PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **180**, 231–235 (1985)

Stimulation of Intestinal Adenylate Cyclase by Cholera Toxin in Malnourished Rats (42169)

IRENE PEREZ-SCHAEL*,† AND JORGE FLORES*,[1]

*Instituto Nacional de Dermatologia, Universidad Central de Venezuela, and †Instituto Nacional de Nutricion, Apartado 4043, Caracas 1010, Venezuela

Abstract. The stimulation of intestinal adenylate cyclase by cholera toxin (CT) was studied in normal and malnourished rats 4 to 24 hr after a 30-min incubation of intestinal loops with the toxin. Whereas in control rats the enzyme activity returned to basal levels after 12 hr of incubation, in malnourished rats the activity of the enzyme remained significantly elevated even after 24 hr of the initial incubation. Malnourished animals had a reduced turnover rate of intestinal cells as determined by thymidine kinase activity. The delayed turnover of intoxicated cells may account for continuous activation of mucosal adenylate cyclase and possibly for prolongation of diarrhea in malnutrition. © 1985 Society for Experimental Biology and Medicine.

The association of diarrhea and malnutrition is one of the most important elements responsible for the high morbidity and mortality rates observed in infants and children in developing countries (1, 2). The adverse effect that this association has on mortality results from the increased severity and duration of diarrhea in malnutrition (3, 4); in addition, the rate of growth of children is heavily impaired by recurrent episodes of diarrhea and other infections (5).

Increased severity of diarrheal episodes in malnutrition may depend at least in part on an impairment of mucosal immunity (6). In this study, we have tested the possibility that the retarded turnover of intestinal epithelial cells observed experimentally in malnourished animals (7, 8) could result in a prolonged activation of the mechanisms by which cholera or other similar toxins produce diarrhea. Since cholera toxin triggers intestinal fluid secretion through stimulation of adenylate cyclase (9) in an irreversible fashion (10, 11) we hypothesized that delayed turnover of intoxicated cells may account for a continuous activation of that enzyme and prolongation of fluid secretion.

Materials and Methods. *Animals.* Two groups of weaned female Sprague–Dawley rats were rendered malnourished by feeding them for a 35-day period with diets containing 3 or 5% casein as the sole source of protein; a control group received a diet containing 16% casein (12). Adequate amounts of carbohydrate, lipid, mineral, and vitamins as well as water were given *ad libitum* to all three groups of rats.

Intoxication of intestinal loops. After overnight fasting the animals were administered 40,000 U penicillin and 34 mg streptomycin 1–2 hr before the experiments. They were anesthesized with ether and the intestine exposed through a midline abdominal incision. A 10 to 12-cm intestinal loop was ligated and divided in two segments preserving the integrity of the mesenteric circulation; 10 μg of purified CT was injected into one of the segments. After 30 min, both segments were extensively washed through a distal aperture. The loops were excised 4,8, 12, or 24 hr later, the mucosal surfaces were scraped off and homogenized in cold 75 m*M* Tris, 12.5 m*M* $MgCl_2$, 1 m*M* EDTA, pH 8.5, and the protein content in the homogenates was determined by the method of Lowry (13).

Enzymatic assays. Adenylate cyclase activity was assayed in the homogenates by the method of Krishna (14). The final composition of the assay mixture was 0.1 m*M* ATP, 0.01 m*M* cyclic AMP, 5 m*M* phosphoenolpyruvate, 5 m*M* $MgCl_2$, 30 m*M* Tris–HCl, pH 8.5, 0.07 U myokinase, 0.7 U pyruvate kinase, and 30 μg/ml enzyme in a total volume of 30 μl. The activity is expressed in picomoles per milligram protein per minute. The Student *t* test

[1] To whom reprint requests should be addressed: Laboratory of Infectious Diseases, NIAID, National Institutes of Health, Bethesda, Md. 20205.

0037-9727/85 $1.50
Copyright © 1985 by the Society for Experimental Biology and Medicine.
All rights reserved.

was applied in each case in which statistical comparisons between two different groups were made.

Thymidine kinase activity was indirectly determined by measuring the incorporation of [*methyl*-^{3}H]thymidine in duodenal epithelial cells of control and malnourished rats (3% protein in the diet) after 3, 24, or 72 hr of an intraperitoneal injection of 1 μCi [*methyl*-^{3}H]thymidine/g weight.

Separation of villus from crypt cells was done by a modification of the methods of Weiser (15) and Harrison and Webster (16). A segment of everted duodenum was slipped over a hollow metal rod. Epithelial cells desquamated from the mucosa were sequentially obtained by inflating the intestinal segment while stirring it at increasing speeds in cold phosphate buffered saline (no Ca^{2+} or Mg^{2+}) containing 1.5 mM EDTA, 0.5 mM dithiothreitol; 11 fractions of approximately the same volume were obtained in this way; a 12th fraction was obtained by scraping off the remaining cells. The cells in each of the 12 fractions were homogenized and assayed for protein concentration, ^{3}H content, and sucrase activity (as a marker for villus cells). To average and compare results from different experiments (in which variable proportions of the desquamated cells were present in the 12 different fractions), the value for total protein content was divided into 12 hypothetical compartments representing 2.5, 2.5, and 5% of the protein (the first 3, respectively) and 10% of the protein (the remaining 9 compartments). The experimental values for ^{3}H content and sucrase activity were then fitted into these 12 hypothetical compartments according to the actual protein content in each of the 12 fractions.

Sucrase activity was determined by the method of Dahlqvist (17). The results are expressed in units of activity per milligram protein (1 unit = 1 μM substrate hydrolyzed per minute).

Results. The mean initial body weights were 55, 53, and 53 g for the animals receiving 3, 5, and 16% protein, respectively; 35 days later their mean body weights were 50, 65, and 126 g for the same groups.

Adenylate cyclase activity was measured at various times after CT incubation in intestinal mucosa scrapings from rats of each group. The results are shown in Fig. 1. Basal adenylate cyclase activity measured in control intestinal loops was not different among the three groups. A significant increase in the adenylate cyclase activity in all three groups was clearly evident 4 hr after incubation with CT. Doubling of the activity was observed in the control group of rats; however, the adenylate cyclase activity in this group was no longer signifi-

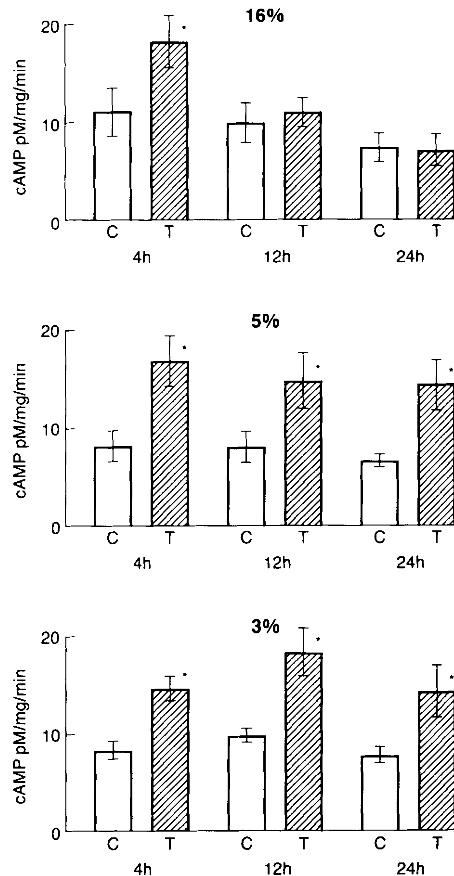

FIG. 1. Effect of cholera toxin on intestinal adenylate cyclase activity in rats receiving 16, 5, or 3% protein in the diet. Adenylate cyclase was measured in mucosal scrapings of intestinal loops 4, 12, and 24 hr after a 30-min incubation with (hatched bars) or without cholera toxin (empty bars). Each bar represents the mean ± SEM of at least five experiments. The Student t test for paired samples was applied for statistical analyses.* $P < 0.01$ as compared with the control enzyme activity measured in untreated intestinal loops (C).

cantly evident after 12 or 24 hr. In contrast, in the two groups of malnourished rats, the adenylate cyclase activity was still elevated after 12 and 24 hr (longest time studied) of incubation.

In separate experiments, the clearance of [methyl-^{3}H]thymidine incorporated into intestinal epithelial cells was estimated as an indirect measure of the turnover rate of those cells. Figure 2 shows the results obtained when [*methyl*-^{3}H]thymidine incorporation was determined after 3, 24, or 72 hr after a single injection in two separate groups of rats. In malnourished rats (3% protein in the diet), after 72 hr of the injection, the amount of labeled thymidine remaining in the epithelial cells represented more than 80% of that present in the intestine of rats sacrificed after 3 hr of injection. In contrast, in control rats, after 24 hr of injection 75% of the amount of label had already been cleared in comparison to control rats examined after 3 hr of thymidine injection.

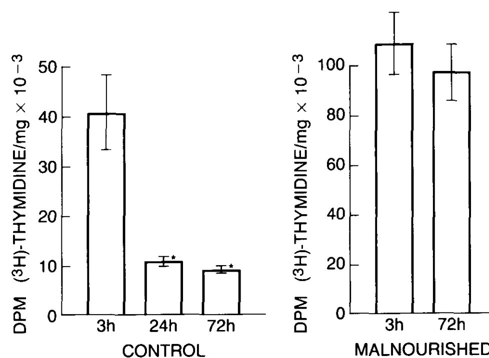

FIG. 2. Thymidine incorporation after 3, 24, or 72 hr of a single injection of [*methyl*-^{3}H]thymidine in control (left) and malnourished (right) rats. The results are expressed as the amount of radioactivity per mg of protein remaining in the epithelial cells obtained from mucosal scrapings. The values in each case represent the means ± SEM of four to six experiments.* $P < 0.01$ as compared with the [3H]thymidine remaining after 3 hr of injection.

When crypt and villi cell fractions of the intestinal mucosa were separated, more than 50% of the thymidine initially incorporated in the epithelium of malnourished rats was still present after 72 hr of injection in fractions corresponding to crypt cells (Fig. 3), whereas in control rats, most of the initial radioactivity found in the crypts 3 hr after injection had essentially cleared from all the fractions after 72 hr.

Discussion. The stimulation of intestinal adenylate cyclase by cholera and *Escherichia coli* heat-labile toxin with the consequent increase in intestinal fluid secretion is thought to be the major pathogenic mechanism by which organisms capable of producing such toxins induce diarrhea. We have tested here the hypothesis that delayed turnover of epithelial cells in malnourished rats may be associated with continuous activation of adenylate cyclase by cholera toxin.

In the present study, a 30-min incubation with CT caused stimulation of intestinal adenylate cyclase in normal and malnourished animals. In the normal rats, the adenylate cyclase activity had returned to basal values after 8–12 hr of the initial incubation with CT. Since the effect of CT on adenylate cyclase is known to be irreversible, a likely explanation for the return of activity to basal level in the control group is that replacement of intoxicated cells by normal cells occurred within this time frame. The fact that after 24 hr 75% of the thymidine incorporated after a single injection was cleared from the epithelial cells in the control rats supports this possibility.

An alteration in the return of CT-stimulated adenylate cyclase activity to baseline was found in the malnourished animals. Adenylate cyclase activation was evident even after 24 hr of the initial exposure to CT. Although we have not measured the half-life of intestinal cells under the conditions of our experiments, it is clear that an altered maturation of crypt cells was detected in the malnourished rats studied confirming the knowledge that malnutrition leads to impairment of intestinal epithelial cell turnover (7, 8). Such delayed turnover may explain the continuance of adenylate cyclase activation by CT observed in the malnourished rats.

Although a direct association between the altered turnover rate of intestinal epithelial cells observed in malnourished rats and the prolongation of intestinal adenylate cyclase activation has not been proven in this study, the results suggest that replacement of intoxicated cells by normal ones may be at least one of the mechanisms by which the duration

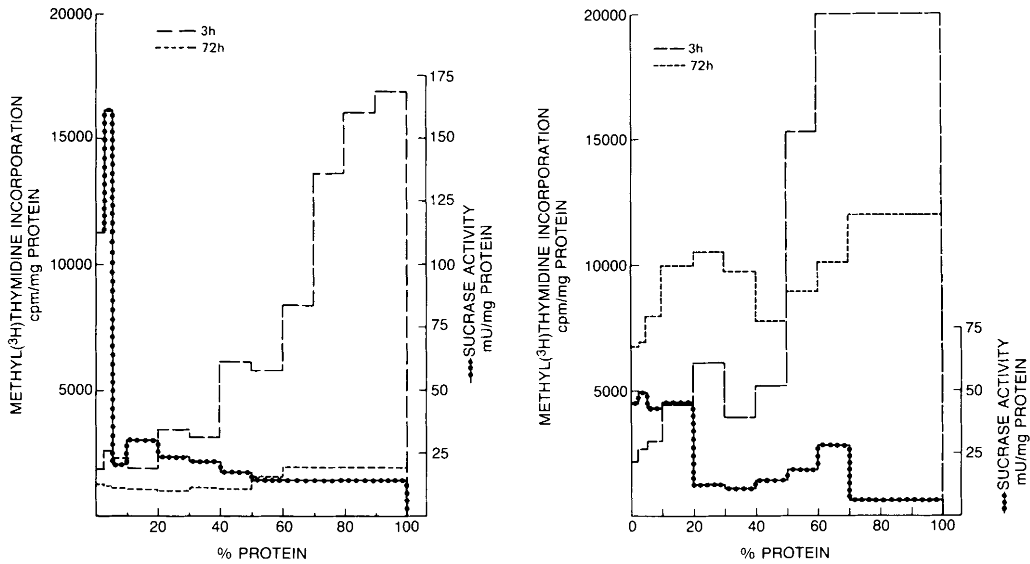

FIG. 3. Incorporation of [*methyl*-^{3}H]thymidine into different cellular fractions of intestinal mucosa of control (left) or malnourished (3% protein in the diet, right) rats. The results represent the mean values obtained from six experiments.

of cholera is limited; impairment of such mechanism—as it occurs during malnutrition—may lead to prolongation of cholera diarrhea.

Prolonged duration of cholera in malnourished subjects could also be explained by impairment of immune mechanisms which may result in a persistent colonization of the intestine by *Vibrio cholerae.* Although no information on the duration of vibrio shedding in malnourished subjects is available, some experimental evidence would suggest that possibility. Thus, the jejunal mucosa of malnourished children has been found to have fewer IgA-containing cells (18); also a diminished SIgA response to oral polio vaccine in malnourished children has been observed (19). Barry and Pierce (6) have shown that protein deficiency in the rat results in a marked diminution of the mucosal immune response to cholera toxin. It is impossible to assess at this time the relative importance of either mechanism (prolonged adenylate cyclase activation and prolonged vibrio shedding) in explaining the longer duration of cholera diarrhea in malnutrition.

This work was supported by the Consejo Nacional de Investigaciones Cientificas y Technologicas (CONICIT, Venezuela) Proyecto S1-0764.

1. Puffer RR, Serrano CV. Patterns of Mortality in Childhood. PAHO Sci Publ No. 262, 1973.
2. Scrimshaw NS, Taylor CE, Gordon JE. Interactions of Nutrition and Infection. WHO Monogr Ser **57,** 1968.
3. Rhode JE, Northrup RS. Taking Science Where the Diarrhoea Is. Ciba Foundation Symposium No. 42. Amesterdam/New York, Elsevier–Excerpta Medica–North-Holland, 1976.
4. Palmer DL, Koster FT, Alam AKMJ, Islam MR. Nutritional status: A Determinant of severity of diarrhea in patients with cholera. J Infect Dis **134:**8–14, 1976.
5. Mata L, Urrutia J, Albertazzi C, Pellecer O, Arellano E. Influence of recurrent infections on nutrition and growth of children in Guatemala. Amer J Clin Nutr **25:**1267–1275, 1972.
6. Barry WS, Pierce NF. Protein deprivation causes reversible impairment of mucosal immune response to cholera toxoid/toxin in rat gut. Nature (London) **281:** 64–65, 1979.
7. Hooper CS, Blair M. The effect of starvation on epithelial renewal in the rat duodenum. Exp Cell Res **14:** 175–181, 1958.
8. Brown HO, Levine ML, Lipkin M. Inhibition of in-

testinal epithelium cell renewal and migration induced by starvation. Amer J Physiol **205**:868–872, 1963.
9. Sharp GWG, Hynie S. Stimulation of intestinal adenyl cyclase by cholera toxin. Nature (London) **229**:266–269, 1971.
10. Beckman B, Flores J, Witkum P, Sharp GWG. Studies on the mode of action of cholera toxin. Effects on solubilized adenyl cyclase. J Clin Invest **53**:1202–1205, 1974.
11. O'Keefe E, Cuatrecasas P. Cholera toxin mimics melanocyte stimulating hormone in inducing differentiation in melanoma cells. Proc Natl Acad Sci USA **71**:2500–2504, 1974.
12. Hested DM, Chang Y. Protein utilization in growing rats. J Nutr **87**:19–25, 1965.
13. Lowry OH, Rosebrough NJ, Farr AL, Randall LJ. Protein measurement with Folin phenol reagent. J Biol Chem **193**:265–275, 1951.
14. Krishna GB, Weiss BB, Brodie BB. A simple sensitive method for the assay of adenyl cyclase. J Pharmacol Exp Ther **163**:379–85, 1968.
15. Weiser MM. Intestinal epithelial cell surface membrane glycoprotein synthesis. An indicator of cellular differentiation. J Biol Chem **248**:2536–2541, 1973.
16. Harrison D, Webster HL. The preparation of isolated intestinal crypt cells. Exp Cell Res **55**:257–260, 1969.
17. Dahlqvist A. Assay of intestinal disaccharidases. Anal Biochem **22**:99–107, 1968.
18. Green F, Heyworth B. Immunoglobulin-containing cells in jejunal mucosa of children with protein-energy malnutrition and gastroenteritis. Arch Dis Child **55**:380–383, 1980.
19. Chandra RK. Reduced secretory antibodies to live attenuated measles and poliovirus vaccines in malnourished children. Brit Med J **2**:583–585, 1975.

Received November 12, 1984. P.S.E.B.M. 1985, Vol. 180.
Accepted May 20, 1985.