, F. (1982) J. Cell Biol. 95, 676-681.
21. Draper, R.K., and Simon, M.I. (1980) J. Cell Biol. 87, 849-854.
22. Leppla, S.H., Dorland, R.B., and Middlebrook, J.L. (1980) J. Biol. Chem. 255, 2247-2250.