DECREASE OF ENTEROTOXIN-INDUCED JEJUNAL FLUID ACCUMULATION BY ACIDS IN DOGS

D.R.Nalin, S.H.Richardson, N.Islam and J.Yardley

Introduction

Mortality from cholera and cholera-like diarrheal diseases can be reduced to less than 1% by replacement of fluid and electrolyte deficits (1). Even when appropriate fluids and antibiotic therapy are given, however, the mean duration of the disease remains 36 hours because the effect of cholera enterotoxin persists after Vibrios are eliminated (2). Analogous observations have been made with regard to the E.coli LT enterotoxin (3). An enterotoxin antagonist capable of quickly reducing diarrhea to negligible levels could simplify therapy by diminishing duration and volume of diarrhea and, consequently, the volume of therapeutic fluids required for treatment. Except for glucose and other agents which enhance absorption, only cycloheximide and ethacrynic acid have been reported to reduce enterotoxin-induced intestinal fluid accumulation when given after fluid accumulation has begun (4,5). Both of these compounds have undesirable effects which preclude a clinical trial in cholera patients.

Since nucleotides which are believed to mediate the action of enterotoxins are hydrolyzed at acid pH's, (6) the effect of low lumenal pH on the intestinal response to enterotoxins was studied in dog jejunal loops.

Methods

Studies were carried out primarily in ligated cannulated 15 cm. dog jejunal loops of a type previously described (7). In addition a series of studies was carried out using loosely ligated "loops" consisting of between one-half and three-quarters of the entire length of the dog small intestine.

After creation of loops and replacement into the peritoneal cavity, loops were exposed to cholera or to E.coli LT enterotoxins, which were administered via plastic canula

and withdrawn after ten minutes. Cholera loops received 0.15 or 0.25 ml. of 2.5 gm.% freshly reconstituted Wyeth 002 cholera toxin (frozen at -20°C and used within 5 days if not used on day of reconstitution.) E.coli loops received 4.0 ml. of one hundred fold concentrated E.coli (strain CRL 10400) culture filtrate (3). Three to four hours later fluid accumulation began and an initial rate of fluid accumulation was measured during a one hour collection period. After this most loops were treated for one-half or for two hours with single doses of acid or control solution (one milliliter per centimeter of loop length). Then the rate of post-acid (or post-control solution) fluid accumulation was measured during an additional four to nine hours. At the termination of studies, loops were biopsied. Most dogs were painlessly sacrificed on termination of studies but in the case of dogs with long, loosely ligated loops, the animals were allowed to recover and biopsies were obtained after two, five, ten or twenty-eight days.

Results

Reduction of lumenal pH to 1 by treatment with 0.3 normal HCl or 300 millimolar Zirconyl chloride ($ZrOCl_2$), which have identical pH's, reduced loop fluid accumulation rates by 60% within one hour compared to pre-treatment rates in the same loops. In contrast the fluid accumulation rates in control loops actually increased significantly during the same period. Data on typical studies conducted in nine dogs (six loops were constructed in most animals) are presented in Table 1. Periods A. B, and C in this table represent the initial post-enterotoxin period (A), the first hour post-acid period (B) and the later (4-9 hours) post-acid period (C).

The decrease in fluid accumulation persisted for the duration of the studies, which lasted up to nine hours after treatment with acid. In addition to HCl and $ZrOCl_2$, 0.3 normal H_2SO_4 or HNO_3 were also tested and found effective. The duration of exposure of loops to acid was thirty minutes for all loops presented in Table 1.

Data from studies in which the exposure period to acid or to control solutions was two hours are presented in Figures one and two. These data show that while 300 millimolar

$ZrOCl_2$ reduced fluid accumulation when given as a single dose of 15 ml. (1 ml./cm. loop length) a single dose of 100 millimolar HCl was not markedly effective. In control loops fluid accumulation increased as in previous studies. Single doses of 100 millimolar $ZrOCl_2$, like 100 millimolar HCl, had no marked effect; and Zr Citrate (even up to 300 millimoles per liter in the latter case) actually increased loop fluid accumulation (Table II).

Additional studies showed that 100 millimolar HCl could be effective when two or three successive 15 ml. doses were administered (to loops with over 0.1 ml./min. of pre-acid fluid accumulation) as needed to maintain loop pH at one for sixty minutes. In ten such loops fluid accumulation decreased from 0.22 ± 0.07 to 0.13 ± 0.03 ml./min./loop after the one hour HCl treatment (mean ±.S.D., p = less than 0.01).

Table III shows that pre-mixture of 0.25 ml. of 002 toxin with 5 ml. of 0.3 normal HCl (followed by neutralization of the mixture to PH 7.0 with 5% $NaHCO_3$) could completely block the toxin effect on loop fluid accumulation. Pre-exposure of loops to 300 millimolar HCl or $ZrOCl_2$ was also effective in reducing the loop response to enterotoxin. In all studies included in Table II, loop fluid accumulation was measured from four to six hours after loop exposure to enterotoxin or enterotoxin-acid mixtures.

With the exception of loop fluid protein content, the loop fluid composition before and after acid treatment did not change markedly. Loop fluid protein loss was low before and after acid treatment but rose during the period of actual exposure to acid (Table IV). Some of the protein lost was deposited as a thin white membrance over the mucosal surface which was observed on dissection of loops at the end of studies. The membrane in $ZrOCl_2$ loops was more adherent, granular and evenly distributed over the loop mucosa than in HCl treated loops. Zirconium deposited in the protein made the loop lining radioopaque in $ZrOCl_2$-treated loops, and in convalescent dogs treated this way and observed ("long loops"- 4 animals), the radioopaque material was passed in the stool after several days.

The transient increase in loop protein loss indicated

some degree of mucosal damage and led us to study the accompanying changes in mucosal morphology. Findings were similar in toxin-treated loops exposed to effective doses of HCl or of $ZrOCl_2$, and consisted of damage confined to villous tips associated with dilatation of crypts (which were otherwise of unremarkable appearance). The villous tips showed pale staining (with eosin) and tip cells showed loss of structural integrity and shedding, indicating necrosis at the tips (Figures 3 and 4). In addition to shedding of villous tips, regeneration of tip epithelium was evident as early as 4-6 hours after exposure to acid. (Figure five). Sections from biopsies taken after two, five, ten or twenty-eight days showed virtually complete healing of the loop mucosa as early as day two. (Figure six).

Discussion

The effect of acidity could be due to acid hydrolysis of nucleotides which may be mediators of the enterotoxin effect (8,9). This type of hydrolysis occurs in vitro and is catalyzed by zirconium (6). However, in equimolar concentrations the effect of $ZrOCl_2$ does not seem to be more marked than that of HCl; its only special characteristic is that its radioopacity makes its distribution along the mucosa visible on X ray.

The neutralization of enterotoxin by pre-mixture with acids suggests that a direct effect on membrane-bound toxin could also play a role, assuming that such toxin remains in a location where it is vulnerable to changes in lumenal pH. A previous report showed that permeability factor of cholera toxin was more sensitive to HCl (pH2) than loop activity factor, but the concentration required to decrease loop activity was not studied (10).

While elucidation of the mechanism of the effects reported here requires further study, the microscopic changes in loops treated with acids after the onset of fluid accumulation suggest that mucosal damage, possibly localised chiefly at the villus tips, could be an important factor leading to reduction in enterotoxin-induced fluid loss in these loops.

The rapid healing process, already evident 4 hours after acid treatment may replace shed toxin damaged cells with new cells and this could account for the rapid return to normal intestinal absorption. A previous report suggested that the crypt cells might be chiefly responsible for fluid loss (11). After acid treatment the crypts, although dilated, appear morphologically intact; this suggests that up to 60% of the fluid loss may come from cells at the tips of the villi. However, crypt dilatation was prominent only when acid was given after fluid accumulation had begun; loops exposed to acid alone (no enterotoxin) had little or no crypt dilatation. This suggests that at least part of the fluid is being produced in the crypts. Plugging of the crypts (due to edema and debris) may increase crypt pressure and result in dilatation due to continued fluid accumulation in the crypt lumen. Back pressure might then inhibit further fluid accumulation.

The reduction in response to enterotoxin following pre-exposure of loops to acid could possibly be explained by an alteration in membrane binding sites for toxin; it is also possible that the acid-induced protein membrane over the mucous may simply block access of toxin to intact membrane binding sites.

The rapid return to normal of mucosal morphology after acid treatment and the healthy condition of ten animals observed in convalescence indicate the superficial nature of the acid-induced charges. In previous studies HCl (100 millimoles per liter) has been infused by plastic tube into the small intestine of human volunteers without ill effect (11,12). The effectiveness of 100 millimolar HCl in reducing fluid accumulation when infused over sixty minutes suggests a possible therapeutic role for dilute HCl in cholera and related voluminous diarrhoeal diseases. In addition this study suggests that other agents which can plug crypts or cause rapid shedding of tip epithelium should be tested for potential therapeutic value in cholera.

References

1. Nalin, D.R. 1974. Mortality from cholera and other diarrhoeal diseases at a cholera hospital. Trop. Geo. Med. 24: 101-106.

2. Greenough, W.B. III, Rosenberg, I.S., Gordon, R.S,Jr., Davies, B.I. and Benenson, A. 1964. Tetracycline in the treatment of cholera. Lancet, 355-357.

3. Nalin, D.R., Bhattacharjee, A.K. and Richardson, S.H, 1974. Diarrhoea resembling cholera induced by E.coli culture filtrate. J.Inf.Dis. In press (Presented in Proceddings of the 1973 U.S.-Japan Cooperative Science Panel Meetings, Grand Canyon, Colorado).

4. Harper, D.T. Jr., Yardley, J.H. and Hendrix, T.R. 1970. Reversal of cholera exotoxin induced jejunal secretion by cycloheximide. Johns Hopk. Med. J. 126: 258-266.

5. Carpenter, C.C.J., Curlin, G.T. and Greenough, W.B. 1969. Response of canine Thiry-Vella Jejunal loops to cholera exotoxin and its modification by ethacrynic acid. J. Inf. Dis. 120: 332-338.

6. Bamann, E., Trapmann, H. and Oechsner, B. 1962. Decomposition of phosphoric acid esters by metal ions in the acid pH range. Arch. Pharm. (Berlin) 295/67: 663-670.

7. Nalin, D.R., Ally, K., Hare, K., 1972. Effect of cholera toxin on jejunal osmoregulation of mannitol solutions in dogs. J. Inf. Dis. 125(5): 528-532.

8. Field., M. 1971. Intestinal secretion: effect of cyclic-AMP and its role in cholera. N.E.J.M. 284: 1137-1144.

9. Evans, D.J.Jr., Chen, L.C. and Curlin, G.T. 1972. Stimulation of adenyl cyclase by Escherichia coli enterotoxin. Nature (New Biology) 236: 137-138.

10. Bhattacharjee, A.K. and Mosley. W.H. 1973. Selective destruction of skin permeability activity in cholera toxin: effect of acid on cholera enterotoxin. Infec. Imm. 8: 133-136.

11. Winship, D.H., Caflisch, C.R. and Schulte, W.J. 1972. Acid loss in human duodenum. J. Appl. Physiol. 32, 5, 585-590.

12. Johnston, D. and Duthie, H.L. 1964. Effect of acid in the duodenum on histamine-stimulated gastric secretion in man. Gut 5, 573-578.

Caption to Table One

Effect of single exposure to 15 ml. of $ZrOCl_2$ (300 mm./L.) or 0.3N HCl persisted over 7-9 hour observation period in 40 loops of 9 dogs (6 cholera, 3 E.coli) but in the same dogs fluid accumulation continued unabated in 12 loops treated with toxin alone. (Data in this table not included in Figures I and II). Exposure period was shortened from 60 to 30 min. in 22 of 34 cholera loops and all E.coli loops. Cholera loops each received a high (0.25 ml.) dose of 002 toxin and E.coli loops received 6 ml. of concentrated culture filtrate. A = initial post-toxin fluid collection period; B = 1 hour after and C = 7-9 hours after $ZrOCl_2$ or HCl treatment; when tested by Wilcoxon's matched pairs ranked sign test, P (A minus B) and (A minus C): > 0.1 and > 0.10 (cholera toxin alone); > 0.7 and < 0.05 (E.coli toxin alone); < 0.01 and < 0.01 (cholera + $ZrOCl_2$); < 0.05 and < 0.05 (E.coli + $ZrOCl_2$); < 0.01 and < 0.01 (cholera + HCl); < 0.05 and > 0.10 (E.coli plus HCl).

Caption to Table Two

Fluid accumulation induced by 0.15 or 0.25 ml. of 002 cholera enterotoxin was measured before and after exposure of loops to low concentration of $ZrOCl_2$ or high concentration of Zr Citrate. At a concentration of 100 millimoles per liter $ZrOCl_2$ had little or no effect in reducing fluid accumulation; Zr Citrate increased fluid accumulation.

Caption to Table Three

Effect of pre-mixing. I. Toxin and acid. Mixture was incubated at room temperature for five minutes and placed in loop after neutralization to pH 7 with 5% $NaHCO_3$. Toxin premixed with acid this way caused no fluid accumulation.

II. Exposure of loops to 300 millimolar HCl or $ZrOCl_2$ before enterotoxin reduced loop fluid outputs compared to outputs in loops treated with enterotoxin alone. Toxin doses were 0.25 ml. of 002 or 4 ml. of 100 fold concentrated 10400 filtrate.

Caption to Table Four

Loop protein loss measurements before, during and after exposure to 300 millimolar HCl or $ZrOCl_2$ showed an increase in loop protein loss primarily during actual exposure to low pH. Post-exposure levels were measured one hour after terminating exposure to acid pH.

Caption to Figure I

Paired Data on 52 loops (mean $\pm$ S.D.) of 9 dogs treated with 200 or 300 mm./L. $ZrOCl_2$ or control solutions for 2 hours exposure period. A = Baseline fluid accumulation rate 2-3 hours after toxin (control period); B = rate 2-3 hours after $ZrOCl_2$ or control solution (4-6 hours after toxin) and C = rate 4-6 hours after $ZrOCl_2$ or control solution (6-9 hours after toxin). P values for differences (A-C) and A-B) = <0.01 for toxin alone, $ZrOCl_2$ and NaCl (600 mmol./L.) when tested either by Wilcoxon's matched pairs signed rank test (SRT) or Student's t test. For 0.1N HCl and normal saline groups $P = < 0.05$ and $P = > 0.05$, respectively (SRT). $ZrOCl_2$ group includes all loops treated with 200 and 300 mmole/L. $ZrOCl_2$.

Caption to Figure II

Paired Data on 36 loops (mean $\pm$ S.D.) of 6 dogs treated with 300 mm./L. $ZrOCl_2$ or control solutions for a 2 hour exposure period. A = Baseline fluid accumulation rate 4-5 hours after toxin (control period); B = rate 2-3 hours after $ZrOCl_2$ or control solution (6-7 hours after toxin) and C = rate 4-6 hours after $ZrOCl_2$ or after control solution (8-9 hours after toxin). P values for differences (A minus C) and (A minus B), respectively: < 0.004 and < 0.005 ($ZrOCl_2$); < 0.031 and < 0.016 (toxin alone); <0.016 and 0.016 (0.1N HCl and ASA) and $\cong 0.047$ and $\cong 0.047$ (NaCl 600 mmole./L.) when tested by Wilsoxon's matched pairs signed rank test. P. for comparisons of (A minus C) or (A minus B) between $ZrOCl_2$ and toxin alone, 0.1N HCl and ASA, and NaCl (600 mmole./L.) <0.001 in all cases except for the comparison of (A minus B) in $ZrOCl_2$ and hypertonic NaCl groups, where $p<0.01$ (Student's t test). *S.D. for all 6 loops.
ASA = Acetylsalicylic acid.

Caption for Figure III

Intestine exposed to 300 millimolar HCl (1 ml. per cm. loop length) after induction of cholera. Upper 1/3rd. of the villi show loss of epithelium and weak staining of their cores due to necrosis and/or lysis of nuclei consistent with low pH environment. Lower villi and crypts are intact. Crypt lumens are prominently dilated. Findings in HCl or $ZrOCl_2$ treated loops were similar and no difference was found after HCl or $ZrOCl_2$ in cholera or E.coli enterotoxin treated loops. Dilatation of crypts was most marked in areas where overlying villi had undergone greatest damage. These observations suggested that dilatation resulted from obstruction of crypt openings secondary to edema and inflammation of the lower villi in conjunction with some continued secretion of fluid from the crypts. Hematoxylin and eosin X70.

Caption for Figure IV

Typical loop not treated with enterotoxin but exposed to 300 millimolar HCl (1 ml. per cm. loop length) for 30 minutes. Same changes in Figure III except that crypt dilatation is less marked.

Caption for Figure V

Detail of Figure IV showing early epithelial regeneration. Tip cells have flattened out as part of reepithelialization process.

Caption for Figure VI

Biopsies taken on day two after exposure to cholera toxin followed by 300 mm. HCl show that mucosal morphology has returned to normal.

PROCEEDINGS OF THE 9TH MEETING OF THE SCIENTIFIC REVIEW AND TECHNICAL ADVISORY COMMITTEE OF THE CHOLERA RESEARCH LABORATORY

(a)

and

REPORTS OF THE COLLABORATIVE STUDIES BETWEEN CENTER FOR MEDICAL RESEARCH AND CHOLERA RESEARCH LABORATORY

For the

YEAR 1974

Dacca, Bangladesh