Pathogenesis of Experimental Cholera: Choleragen-Induced Rat Foot Edema; a Method of Screening Anticholera Drugs (34318)

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Recently, advances in understanding the pathophysiology of cholera have led to dramatic improvements in treatment with consequent reduction of specific mortality, in adequately treated patients, virtually to nil (1). The treatment, however, depends primarily on replacement of the tremendous amounts of water and electrolytes lost in the cholera stool. Adjunctive therapy with appropriate antibiotics reduces the course of the disease and the amount of fluid required in treatment. Replacement therapy, while of great benefit, is inefficient, expensive, and poses huge logistical problems in providing adequate amounts of intravenous fluids—estimated at 20 liters/patient (1)—to the developing areas which are afflicted with cholera epidemics. These observations point out the need for a specific method of treatment: one directed toward reversal of, or interference with, the specific metabolic lesion in cholera rather than replacement of the materials lost as a consequence thereof.

Concomitantly, research on the pathogenic mechanism in several different laboratories (2–6) has led to the probable conclusion that the diarrhea of cholera is mediated by an exo-enterotoxin elaborated by cholera vibrios *in vitro* and in the small bowel of cholera patients. The mode of action of this toxin in evoking the fluid and electrolyte movement observed in cholera is not yet clearly understood. However, the enterotoxin, given the name, "choleragen," by the investigators who first separated it from culture filtrates (4), has recently been isolated in apparently pure form (7). This isolated choleragen, or cholera enterotoxin, which causes choleraic diarrhea in suckling rabbits and enterosorption in ligated ileal loops of adult rabbits, was also shown to be an extremely potent vascular permeability factor causing a delayed, sustained, erythematous, edematous, indurated lesion, permeable to colloidal carbon particles (8), following intradermal inoculation (7). These observations led the authors to postulate (7) that, while it could not yet be said that the diarrhea of cholera is the result of increased vascular permeability, choleragenicity and increased vascular permeability are intimately associated phenomena suggesting the potential value of experimental inquiry into a common mechanism. A study of the effect of inhibitors on the permeability reaction and on choleragenicity could provide useful information toward this end and, at the same time, might reveal a drug or drugs which could be useful in treatment of cholera patients.

However, each of the systems which has been used for bioassay of cholera enterotoxin, the suckling rabbit, the ligated ileal loop, the skin reaction, and, lately, the canine model (9), suffers some disadvantage which renders it less than suitable for screening of any substantial number of potential drug antagonists. On the other hand, the rat foot edema test (10, 11), which is commonly employed by pharmacologists for assay of anti-inflammatory drugs, has many attributes to suggest its potential value for our purposes. It provides numerical, rather than attribute-type, data; it has a high degree of reproducibility, and is both convenient and economical. Accordingly, we attempted to determine whether the rat foot edema technique would be useful in bioassay of choleragen and of

inhibitors of its effects. The present paper describes our preliminary findings.

Materials and Methods. Young female Holtzman rats, approximately 150 g, housed in air-conditioned quarters with food and water *ad libitum* were employed in this study. The rats, under light ether anesthesia, were inoculated with Millipore-filtered choleragen, 0.1 ml, in the plantar tissue of the right hind paw; the left hind paw received the diluent, sterile 0.85% NaCl, as a control. Inocula were administered by means of sterile 1-ml disposable glass tuberculin syringes fitted with disposable 26-gauge needles. Care was taken to avoid local hemorrhage. One-half hr later, and at subsequent intervals, the volume of the injected feet was determined by immersing each foot up to the top of the hindmost callosity into a mercury bath connected to a pressure transducer (Statham Company, Hato Rey, Puerto Rico) and a model 312 Sanborn transducer amplifier indicator (Sanborn Company, Waltham, Mass.). The apparatus was calibrated before and during each series of measurements by immersion of a calibrated syringe plunger. A minimum of three rats was used for each dose employed and the results are presented as the mean of the changes in volume (edema) relative to the initial readings. Cycloheximide (Actidione, Lot 4172, Nutritional Biochemicals Corp., Cleveland, Ohio.) was selected for use in this initial study after preliminary experiments revealed a long latent period before edema following choleragen administration. It has recently been reported (12) to inhibit the effect of cholera toxin in rabbit jejunal loops. The drug was administered intraperitoneally at the times and doses described in "Results." Saline was administered to control animals. Purified choleragen was prepared by a modification (Finkelstein and LoSpalluto, to be published) of the previously described technique (7). Briefly, in the modified procedure, selective filtration through membrane filters and an additional gel filtration step were used in lieu of ammonium sulfate precipitation and DEAE-cellulose chromatography. Two lots were used. One was stored in the frozen state as a dilute stock solution which was

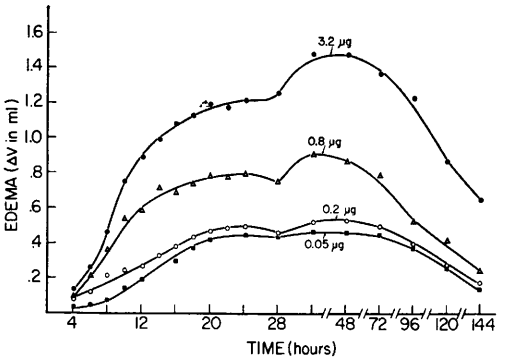

FIG. 1. Rat foot edema in response to varying doses of choleragen.

thawed and further diluted on the day of use and refrozen. An aliquot was also maintained under refrigeration as a 1.3 mg/ml solution in 0.4 *M* ammonium formate with 0.02% sodium azide and diluted on the day of use. The second lot was a more recent preparation stored under refrigeration in the liquid state at 1 mg/ml of the formate buffer, and di-

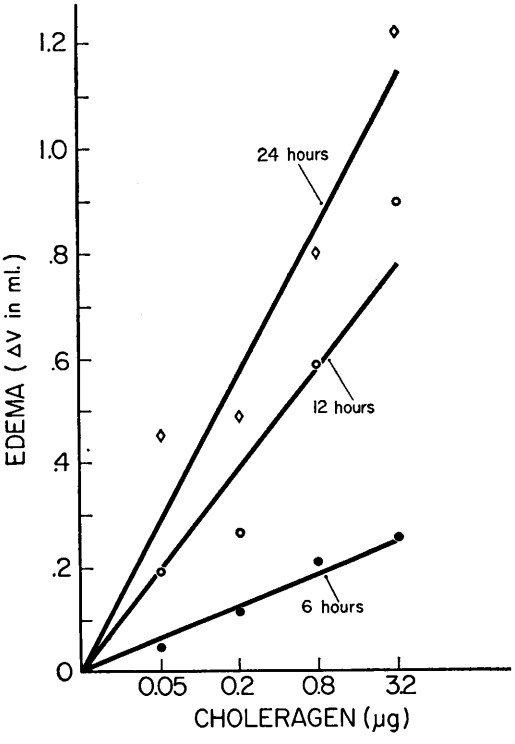

FIG. 2. Choleragen dose/edematous response relationship at selected time intervals.

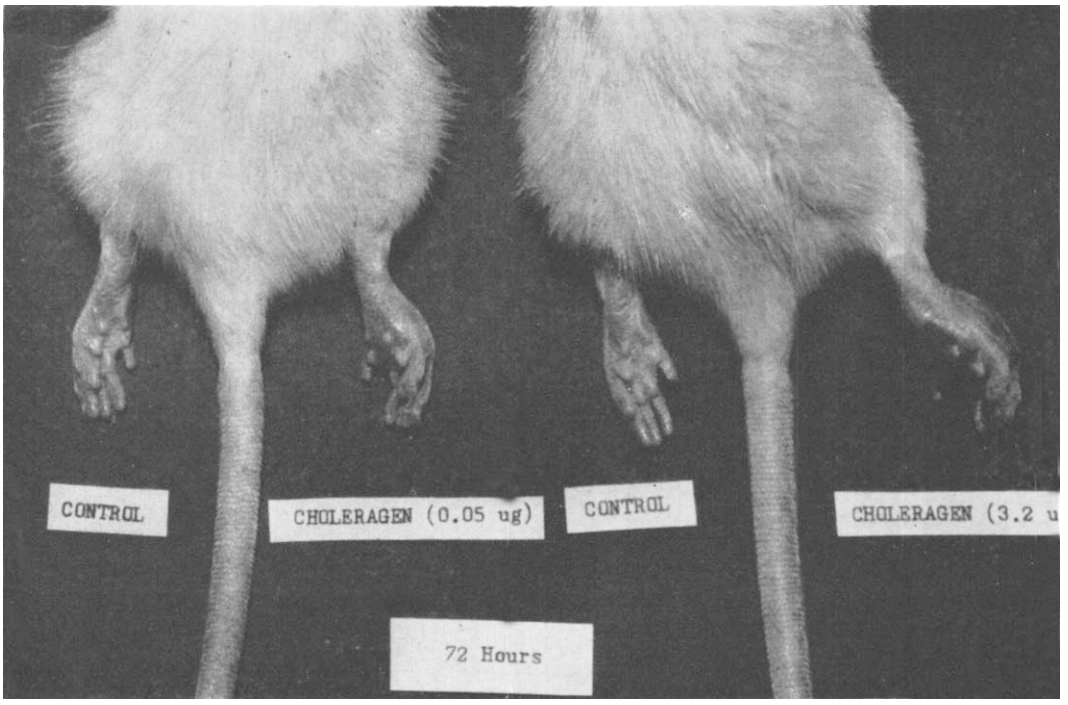

FIG. 3. Gross appearance of rat feet 72 hr after choleragen or control inoculation: (L. to R.) control; choleragen 0.05 μg; control; choleragen 3.2 μg.

luted on the day of use. The suckling rabbits used were handled and the mean choleragenic scores were determined as described previously (7) with the exception that the choleragen was administered to unlavaged rabbits in 5 ml of 0.1 *M* Tris buffer, pH 8.0 (Keusch, G. T., personal communication). This procedure has been found to give results equivalent to those of our previous technique. The scoring technique involves numerical grading of the diarrhea and excessive fluid in the bowel. An animal which succumbs to diarrhea is scored as 10; one with no response as 0. A mean choleragenic score of 5 indicates that the experimental group had markedly excessive fluid in the intestines and that at least some of the animals manifested diarrhea. This value is most reproducible as it is in the middle of the linear portion of the curve relating mean choleragenic score to dose of choleragen.

Results. Choleragen, in small doses, was found to produce a characteristic, and apparently unique, edema in the inoculated rat foot. The response was dose related (Fig. 1), beginning to appear after an initial lag of approximately 4 hr, peaking at approximately 24 hr, with a second peak, apparent with the upper levels of the dose range employed, at approximately 48 hr. The edematous response was sustained for a long period: paws inoculated with higher doses had still not returned to normal size by 144 hr. Control paws showed essentially no changes over the interval of observation except for a slight increase in volume over extended intervals which is likely due to growth of the animals. The volume of normal paws of the rats used in this investigation averaged approximately 1 ml so, as Fig. 1 shows, the choleragen-induced edema can result in more than doubling of the normal volume. The edematous response at different time intervals appears to be nearly linearly related to the dose of choleragen employed, plotted logarithmically, as indicated in Fig. 2. The gross appearance of choleragen-treated and control paws, at 72 hr, is presented in Fig. 3. With higher doses, the inflamed paw is quite red while this change is not so apparent at lower doses.

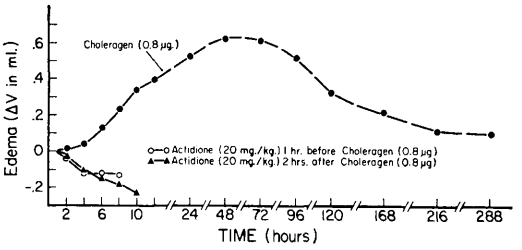

FIG. 4. Effect of Actidione (cycloheximide), 20 mg/kg, on choleragen-induced rat foot edema; all rats receiving this dosage of drug succumbed at, or shortly after, the last observation plotted.

Histological studies of the changes induced by choleragen are in progress.

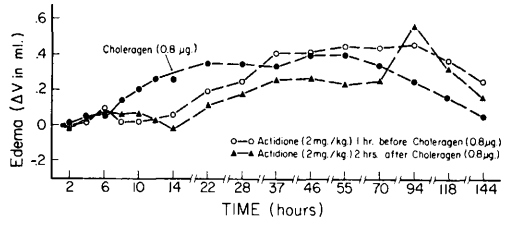

FIG. 5. Effect of Actidione (cycloheximide), 2 mg/kg, on choleragen-induced rat foot edema.

Actidione, 20 mg/kg administered 1 hr before or 2 hr after choleragen, had a pronounced inhibitory effect on the edematous response (Fig. 4), but resulted, at that dosage, in death of the experimental animals. A lower dose, 2 mg/kg, allowed survival of the animals and prevented development of the edema for approximately 14 hr (Fig. 5). Subsequent experiments revealed that Actidione, given in doses of 2 mg/kg initially and 10 hr after choleragen, delayed onset of significant edema for 36 hr after which it reached a peak less than that of the controls (Fig. 6). On the other hand, Actidione administered in a single dose of 2 mg/kg 10 hr after choleragen had essentially no effect on the edema (Fig. 6).

To determine whether these results are applicable to a system in which the manifestation of choleragen's action is outpouring of fluid into the gut, Actidione, 2 mg/kg, was administered to a group of 4 infant rabbits at the time of gastric challenge with 5 μg of choleragen. A group of 3 saline-inoculated rabbits fed choleragen was used as controls.

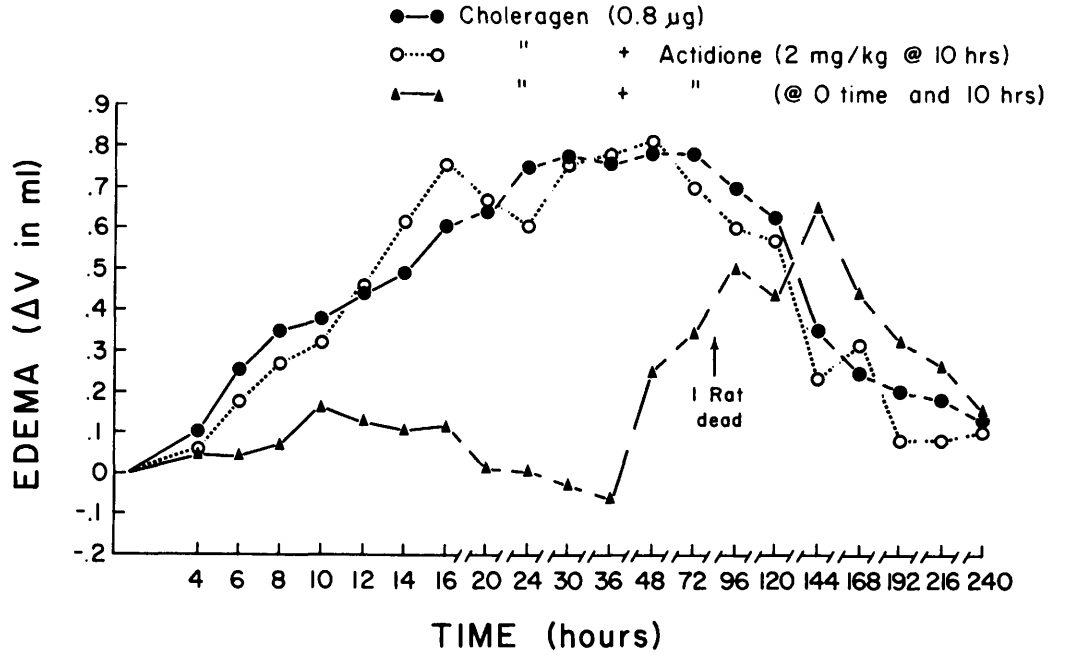

FIG. 6. Effect of Actidione (cycloheximide), 2 mg/kg, administered twice, at 0 time and at 10 hr, or once, at 10 hr, on choleragen-induced rat foot edema. (one rat in the treated group of 3 died after the 72-hr readings.) A fresh preparation of choleragen was used in this challenge.

Eighteen hr later, the control animals had succumbed to choleraic diarrhea for a mean choleragenic score of 10, the maximum possible response. In the treated group, which were all moribund from the Actidione at 18 hr, none of the animals had diarrhea, two had marked excess fluid in the gut and the other two had minimally excessive fluid for a mean choleragenic score of 2.8. Parallel studies indicated that a mean choleragenic score of 5 is produced with approximately 1 μg of this lot of choleragen. The slight response observed in the treated animals may be attributable to breakthrough after decline of the effect of Actidione as observed in the rat foot experiments.

It may be noted (compare Fig. 1, 4, and 5) that some decline in edematous response occurred in rats challenged later in the course of the investigation. This is probably the result of loss of activity of the choleragen stock solution which was stored in the frozen state and thawed and refrozen after making dilutions for use in the experiments described. An aliquot of the stock solution which was stored in the liquid state under refrigeration for 2 months gave results essentially identical with Fig. 1, as did a freshly prepared lot of choleragen such as that used in the experiment depicted in Fig. 6.

Discussion. The present observations suggest that the rat foot edema assay may provide a valuable means of screening drugs for anticholeragen activity. The test is highly sensitive: edema is produced following doses of 0.05 μg, and probably less, of the purified choleragen preparations used. The response is quite uniform and reproducible, and statistically valid data can be obtained with relatively few animals. The model is a convenient one requiring only minimal manipulation. As in other systems (8, 13, 14), the response to choleragen in the rat paw occurs after an initial delay of from 2 to 4 hr and then is evident over a prolonged period. It is of interest that the first drug tested for its anticholeragen effect in this system, cycloheximide, which was selected for study because of the delayed response to choleragen and because of its recently reported (12) effect on choleragen-induced fluid movement into the lumen of the small bowel of adult rabbits, was effective in preventing or delaying edema in the rat foot and also had a protective effect against experimental cholera in the infant rabbit model. This is consistent with, but does not prove, the hypothesis that the same basic pathogenetic lesion is operative in all three systems.

The mechanism by which choleragen induces fluid movement into the gut, or edema in rat feet or skin, is still not clear. If the cycloheximide is acting according to its reported mode of action in inhibiting *de novo* protein synthesis, then the findings reported herein are in general accord with those of Serebro *et al.* (12) who postulated a choleragen-induced, host-produced protein mediator of intestinal secretion, with the significant exception that, since rat paws are not *secretory* organs, a host-produced protein *permeability factor* may be the more likely explanation. Our, and their, observations suggest that once the hypothetical intermediate is produced, treatment with cycloheximide has no beneficial effect. Thus it is highly unlikely that cycloheximide or related drugs will find application in the treatment of cholera patients who present with already established lesions. What is needed is a pharmacological agent which will reverse the already established process. Hopefully, such a drug may emerge from studies of the nature reported herein.

The profile of the edema produced in response to choleragen in the rat foot was quite distinct from the responses observed (10, 11) following administration of carrageenin, yeast, bradykinin, histamine, or serotonin each of which produced a more rapid response of much shorter duration. Although preliminary evidence suggests (14) that the vasoactive plasma kinins are not involved in the infant rabbit cholera model additional study should be directed toward this point. It is of interest that choleragen does not appear to be readily degraded biologically and can persist in tissue to produce marked effects after the disappearance of the blocking effect of cycloheximide. This observation

may have a bearing on any treatment regimens which may develop following further study of potential inhibitors.

Summary. This preliminary study suggests that the rat foot edema test may provide a useful means of screening pharmacological agents potentially capable of preventing or reversing the specific metabolic lesions induced by cholera enterotoxin (choleragen). The first inhibitor selected for study, cycloheximide, prevented or delayed the onset of choleragen-induced edema in the rat foot test and also prevented the development of choleraic diarrhea in the infant rabbit assay. However, it did not alter the course of the established lesion in the rat foot. These observations suggest that a choleragen-induced, host-produced protein mediator may be involved. Additional studies, involving this model, directed toward understanding the pathogenic mechanism of cholera toxin and to develop potentially more effective methods of treatment of cholera patients, are in progress.

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1. Phillips, R. A., Ann. Internal Med. **65**, 922 (1966).

2. De, S. N., Nature **183**, 1533 (1959).

3. Dutta, N. K., Panse, M. V., and Kulkarni, D. R., J. Bacteriol. **78**, 594 (1959).

4. Finkelstein, R. A., Norris, H. T., and Dutta, N. K., J. Infect. Diseases **114**, 203 (1964).

5. Craig, J. P, Nature **207**, 614 (1965).

6. Burrows, W., Ann. Rev. Microbiol. **22**, 245 (1968).

7. Finkelstein, R. A. and LoSpalluto, J., J. Exptl. Med. **130**, 185 (1969).

8. Finkelstein, R. A., Nye, S. W., Atthasampunna, P., and Charunmethee, P., Lab. Invest. **15**, 1601 (1966).

9. Sack, R. B., Carpenter, C. C. J., Steenburg, R. W., and Pierce, N. F., Lancet **2**, 206 (1966).

10. Winter, C A., Risley, E. A., and Nuss, G. W., Proc. Soc. Exptl. Biol. Med. **111**, 544 (1962).

11. Van Arman, C. G., Begany, A. J., Miller, L. M., and Pless, H. H., J. Pharmacol. Exptl. Therap. **150**, 328 (1965).

12. Serebro, H. A., Iber, F. L., Yardley, J. H., and Hendrix, T. R., Gastroenterology **56**, 506 (1969).

13. Keusch, G. T., Atthasampunna, P., and Finkelstein, R. A., Proc. Soc. Exptl. Biol. Med. **124**, 822 (1967).

14. Finkelstein, R. A., Texas Rept. Biol. Med. **27 (Supplement 1)**, 181 (1969).

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