Rabbit Small-Bowel Response to Cell-Free Filtrate of Choleraic Fluid[1] (34735)

D. E. SCHAFER,[2] D. BANERJEE, U. BHARGAVA, S. SENGUPTA, B. SIRCAR, AND A. K. THAKUR
(Introduced by F. B. Bang)
The Johns Hopkins University Center for Medical Research and Training and the Calcutta University Science College, Calcutta, India

Sterile extracts of *Vibrio cholerae* cultures induce a form of intestinal secretion when placed in contact with the intestinal mucosa (3). Human cholera stools and intestinal fluids from animals experimentally infected with *V. cholerae* contain a similar enterotoxic activity. We have investigated the short-term response of the rabbit small bowel to treatment with fluids of the latter type. Intestinal loops of donor rabbits were inoculated with cultures of *V. cholerae*. Effluent harvested from the infected loops was sterilized by filtration and the filtrate was reinjected intraluminally into ligated intestinal loops of other rabbits. As a part of this investigation, the present study was carried out to ascertain some general characteristics of the secretory response: the volumes and electrolyte contents (potassium, chloride) of fluids accumulating in filtrate-injected loops were determined at various times, filtrate dilutions, and positions in the jejunoileum. A preliminary report has appeared elsewhere (8).

Methods. Mature rabbits of either sex and undetermined breed (mainly short-haired albinos) were used, with a mean weight at experiment of 1.2 ± 0.2 (SD) kg. Animals were received in the morning after feeding and were offered only water until use 24 or 48 hr later.

Ligated intestinal loops were prepared antiseptically by a version of the method described by De and Chatterje (2). Laparotomy was performed after procaine anesthesia of the anterior abdominal wall and peritoneum. Loops 15 cm in length were formed by ligating at both ends with silk suture introduced between the bowel wall and the vascular arcade. Loops were separated by intervening segments of 4 to 6 cm in length, which served as untreated controls as well as for greater isolation of loops. The entire jejunoileum was divided thus, from just below the duodenum to about 10 cm above a site adjacent to the tip of the appendix. Five to seven loops were made in each animal. Into the lumen of the completed loop, 2 ml of test fluid was slowly injected directly through the intestinal wall, with a 26-gauge needle. The abdomen was closed with muscle sutures and skin clips.

At a set time the animal was killed and the jejunoileum was removed. Segments between loops were examined for the presence of fluid in the lumen. The postmortem length of each loop was recorded and its contents were drained into a graduated cylinder. Physical characteristics of the bowel and secretion fluid were noted and effluent volume was recorded. Fluids with volumes of 3 ml or more were centrifuged and the supernatants were analyzed for potassium by flame photometry and chloride by coulometry.

Intestinal fluid filtrate was prepared in this and other experiments by the following method: A strain of *V. cholerae* designated Inaba 16, supplied by Prof. S. N. De, was chosen after a series of equivocal experiments

[1] This investigation was supported in part by U.S. Public Health Service Research Grant No. H.26. 6114 from the National Institutes of Health and in part by funding under Public Law 480, section 104(c), agreement no. 5X4317.

[2] Present Address: Veterans Administration Hospital, Minneapolis, Minnesota 55417.

with strains isolated from recent cholera cases. The entire jejunoileum of an adult rabbit was rinsed and closed at both ends by ligation, and the lumen of the closed loop was inoculated at from 2 to 8 sites, with a total of 5 to 10 ml of a 14-hr culture of *V. cholerae* in alkaline 2% peptone water. After 6 to 10 hr, the inoculated loop was mildly distended with secretion fluid, resembling that subsequently obtained in response to cell-free filtrate (below), except that the former generally contained little insoluble mucus.

We inoculated the largest possible loops of jejunoileum, 1.0 to 1.3 m in length, and pooled fluid harvested from 8 to 12 rabbits. Typical yields per animal were 60 to 80 ml. Pooled fluid was centrifuged at about 2500*g* for 30 min and frozen for storage. Subsequently the frozen material was thawed and centrifuged for 30 min at 20,000*g* at 4°. It was then sterilized by suction filtration through 0.22-μ pore size (GS) Millipore filters. About 10 to 15 ml could be sterilized with a single filter. The resulting clear fluid could be stored frozen indefinitely without measurable loss of activity. Another successful method of sterilization with respect to *V. cholerae* was alternate freezing and thawing, through 2–8 cycles. In all cases, sterility with respect to *V. cholerae* was confirmed by duplicate plating on bile salt agar.

The fluid used in the present series was sterilized by filtration, divided into 20-ml aliquots, and frozen until use. Experiments were conducted with aliquots thawed and refrozen not more than once prior to use. Studies in 64 loops of 16 rabbits showed that there was no detectable reduction in enterotoxic activity of the filtrate following a single freeze–thaw cycle.

Each of 76 rabbits was administered 3 or 4 serial 10-fold dilutions of filtrate in isotonic saline, together with two saline controls in the extreme end loops. Loops were numbered from the jejunal end. In one subseries undiluted filtrate, 1:10, 1:100, and 1:1000 dilutions were administered to loops II, III, IV, and V, respectively. In the other subseries the order was reversed, the same concentrations being administered to loops V, IV, III, and II, respectively. Experiment durations were 2, 4, 6, 8, 10, and 12 hr. In 12-hr experiments, undiluted filtrate was not administered.

Results. In the present series only the two highest concentrations of filtrate (undiluted and diluted 1:10) evoked significantly higher mean secretion volumes than did isotonic saline, and only at times greater than 2 hr (Table I). A substantial number of individual loops injected with filtrate diluted 1:100 or 1:1000, or with saline, also contained some fluid at the time of observation. Among saline-injected controls the mean volume of accumulated fluid was greater in the ileum (loop VI) than in the jejunum (loop I); 19 of 76 ileal loops contained more than 1 ml.

Certain conditions appeared to predispose to abnormally low or high secretion volumes. For example, two animals, both sacrificed at 8 hr, showed "thick-walled" intestines at operation; these yielded mean volumes of only 2.8 ml in response to undiluted filtrate, and 0.4 ml in response to the 1:10 dilution. A third animal, in the 12-hr group, was considered at the initial operation to show impaired intestinal blood flow. This animal produced a volume of only 0.7 ml in response to the 1:10 dilution. On the other hand, control loops adjacent to untreated intervening segments that contained fluid usually contained fluid themselves.

To correct for "false positive" and "false negative" responses (see Discussion) we recalculated the mean volumes after rejecting the following questionable loops: (a) all loops in the three animals described in the preceding paragraph; (b) any loop adjacent to an untreated intervening segment reported as "containing fluid"; (c) where the condition of intervening segments was not recorded, all loops from any animal in which either saline-injected loop contained 1 ml or more of fluid. Altogether, 115 of 428 loops (27%) were rejected for purposes of this recalculation. The recalculated mean volumes were lower not only for saline-injected loops, as required, but also for most positive loops receiving 1:100 and 1:1000 dilutions of filtrate. The mean responses to undiluted and 1:10 diluted filtrate, however, were essentially the same as in Table I.

TABLE I. Unadjusted Volumes of Choleraic and Control Loop Effluents.

Loop no.	Solution injected	Accumulated volume (mean ± standard deviation; ml) at the following time after injection (hr): 2	4	6	8	10	12
		A. Dose decreasing aborally					
		(6)[a]	(5)	(9)	(7)	(6)	(7)
I	Saline	0.2 ±0.3 (6)[b]	0.0 ±0.0 (4)	0.1 ±0.3 (9)	0.0 ±0.0 (7)	0.0 ±0.0 (6)	0.0 ±0.0 (7)
	Filtrate						
II	1:1	0.8 ±0.7 (6)	5.4 ±1.5 (5)	10.9 ±2.9 (9)	13.2 ±5.0 (7)	14.9 ±4.0 (6)	—
III	1:10	0.6 ±0.7 (6)	2.5 ±1.4 (5)	5.0 ±1.7 (9)	9.3 ±4.4 (7)	10.7 ±4.4 (6)	13.5 ±6.2 (6)
IV	1:100	0.4 ±0.4 (6)	0.6 ±0.7 (5)	1.9 ±2.4 (9)	2.8 ±2.1 (7)	2.5 ±1.7 (6)	3.9 ±3.9 (7)
V	1:1000	0.4 ±0.2 (6)	0.8 ±1.0 (5)	2.0 ±2.3 (9)	1.6 ±1.7 (7)	1.0 ±1.6 (6)	2.9 ±3.4 (7)
VI	Saline	0.1 ±0.2 (6)	0.4 ±0.7 (5)	1.6 ±1.8 (9)	1.0 ±1.6 (7)	0.4 ±0.8 (6)	3.6 ±5.7 (7)
		B. Dose increasing aborally					
		(6)	(7)	(7)	(7)	(4)	(5)
I	Saline	0.4 ±0.3 (3)[b]	0.0 ±0.0 (3)	0.0 ±0.0 (5)	0.0 ±0.0 (5)	0.0 ±0.0 (4)	0.4 ±0.8 (5)
	Filtrate						
II	1:1000	0.6 ±0.5 (6)	0.3 ±0.6 (7)	0.3 ±0.6 (7)	0.4 ±0.5 (7)	0.0 ±0.0 (4)	1.9 ±3.3 (5)
III	1:100	0.8 ±0.5 (6)	0.6 ±1.2 (7)	1.6 ±1.8 (7)	1.2 ±1.3 (7)	0.1 ±0.1 (4)	3.7 ±5.2 (5)
IV	1:10	0.9 ±0.5 (6)	2.5 ±1.7 (7)	5.9 ±2.9 (7)	5.4 ±3.3 (7)	11.6 ±3.0 (4)	15.5 ±4.1 (5)
V	1:1	1.4 ±0.6 (6)	5.2 ±1.7 (7)	8.6 ±2.6 (7)	10.2 ±4.0 (6)	17.3 ±3.5 (4)	—
VI	Saline	0.8 ±0.7 (6)	0.1 ±0.2 (7)	1.4 ±1.9 (7)	0.7 ±0.9 (5)	0.2 ±0.3 (4)	2.4 ±4.4 (5)

[a] Total number of animals.

[b] Number of loops.

On comparing the mean volumes in corresponding jejunal and ileal loops treated with undiluted filtrate (loops II and V) we found that none of the differences was significant at the 5% level; the same was true for loops III and IV, treated with filtrate diluted 1:10. Considering all loops receiving the same dose of filtrate as having been taken from a single population, therefore, we obtained adjusted overall mean values (Table II). In response to undiluted filtrate the maximum secretion rate of 0.15 ml/cm-hr was observed between 2 and 8 hr. After filtrate diluted 1:10 the maximum secretion rate was almost as great, 0.13 ml/cm-hr, but was not reached until 4 hr later, between 6 and 12 hr. Secretion volumes per unit length were calculated on the assumption of a length of 15 cm. Secretion volumes per unit actual final length of loop were also calculated; the results were essentially the same except at longer times, when the loops tended to be stretched by secretion fluid.

Effluent contained a variable amount of undissolved mucus and sometimes a few erythrocytes. On centrifugation, red cells were found to comprise at most 1% of the total volume, but mucus occupied 5 to 10%, and occasionally somewhat more. Of loops receiving either undiluted or 1:10 diluted filtrate,

TABLE II. Adjusted Volumes of Choleraic Loop Effluents.

Filtrate dilution	Adjusted volume (mean ± standard error; ml) at the following time after injection (hr): 2	4	6	8	10	12
1:1	1.0 ±0.2 (10)[a]	5.0 ±0.4 (11)	9.0 ±1.1 (9)	14.2 ±1.1 (9)	15.9 ±1.3 (10)	—
1:10	0.7 ±0.2 (10)	2.4 ±0.5 (11)	4.2 ±0.4 (9)	8.7 ±1.3(10)	11.1 ±1.3 (10)	16.0 ±1.7 (7)

[a] Number of loops.

76 loops in 52 rabbits yielded sufficient fluid for electrolyte analysis. These fluids were considered representative of the response to the filtrate. Fluids collected from four control loops, all ileal, in two 6-hr and two 12-hr animals, were also analyzed; the mean volume of effluent in this group was 9.1 ± 3.5 (SD) ml/loop. Mean potassium concentrations for the experimental and control fluids, respectively, were 4.2 ± 1.0 (SD) and 4.6 ± 0.5 meq/liter. The corresponding chloride concentrations for the two groups were 58 ± 11 and 57 ± 5 meq/liter.

Analysis revealed no consistent effect of filtrate dilution or loop location on either potassium or chloride content. Both potassium and chloride, however, showed an upward trend with time and effluent volume, this being more marked in the case of chloride. Specifically, for the 13 samples whose volume was less than 6.6 ml the mean chloride concentration was 50.6 ± 2.0 (SEM) meq/liter. By contrast, for the 32 samples over 12.5 ml in volume the corresponding value was 64.6 ± 2.2 meq/liter. Overall, the positive correlation between chloride and volume was highly significant ($r = +0.44$, $N = 76$, $p = 0.0002$). The chloride increase appeared mainly over the lower and middle volume range, up to about 16.5 ml ($r = +0.60$, $N = 67$, $p < 0.0001$); at higher volumes the mean chloride decreased somewhat, but not significantly.

Discussion. The pattern of fluid secretion in response to sterile filtrate of fluid produced *in vivo* was similar to that obtained with filtrates of *in vitro* cultures (5, 7). In particular, the latent period of the response is not greatly altered, if at all, by prior intestinal incubation. Although a highly heterogeneous rabbit population was used, the standard errors of the adjusted mean secretion volumes obtained were comparable to those obtained with a more hemogeneous population (5).

The incidence in the ileum—as contrasted to the jejunum—of "false positive" saline-injected loops and untreated intervening segments might be the result of "spontaneous" secretion in the ileum. Secretion without apparent stimulation does occur in the rabbit duodenum (4) and appendix (10). Others, however, have reported the absence of secretion from ileal control loops (5–7). Remote effects of absorbed enterotoxin (9) in the present experiments cannot be entirely ruled out, but it is probable that the enterotoxic activity of our preparation was too low for these to be detectable. In a study performed elsewhere (unpublished observations) we have since found that positive ileal controls could be practically eliminated by prior treatment of the animal for 1–2 weeks with oral sulfamethazine. The only pathogens investigated were coccidia; the incidence of severe jejunoileal coccidiosis in our rabbit population was found to be essentially 100% and this incidence was drastically reduced by the sulfamethazine treatment. A causal relationship of coccidiosis to ileal secretion was not established.

Two abnormal conditions were associated with strikingly reduced secretion volumes in three rabbits of the present series: (a) conspicuous thickening of the bowel wall, and (b) sharply diminished intestinal blood flow (blue color, reduced temperature). Our accumulated experience to date, with over 4000 loops in over 1000 rabbits, has confirmed that in either of these conditions intestinal secretion in response to *V. cholerae* enterotoxin is usually very much diminished. At present we have no further information regarding the nature or etiology of the first condition, thickening of the wall. As for the second condition, there is evidence that a marked diminution of intestinal blood flow does not appreciably alter an ongoing secretory response to the enterotoxin in the dog (1). Our observations indicate, however, that if blood flow is impaired at the time of enterotoxin administration the effect of the toxin is reduced, possibly because it is not well absorbed.

The mean effluent potassium concentration obtained here is slightly lower than those found by Norris and colleagues (6, 7) but agrees with that reported by Leitch *et al.* (5). The mean chloride concentration and the increase in chloride with time appear to be consistent with data from these sources (5, 7). The red cells present in some of the secretion fluids clearly were not characteristic

of the secretion proper. Their presence was interpreted as a sign of slight injury at the site of ligation, injection, or postmortem opening of the loop. Such injury was considered to have a negligible effect on the volume and composition of secretion.

It has been reported (4) that the upper jejunoileum of rabbits secretes less fluid in a given time than the lower in response to a given enterotoxic dose, and we have repeatedly made similar observations (unpublished). In the present series no evidence was found for such a difference. The reasons for this discrepancy, and the question of changes in anion content of choleraic secretions with time, have been investigated further and will be dealt with in separate communications.

Summary. Intestinal secretion fluid from donor rabbits experimentally infected with *V. cholerae* was sterilized by filtration. The filtrate was administered intraluminally to ligated small-intestinal loops of recipient rabbits. The volume of fluid accumulating in such loops was determined at various times, filtrate dilutions, and loop positions in the jejunoileum. Effluents were analyzed for potassium and chloride content. The time course of the volume response to filtrate (undiluted and diluted 1:10) was similar to that reported for experiments with crude enterotoxins prepared *in vitro*, as were mean effluent potassium and chloride concentrations. Regional variations in effluent volume and composition were not evident. Effluent chloride content increased substantially with time.

We thank Dr. G. W. Lewis for his part in preliminary experiments, Dr. D. Barua, Dr. R. A. Phillips, and Dr. E. A. Johnson for advice and assistance, and Dr. S. R. Maitra for generously providing facilities for this investigation.

1. Carpenter, C. C. J., Greenough, W. B., III, and Sack, R. B., J. Infec. Dis. **119**, 182 (1969).

2. De, S. N., and Chatterje, D. N., J. Pathol. Bacteriol. **66**, 559 (1953).

3. De, S. N., Ghose, M. L., and Sen, A., J. Pathol. Bacteriol. **79**, 373 (1960).

4. Leitch, G. J., and Burrows, W., J. Infec. Dis. **118**, 349 (1968).

5. Leitch, G. J., Iwert, M. E., and Burrows, W., J. Infec. Dis. **116**, 303 (1966).

6. Norris, H. T., Curran, P. F., and Schultz, S. G., J. Infec. Dis. **119**, 117 (1969).

7. Norris, H. T., and Majno, G., Amer. J. Pathol. **53**, 263 (1968).

8. Schafer, D. E., and Lewis, G. W., Proc. Cholera Res. Symp. (Honolulu). U.S. Pub. Health Serv. Publ. No. **1328**, 160 (1965).

9. Serebro, H., Hendrix, T, Iber, F., Royall, R., and McGonagle, T., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **27**, (2), 574 (1968).

10. Wangensteen, O. H., Buirge, R. E., Dennis, C., and Ritchie, W. P., Ann. Surg. **106**, 910 (1937).

Received Sept. 8, 1969. P.S.E.B.M., 1970, Vol. 134.