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Inhibition of Cholera Toxin-Stimulated Intestinal Epithelial Cell Adenylate Cyclase by Adenosine Analogs[1] (39342)

TERRY V. ZENSER[2]

(Introduced by R. W. Wannemacher, Jr.)

United States Army Medical Research Institute of Infectious Diseases, Frederick, Maryland 21701

Cholera toxin elaborated by *Vibrio cholerae* binds essentially irreversibly to its membranous receptor within minutes of contact with intestinal cells (1). The resulting increase in intestinal fluid and electrolyte secretion is mediated by toxin activation of the intestinal adenylate cyclase-cyclic AMP system (2). Cholera toxin is also able to activate adenylate cyclases in a variety of nonintestinal mammalian tissues (3-5). Effective therapy for cholera now consists of replacement of fluid and electrolytes and administration of antibiotics (6). Although this therapy is very successful when initiated early, logistics is a major problem in treating patients in underdeveloped areas. Therefore, a simpler method of treating cholera is needed. Theoretically, agents which lower intestinal cyclic AMP content could either complement or, perhaps, replace current therapy.

Although various attempts have been made to blunt the cholera toxin response, little success has been achieved. Antibodies to cholera toxin which are effective in removing free toxin from solution cannot remove the toxin from its membranous receptor (7). Similarily, ganglioside G_{M1}, the toxin's membranous receptor, is effective in binding free but not bound toxin (8). A natural occuring toxoid is capable of inhibiting cholera toxin binding and thereby toxin action (9). However, since the toxoid acts by forming an inactive complex with the membrane receptor, it must be administered prior to the toxin making it unsuitable for therapeutic use. Similarily, ethacrynic acid (10) and cycloheximide (11) have been shown to reverse some of the effects of cholera toxin, but neither is suitable as a therapeutic agent for use in humans. These results suggest that other pharmacological agents might be found which would reverse cholera toxin effects and be safe for human use. In this regard, recent studies have shown that adenosine and related analogs are capable of inhibiting adenylate cyclase in a variety of systems (12-15). Therefore, the ability of these agents to inhibit cholera toxin activation of human embryonic intestinal epithelial cell adenylate cyclase was investigated.

Materials and methods. [α-^{32}P]ATP, [^{3}H]cyclic AMP, and Riafluor were purchased from New England Nuclear, Boston, Mass. Cholera toxin was purchased from Schwarz/Mann. 2',5'-Dideoxyadenosine and 2'-fluoroadenosine were kindly supplied by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. All other chemicals were purchased from standard sources in the highest available grade.

Tissue culture preparation. Human embryonic intestinal epithelial cells, CCL-6, were obtained from the American Type Culture Collection, Rockville, Md. Cell monolayers were grown to confluency in sterile 60- or 150-mm petri dishes. Growth media consisted of Hanks basal minimal essential medium supplemented with 15% fetal calf serum (16).

Preparation and assay of CCL-6 cell adenylate cyclase. After incubating cells in 150-mm petri dishes for 18 hr at 37° with 0.7 μg/ml of cholera toxin, monolayers were washed twice with 10 ml of RPMI-1640. Three dishes of cells were then suspended

[1] In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council. The facilities are fully accredited by the American Association of Accreditation of Laboratory Animal Care.

[2] Present address: Departments of Biochemistry and Medicine, VA Hospital, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15240.

by mechanical scraping in a total of 20 ml of 10 m*M* Tris–HCl (pH 7.6), containing 2.0 m*M* $MgSO_4$, 0.5 m*M* EDTA, and 1.0 m*M* dithiothreitol. After homogenizing, the suspension was diluted to 30 ml, passed through gauze, and centrifuged at 3000 *g* for 10 min. This pellet was homogenized by hand in 1.5 ml of buffer. Twenty-five microliters of homogenate (150–200 μg protein) were assayed for adenylate cyclase activity.

Adenylate cyclase activity was measured by the conversion of [α-^{32}P]ATP into [^{32}P]cyclic AMP as previously described using the alumina column method (17). Under these assay conditions, both stimulated and nonstimulated cyclase activities were linear during a 10-min incubation at 30°. Protein concentrations were estimated by the Lowry method (18) using bovine serum albumin as a standard.

Determination of cyclic AMP. Cells were washed twice with 2 ml of RPMI-1640 to remove serum and incubated at 37° with 2 ml of RPMI-1640 containing 100 ng/ml of cholera toxin in 60-mm petri dishes for 6 hr. Preliminary studies showed no major differences between 6-hr compared to 18-hr incubations. After exposure to cholera toxin for 5.5 hr, test agents were added. At 5.75 hr, 1 ml of RPMI-1640 containing 3 m*M* 1-methyl-3-isobutylxanthine (MIX) was added. The incubation was terminated at the end of 6 hr by removing the media and adding 1 ml of boiling 50 m*M* sodium acetate buffer, pH 4.0, containing 2000 cpm of [^{3}H]cyclic AMP to assess recovery (approx 75%). Cells were scraped into size AA Thomas homogenizers, homogenized, and heated at 100° for 10 min. The cooled homogenates were centrifuged at 3000 rpm for 10 min. Aliquots (50 μl) were then assayed directly for cyclic AMP content by the binding-protein method of Gilman (19) as modified by Mashiter *et al.* (20). Cell pellets were dissolved with 0.2 *N* NaOH and the protein concentration was determined by the Lowry protein method (18). Data is expressed on the basis of total picomoles of cyclic AMP per plate, but similar results are obtained on a per milligram of protein basis.

Results. Six hours after addition of cholera toxin to CCL-6 cells, the cyclic AMP content dramatically increased by 85 ± 6 pmole/plate (Table I, Expt. 1). Of the compounds tested, only 2′,5′-dideoxyadenosine and 2′-deoxyadenosine successfully suppressed cholera toxin-mediated increases in cyclic AMP content. 2′,5′-Dideoxyadenosine was the more potent inhibitor. As shown in Table I, Expt. 1, 2′,5′-dideoxyadenosine inhibited cholera toxin by nearly 70% while 2′-deoxyadenosine inhibited by only 25%. 2′,5′-Dideoxyadenosine was able to effectively inhibit cholera toxin from 5 to 60 min after addition to cholera toxin-treated cells; shorter and longer times were not examined.

TABLE I. EFFECT OF VARIOUS ADENOSINE ANALOGS ON CHOLERA TOXIN-MEDIATED INCREASES IN CCL-6 CELL CYCLIC AMP CONTENT.[a]

Additions	Concn. (μM)	Picomoles cyclic AMP/ plate ± SEM
Experiment 1		
None		104 ± 6
2′,5′-Dideoxyadenosine	130	46 ± 4
	70	71 ± 4
	35	97 ± 5
Adenosine	130	116 ± 9
2′-Deoxyadenosine	130	83 ± 3
2-Chloroadenosine	130	121 ± 4
2-Fluoroadenosine	130	114 ± 9
7-Deazaadenosine	130	97 ± 8
Experiment 2		
None		142 ± 8
2′,5′-Dideoxyadenosine	130	78 ± 7
Adenosine 5′-sulfate	130	135 ± 3
3′-Deoxyadenosine	130	129 ± 12
Dimethylallyladenosine	130	155 ± 6
1-Methyladenosine	130	149 ± 10

[a] Cholera toxin (100 ng/ml) was added at zero time to CCL-6 cells. Test agents were present during the last 30 min, and 1-methyl-3-isobutylxanthine during the last 15 min of a 6-hr incubation. Controls contained 19 ± 2 and 12 ± 2 pmole of cyclic AMP per plate for Experiments 1 and 2, respectively. $N = 4$.

The ability of 2′,5′-dideoxyadenosine to directly inhibit CCL-6 cell adenylate cyclase was examined next. At 500 μ*M*, the adenosine analogs tested caused a 95 to 20% inhibition of cholera toxin-stimulated adenylate cyclase activity (Table II). 2′,5′-Dideoxyadenosine was again the most potent inhibitor. Shown in Fig. 1 is the concentration-dependent inhibition of cholera toxin activation of adenylate cyclase by 2′,5′-dideoxyadenosine. Half-maximal inhibition was seen at 16 μ*M*. To determine the extent of 2′,5′-dideoxyadenosine inhibition of adenylate cyclase activity, the NaF-activated enzyme was also examined (Table

TABLE II. EFFECT OF VARIOUS ADENOSINE ANALOGS ON CHOLERA TOXIN-STIMULATED CCL-6 CELL ADENYLATE CYCLASE ACTIVITY.[a]

Additions	μM	Picomoles cyclic AMP formed/mg/10 min ± SEM
None		670 ± 8
2′,5′-Dideoxyadenosine	500	40 ± 10
Adenosine	500	354 ± 9
2′-Deoxyadenosine	500	180 ± 6
2-Chloroadenosine	500	550 ± 24

[a] Cells grown on 150-mm petri dishes were incubated with 0.7 μg/ml of cholera toxin. After 18 hr, membranes were prepared and exposed to the adenosine analogs during the 10-min assay of cyclase activity at 30°. The adenylate cyclase activity of cells not treated with cholera toxin was 18 ± 2 pmole cyclic AMP/mg/10 min. $N = 4$.

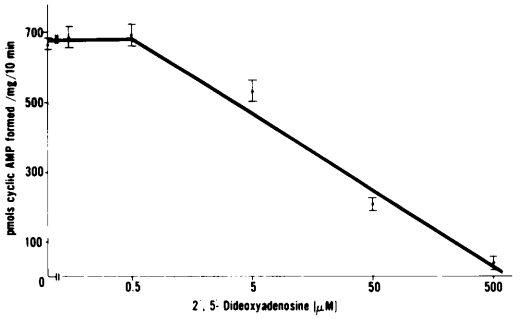

FIG. 1. Concentration-dependent inhibition of cholera toxin-stimulated CCL-6 cell adenylate cyclase by 2′,5′-dideoxyadenosine. Confluent cells were exposed to 0.7 μg/ml of cholera toxin at 37° for 18 hr. A membrane fraction was then prepared and cyclase activity determined in the presence of the indicated concentrations of 2′,5′-dideoxyadenosine $N = 4$.

III). As previously shown by Kantor *et al.* (21), NaF stimulated CCL-6 cell adenylate cyclase to a larger extent than cholera toxin. 2′,5′-Dideoxyadenosine inhibited both stimulated enzymes. There was greater inhibition of the cholera toxin than NaF-stimulated enzyme.

Discussion. This study demonstrates a potential usefulness of certain adenosine analogs in blunting the increases in intestinal cell cyclic AMP caused by cholera toxin. Of the adenosine analogs tested, 2′,5′-dideoxyadenosine was the most effective inhibitor. Similarly, Fain *et al.* (13) have shown this compound to be the most effective adenosine inhibitior of fat cell adenylate cyclase. Inhibition was not due to a nucleotide intermediate since 2′,5′ dideoxyadenosine is not suitable for such a conversion (22). Although CCL-6 cell adenylate cyclase activity has been shown to be elevated by 2 hr after addition of toxin (16), inhibition by 2′,5′-dideoxyadenosine in intact cells was observed at 6 hr after toxin addition, well after initial activation of adenylate cyclase occurs. Inhibition was apparent by 5 min after addition of inhibitor and could be sustained for at least 1 hr. This lowering in the cyclic AMP content of cholera toxin-treated cells could not be explained by excretion of cyclic AMP into the media since no change in the CCL-6 cell media cyclic AMP content was observed. Furthermore, in additional studies not shown, 2′,5′-dideoxyadenosine inhibition of cholera toxin was also observed in murine thymocytes where cells plus media were analyzed.

Inhibition of cholera toxin-mediated increases in cellular cyclic AMP content was reflected in a corresponding decrease in CCL-6 cell adenylate cyclase activity. The NaF-, as well as cholera toxin-, stimulated cyclase was inhibited. Differences in the mechanism of adenylate cyclase activation by NaF and cholera toxin have been reported (23–25) and are reflected in the insensitivity of the NaF-stimulated cyclase to 2′,5′-dideoxyadenosine inhibition compared to the toxin-stimulated enzyme. Various adenosine analogs have also been shown to inhibit adenylate cyclase in rat brain (12), liver (15), fat cells (13), and guinea pig lung (14). This supplementary evidence along with the high concentration of cyclic phosphodiesterase inhibitors pres-

TABLE III. EFFECT OF 2′,5′-DIDEOXYADENOSINE ON CHOLERA TOXIN- AND NaF-STIMULATED CCL-6 CELL ADENYLATE CYCLASE ACTIVITY.[a]

		Picomoles cyclic AMP formed/mg/10 min ± SEM	
Additions	μM	Cholera toxin	10 mM NaF
None		730 ± 50	1350 ± 94
2′,5′-Dideoxy-adenosine	500	83 ± 6	580 ± 55
	50	220 ± 12	870 ± 64

[a] Membranes were prepared from cells exposed to either 0.7 μg/ml of cholera toxin or diluent (saline) at 37° for 18 hr. 2′,5′-Dideoxyadenosine and NaF were present only during the 10-min cyclase assay at 30°. Adenylate cyclase activity of cells not treated with cholera toxin was 24 ± 2 pmole cyclic AMP/mg/10 min. $N = 4$.

ent during these experiments and the irreversibility of the cholera toxin binding suggest that the actions of 2′,5′-dideoxyadenosine are at least due in part to direct inhibition of adenylate cyclase and not activation of cyclic phosphodiesterase or dissociation of cholera toxin from its receptor. However, realization of the therapeutic usefulness of 2′,5′-dideoxyadenosine and related compounds awaits demonstration of the concomitant ability of these test agents to lower cholera toxin-mediated intestinal ileal cyclic AMP content and fluid accumulation.

Summary. The ability of various adenosine analogs to inhibit cholera toxin activation of the intestinal epithelial cell adenylate cyclase–cyclic AMP system was investigated. After incubation of cells with cholera toxin for 6 hr, large increases in cellular cyclic AMP content were observed. Addition of 2′,5′-dideoxyadenosine during the last 30 min of this 6-hr incubation resulted in 70% reduction in elevated cyclic AMP content. Other analogs were not effective inhibitors. 2′,5′-Dideoxyadenosine was also a potent inhibitor of cholera toxin-activated intestinal cell adenylate cyclase activity with half-maximal inhibition occuring at 16 μM. NaF-stimulated cyclase was less susceptible to inhibition. The data suggest that inhibition by 2′,5′-dideoxyadenosine is due at least in part to direct inhibition of the cholera toxin-activated intestinal adenylate cyclase activity.

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1. Cautrecasas, P., Biochemistry **12,** 3567 (1973).
2. Schafer, D. E., Lust, W. D., Sircar, B., and Goldberg, N. D., Proc. Nat. Acad. Sci. **67,** 851 (1970).
3. Zenser, T. V., and Metzger, J. F., Infec. Immunity **10,** 503 (1974).
4. Wolff, J., Temple, R., and Cook, G. H., Proc. Nat. Acad. Sci. **70,** 2741 (1973).
5. Bourne, H. R., Lehrer, R. I., Lichtenstein, L. M., Weissman, G., and Zurier, R., J. Clin. Invest. **52,** 698 (1973).
6. Carpenter, C. C. J., Bull. N.Y. Acad. Med. **47,** 1192 (1971).
7. Boyle, J. M., and Gardner, J. D., J. Clin. Invest. **53,** 1149 (1974).
8. Cuatrecasas, P., Biochemistry **12,** 3558 (1973).
9. Pierce, N. F., J. Exp. Med. **137,** 1009 (1973).
10. Carpenter, C. C. J., Curlin, G. T., and Greenough, W. B., J. Infec. Dis. **120,** 332 (1969).
11. Serebro, H. A., Iber, F. L., Yardley, J. H., and Hendrix, T. R., Gastroenterology **56,** 506 (1969).
12. McKenzie, S. G., and Bar, H. P., Canad. J. Physiol. Pharmacol. **51,** 190 (1973).
13. Fain, J. N., Pointer, R. H., and Ward, W. F., J. Biol. Chem. **247,** 6866 (1972).
14. Weinryb, I., and Michel, I. M., Biochim. Biophys. Acta **334,** 218 (1974).
15. Moriwaki, K., and Foa, P. P., Experientia **26,** 22 (1970).
16. Kantor, H. S., Tao, P., and Wisdom, C., Infect. Immunity **9,** 1003 (1974).
17. Zenser, T. V., Biochim. Biophys. Acta **404,** 202 (1975).
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193,** 265 (1951).
19. Gilman, A. G., Proc. Nat. Acad. Sci. **67,** 305 (1970).
20. Mashiter, K., Mashiter, G. D., Hauger, R. L., and Field, J. B., Endocrinology **92,** 541 (1973).
21. Kantor, H. S., J. Infec. Dis. **131** (Suppl.), S22 (1975).
22. Trysted, G., and Sartorelli, A. C., Biochim. Biophys. Acta **155,** 619 (1968).
23. Field, M., Proc. Nat. Acad. Sci. **71,** 3299 (1974).
24. Bennett, V., O'Keefe, E., and Cuatrecasas, P., Proc. Nat. Acad. Sci. **72,** 33 (1975).
25. Petrella, V. J., and Zenser, T. V., Biochim. Biophys. Acta **421,** 237 (1976).

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