

Improved Protection Against Cholera in Adult Rabbits with a Combined Flagellar-Toxoid Vaccine

I. GARY RESNICK, CHARLES W. FORD, GREGORY M. SHACKLEFORD, AND L. JOE BERRY*

Department of Microbiology, The University of Texas, Austin, Texas 78712

Ligated ileal loops of adult rabbits were used to evaluate the prophylactic potential against cholera of a combined vaccine consisting of toxin-free crude flagella (CF) and glutaraldehyde-derived cholera toxoid (TV). The resulting fluid accumulation ratios were compared with those in rabbits immunized with saline (controls) and with CF and TV alone. Data for single vaccines confirmed the superior protection effect of CF over TV. In rabbits vaccinated with both CF and TV, maximal fluid accumulation ratios were not obtained with a challenge dose as high as 5×10^9 colony-forming units (CFU). Two rabbits similarly immunized failed to produce positive loops with challenges of 6.3×10^8 and 5×10^9 CFU, respectively. The vibriocidal titer of serum from rabbits immunized with a killed commercial vaccine in addition to those listed above was determined at intervals for a period of 164 days. No vibriocidal activity was detected in serum of control rabbits and of rabbits vaccinated with TV. Serum from animals given CF or commercial vaccine had similar vibriocidal titers even when the test bacteria were nonflagellated. Protection against challenge as evaluated by ileal loops did not, however, correlate with vibriocidal titer.

The pathogenesis of cholera is due primarily to the elaboration of a well-characterized toxin whose mode of action is known except for certain details (7, 10). Toxigenicity alone does not account completely for the development of symptoms in laboratory animal models, however, since cells lacking motility are dramatically less virulent than the motile wild types from which they are derived (9, 20, 21). In contrast to other diseases caused by toxigenic organisms, where toxoids serve as highly efficacious vaccines, cholera toxoid has not yielded the level of immunity that might be anticipated. Recent reports (17, 19) indicate that concurrent immunization with toxoid and somatic antigens provides a synergistic protective effect believed to be due to disruption of the pathogenic process at two distinct steps.

The role of flagella and motility in determining the virulence of *Vibrio cholerae* was first described by Guentzel and Berry (9). Although the exact point(s) at which motility is critical to successful infection is not known, the fact that motile cells are required has been established in infant mice and ligated ileal loops of adult rabbits (9, 21). The information obtained with infant mice prompted Eubanks et al. (6) to attempt, successfully, to passively protect these mice against challenge with live virulent *V. cholerae* by vaccinating females just before mating with a preparation composed of sheathed flagella and

vesicles (referred to as crude flagella or CF). A similar preparation was used by Yancey et al. (22) to protect adult rabbits against fluid accumulation in ligated ileal loops. The immunogen primarily responsible for this protection was shown to be associated with the flagellum. Whether the flagellar core or the sheath material (or both) was the active immunogen was not definitely determined. In all of these studies, however, the level of protection afforded by CF exceeded that obtained with toxin, toxoid, or killed whole-cell vaccines. These latter substances are effective, nevertheless, against challenge with live vibrio and culture supernatants, as evidenced by studies in infant rabbits and in rabbit ileal loop models (8, 10, 21). Because it seems realistic to assume that antitoxic immunity and antflagellar immunity are interrupting different steps in the pathogenic sequence of events, a vaccine combining these two substances might be expected to have an additive effect.

In this report, the efficacy of a combined CF-toxoid vaccine was examined in the adult rabbit ileal loop model. Vibriocidal levels stimulated by immunization of rabbits with a bivalent killed whole-cell vaccine, with toxoid, and with CF were determined over a 164-day period since some workers believe this indicates the immune status of a population (13, 14) and of individual animals (16). Mosley et al. (15), however, caution

that the correlation between vibriocidal levels and protection is applicable to group-specific and not type-specific immunity.

MATERIALS AND METHODS

Bacterial strain. The highly virulent strain of *V. cholerae* CA401 (classical Inaba) was used unless otherwise indicated. The nonmotile mutants derived from strain CA401, M-1 and M-4, were previously described by Guentzel and Berry (9). The cultures were maintained in a lyophilized state at 4°C and revived before use. Growth conditions and media for cultures of test organisms were as indicated below.

Rabbits. Adult female rabbits weighing 1 to 4 kg were obtained from local breeders.

Cholera toxin. The crude cholera toxin preparation used in these studies was provided by Carl Miller, National Institute of Allergy and Infectious Diseases (lot 001; Wyeth Laboratories).

Vaccines. A commercial cholera vaccine (CV; Lederle Laboratories) prepared from phenolized liquid medium cultures of *V. cholerae* strains Ogawa and Inaba was used. The CV preparation contained 4.0×10^9 cells of each serotype per ml.

An experimental glutaraldehyde-treated cholera toxoid prepared by Rappaport et al. (18) (TV; lot 20201, Wyeth) was provided by Carl Miller, National Institute of Allergy and Infectious Diseases. TV was rehydrated to a concentration of 200 µg/ml with a protamine sulfate-aluminum chloride diluent containing 3.75 mg of aluminum chloride, 0.5 mg of protamine sulfate, and 0.05 mg of thimerosal per ml.

CF was prepared by a modification of the method of Eubanks et al. (6). Briefly stated, CA401 cells from an 8-h brain heart infusion (BHI) agar slant were suspended in phosphate-buffered saline with 1% gelatin (PBSG; 0.1 M potassium phosphate, pH 7.2, 0.15 M NaCl, and 1% gelatin) and inoculated into a 1-liter flask containing 200 ml of BHI broth. After 14 to 18 h of incubation at 37°C on a rotary shaker, this culture was added to 5 liters of BHI broth in a 10-liter carboy. The batch culture was incubated at 37°C with aeration until an absorbance reading at a wavelength of 540 nm reached 0.33 as measured against a BHI broth blank (midexponential growth). The cells were then harvested ($6,000 \times g$ for 10 min) and suspended in 150 ml of phosphate buffer-calcium (PC; 0.1 M potassium phosphate and 1 mM CaCl₂, pH 7.2). Flagella were sheared from the cells in a Waring blender for 20 s. Cells and large debris were removed by centrifugation twice at $16,000 \times g$ for 30 min. Flagella and smaller debris were collected from the supernatant fluid by centrifugation at $85,000 \times g$ for 1 h. The pellet was resuspended overnight in PC buffer and submitted to three cycles of differential centrifugation. The final pellet was suspended in 5 ml of PC containing 5% Formalin, stored at 4°C, and used within 2 weeks. The protein concentrations of several CF preparations ranged from 0.6 to 0.85 mg/ml.

Toxin analyses. The presence of cholera toxin was confirmed by using the adult rabbit ligated ileal loop procedure (as detailed below). The adrenal cell assay of Donta et al. (5), sensitive to 5 µg of purified cholera toxin, was used when indicated.

Electron microscopy. The morphology of the particulate materials contained in CF preparations was observed after negative staining with uranyl acetate.

Protein estimation. Protein concentration was estimated by the procedure of Lowry et al. (12) against a bovine serum albumin standard.

Immunization. Rabbits were immunized by subcutaneous injection of the designated antigen(s) on days 0 and 7. The total dose to be delivered on a given day was divided in half, and each flank was injected. CF vaccine and TV were administered in 100-µg amounts unless otherwise stated. CV was given in high dose (0.5 ml, day 0; 1.0 ml, day 7) or low dose (25 µg, day 0; 50 µl, day 7). The high dose of CV represents the manufacturers' suggested vaccination for adult humans; the low dose represents a rabbit weight equivalent. The combined CF-TV vaccine was delivered the same as the others.

Adult rabbit ileal loop model. A modification of the method of De and Chatterje (4) was used. Rabbits were challenged between days 16 and 18 after being fasted for 48 h. The ileum was ligated into 10 segments, 8 cm long, with 2-cm spacers. *V. cholerae* for challenge were harvested in 5 ml of PBSG from 8-h BHI agar slants, washed, and resuspended in 5 ml of PBSG. The loop challenge dose was contained in a volume of 0.5 ml. Positive and negative loops injected with 500 µg of toxin and 0.5 ml of saline, respectively, were included in each rabbit. The amount of fluid accumulated in each was measured after approximately 18 h. The results are reported as fluid accumulation (FA) ratios (volume of fluid accumulated in milliliters per loop divided by 8 cm).

Vibriocidal assay. Vibriocidal titers were determined by the microtechnique of Benenson et al. (2). The antigen used was *V. cholerae* serotype Inaba CA401, except when the nonmotile mutants M-1 and M-4 were used as indicated.

RESULTS

The CF preparations used in this investigation were similar to those of Yancey et al. (22) with one major exception: transmission electron microscopic examination of CF revealed a large number of unsheathed core flagella with many small vesicles, whereas Yancey et al. (22) report a predominance of sheathed structures. This difference may be attributed to the harvest of cells at a later stage of development. The CF preparations contained less than 600 µg per mg of protein of cholera toxin as measured by the adrenal cell assay.

The adult rabbit ileal loop model was used to evaluate the efficacy of a combined vaccine for cholera prophylaxis. The FA ratios measured in loops from rabbits immunized with CF, TV, CF-TV, and saline appear in Fig. 1. The challenge-response curves generated for individual rabbits were linear in most cases. The moderate variation in results from rabbit to rabbit permits analysis by treatment groups. To facilitate this assessment, it was assumed that the FA response

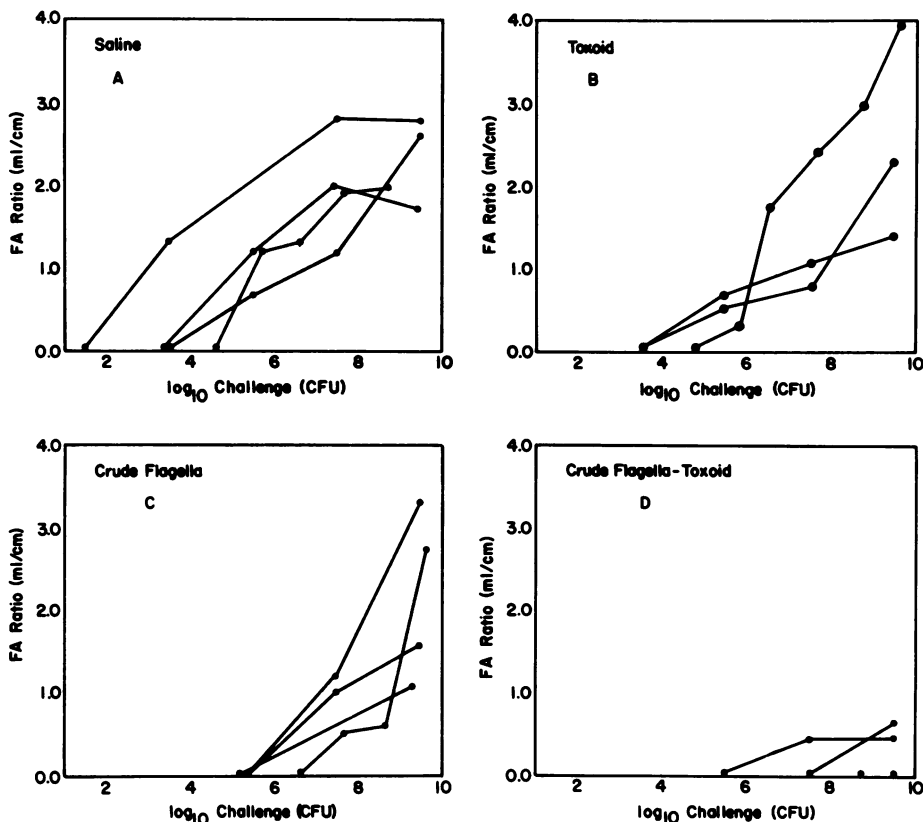


FIG. 1. FA response in ligated ileal loops of individual rabbits given various challenge doses of viable *V. cholerae* CA401. Rabbits were immunized on days 0 and 7 as follows: (A) saline (B) TV; (C) CF; (D) CF-TV. Challenge was carried out on days 16 and 18.

to the entire range of challenge doses could be expressed linearly. The dose-response plots, computed by least-squares analysis, are presented in Fig. 2. In all cases, vaccinated animals required larger numbers of vibrios to yield a positive loop. Animals immunized with TV, when compared with the saline-immunized controls, required approximately 100-fold more *V. cholerae* to produce a detectable fluid accumulation. CF-immunized animals, when compared with TV-immunized animals, required a further 100-fold increase in challenge dose to achieve a detectable fluid accumulation.

The CF-TV-vaccinated rabbits were most resistant to challenge by an amount not easily quantitated. In no case was an FA ratio of 1 reached, and in two rabbits a positive loop could not be produced with a challenge inoculum as large as 6.3×10^8 colony-forming units (CFU).

The level and duration of vibriocidal activity in rabbits vaccinated with CV, CF, TV, and saline over a 174-day period was investigated. The results obtained for CV and CF appear in

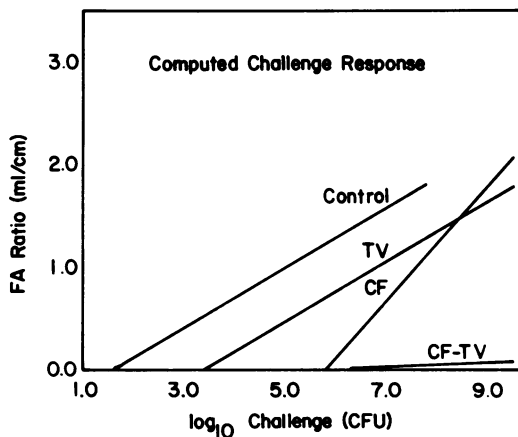


FIG. 2. Computed challenge response by least-squares analysis for *V. cholerae* CA401-challenged rabbits. The vaccine administered, number of rabbits, and *r* values for each treatment group were as follows: saline control (control), four rabbits, *r* = 0.9; TV, two rabbits, *r* = 0.95; CF, four rabbits, *r* = 0.8; CF-TV, four rabbits, *r* = 0.5.

Fig. 3 and 4. No vibriocidal activity was evident in animals that received TV or saline. The two doses of CV chosen represent the manufacturers' recommended human dose (high dose) and an inoculum adjusted to a rabbit's weight based on the human dose (low dose). The high dose of CV contained phenolized *V. cholerae*, 4×10^8 cells on day 0 and 2.4×10^8 on day 7. CF was administered in either 100-, 10-, or 1- μ g amounts on days 0 and 7. Peripheral blood was collected periodically, and vibriocidal levels were determined. The shapes of the curves generated were similar for treatments, and the levels observed after 130 days were alike. The initial period of response varied directly with the amount of an-

tigen administered. The vibriocidal levels in rabbits receiving 100 μ g of CF were similar to those seen with the high dose of CV. The titers obtained from animals immunized with the low dose of CV were much lower and declined sooner than those observed for animals inoculated with 10 and 1 μ g of CF. The response of animals to 1 and 10 μ g of CF was not significantly different. CF was effective in initiating substantial vibriocidal activity with a dose as low as 1 μ g.

The vibriocidal activity, assayed by using the nonmotile mutants M-1 and M-4 of the wild-type strain CA401, for CF (rabbits 500 and 501) and CV hyperimmune sera was determined (Fig. 5). The CV hyperimmune sera showed no sig-

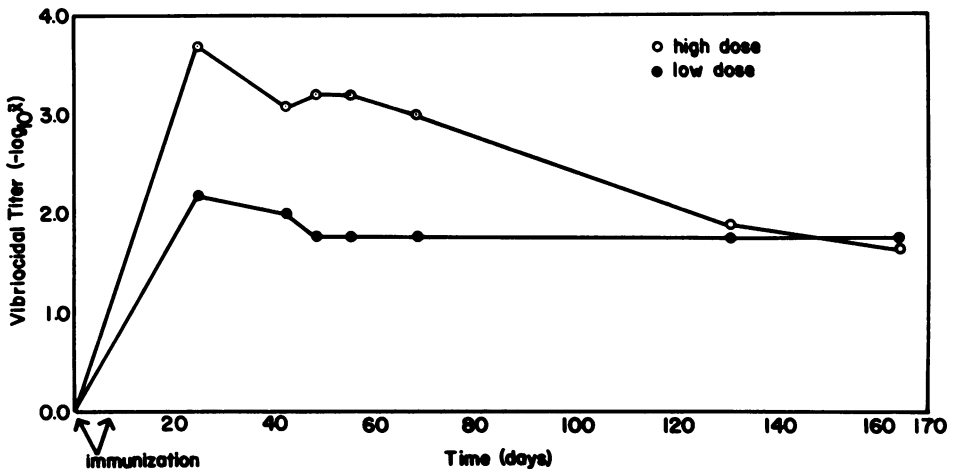


FIG. 3. Mean vibriocidal titers (three rabbits/treatment group) of rabbits immunized with phenol-killed bivalent whole-cell *V. cholerae* vaccine. Symbols: \circ , high dose; \bullet , low dose. Treatment groups received 4×10^9 and 1.2×10^8 cells on day 7, respectively.

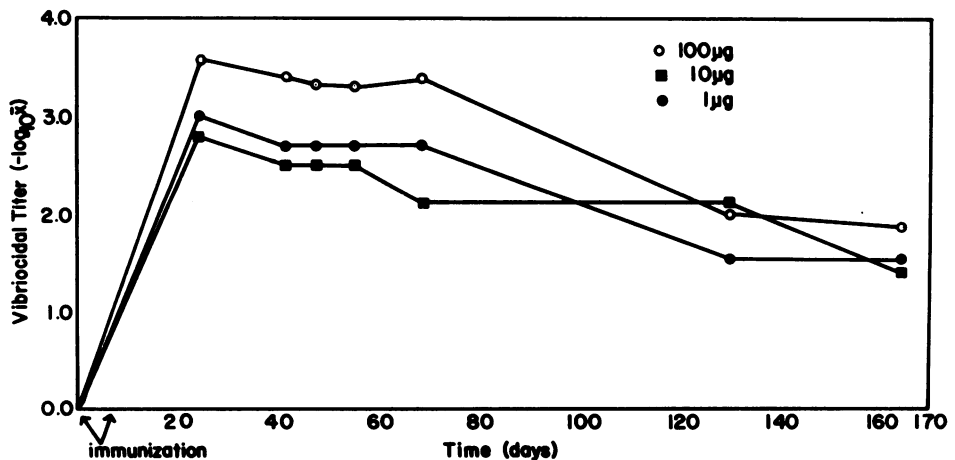


FIG. 4. Mean vibriocidal titers (three rabbits/treatment group) of rabbits immunized with crude flagella vaccine. *V. cholerae* CA401 wild type was the test antigen. Animals were immunized on days 0 and 7 as indicated: 100 μ g (\circ), 10 μ g (\blacksquare), 1 μ g (\bullet).

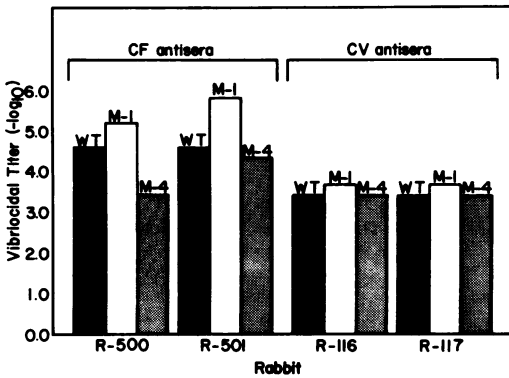


FIG. 5. Comparison of the vibriocidal activity in hyperimmune sera produced by immunization with CF and CV, as assayed with *V. cholerae* CA401 wild type (WT) and the nonmotile mutants M-1 and M-4 as antigens.

nificant difference in cidal activity directed against aflagellated mutants as compared with wild-type cells. However, CF antisera displayed a marked increase in cidal activity against M-1 and a decrease in activity against M-4.

DISCUSSION

The data obtained in this investigation support and expand the conclusions of Yancey et al. (22). They state that "the critical immunogenic component of CF appears to be a flagella-derived protein." These investigators base this statement upon three observations: (i) the protection conferred by CF immunization is superior to that of a commercial bivalent vaccine; (ii) the immunogenicity of CF was destroyed by heat treatment; and (iii) the adsorption of CF immune serum with aflagellated mutant vibrios did not diminish its ability to confer a high level of passive protection. The above three facts diminish the antigenic role of lipopolysaccharide in CF except as an adjuvant.

Rabbits immunized with CF require up to 10^4 more viable *V. cholerae* than saline control animals for initiation of fluid accumulation in a ligated ileal loop. When CF immunization is compared with TV immunization, 100 times as many cells are required for fluid accumulation. Simultaneous immunization with CF and TV resulted in rabbits that were highly refractory to live challenge. CF-TV-immunized rabbits were protected to the extent that a maximal fluid accumulation ratio was not reached with an injection of up to 5×10^9 CFU. Two of the CF-TV rabbits showed virtually no fluid accumulation with up to 6.3×10^8 and 5.0×10^9 CFU, respectively.

The components of a vaccine should be readily

available if the preparation is to enjoy wide usage. The finding that CF composed of bare core flagella, as opposed to sheathed structures, can confer protection is encouraging. The preparation of CF composed of flagellar cores is a relatively simple process, and the cores are stable. The absence of active toxin in CF preparations should eliminate undesirable side effects from vaccination and allow controlled addition of optimal amounts of toxoid.

The extreme protection conferred by CF-TV is not surprising. Flagella are T-cell-independent antigens capable of inducing antibody formation when injected in nanogram quantities (1, 3). Yang et al. (23) reported the production of antibody in rabbits immunized with purified flagellar cores of *V. cholerae* which reacted with intact flagella. The positive role of motility in cholera has been established. In theory, as it appears in practice, a flagellum-based vaccine should be protective. The prominent role of cholera toxin, and the ability of infected hosts to produce neutralizing antibodies against the enterotoxin, suggested the addition of toxoid of the CF vaccine. By interrupting two distinct but related steps in the infectious process, an increased protective effect was expected and observed. Whether the combined protection offered by CF-TV was additive or synergistic could not be determined with the ileal loop model.

The vibriocidal levels induced by CV and CF were observed over a 164-day period to determine the magnitude and duration of response. CF- and CV-immunized animals displayed comparable vibriocidal activities. The cidal activity of CF hyperimmune sera was not flagellum dependent. This is not unexpected considering the presence of vesicles in CF, which in light of these results are at least in part cell wall material. Although a direct relationship between vibriocidal level and protective state has been suggested (13, 14, 16), this does not seem to be the case. Rabbits protected with CV were markedly more susceptible to live challenge than CF-immunized animals (22), whereas the vibriocidal levels for these two groups, as reported here, are similar. Apparently the protective effect of CF is not dependent upon an increase in vibriocidal activity in the gut. The protection afforded by CF immunization could result from an interruption of the association of vibrio with the gut wall due to inhibition of bacterial motility. It appears that the protective humoral response to antigens of *V. cholerae* is complex, with flagellum-associated antigens playing an important role.

In conclusion, the continued evaluation of the CF-TV vaccine is justified. Determinations of the antigen(s) responsible for the protective ef-

fect of CF should be pursued in hopes of developing a suitable vaccine against *V. cholerae* and for further elucidation of the pathogenic process.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service grant AI-10466 from the National Institutes of Health.

We are grateful to Leodocia Pope for her assistance with the electron microscopic examination of the crude flagella.

LITERATURE CITED

1. Ada, G. L., G. J. V. Nossal, J. Pye, and A. Abbot. 1964. Antigens in immunity. I. Preparation and properties of flagellar antigens from *Salmonella adelaide*. *Aust. J. Exp. Biol. Med. Sci.* **42**:267-282.
2. Benenson, A. S., A. Saad, and W. H. Mosley. 1968. Serological studies in cholera. 2. The vibriocidal antibody response of cholera patients determined by a microtechnique. *Bull. W.H.O.* **38**:277-285.
3. Coutinho, A., and G. Moller. 1975. Thymus-independent B-cell induction and paralysis. *Adv. Immunol.* **21**:113-236.
4. De, S. N., and D. N. Chatterje. 1953. An experimental study of the mechanism of action of *Vibrio cholerae* on the intestinal mucous membrane. *J. Pathol. Bacteriol.* **79**:131-140.
5. Donta, S. T., M. King, and K. Sloper. 1973. Induction of steroidogenesis in tissue culture by cholera enterotoxin. *Nature (London) New Biol.* **243**:246-247.
6. Eubanks, E. R., M. N. Guentzel, and L. J. Berry. 1977. Evaluation of surface components of *Vibrio cholerae* as protective immunogens. *Infect. Immun.* **15**:533-588.
7. Finkelstein, R. A. 1975. Immunology of cholera. *Immunology* **69**:137-195.
8. Fujita, K., and R. A. Finkelstein. 1972. Antitoxic immunity in experimental cholera: comparison of immunity induced perorally and parenterally in mice. *J. Infect. Dis.* **125**:647-655.
9. Guentzel, M. N., and L. J. Berry. 1975. Motility as a virulence factor for *Vibrio cholerae*. *Infect. Immun.* **11**:890-897.
10. Holmgren, J., and A. Svennerholm. 1977. Mechanisms of disease and immunity in cholera: a review. *J. Infect. Dis.* **136**:s105-s107.
11. Holmgren, J., A. M. Svennerholm, O. Ouchterlony, A. Anderson, G. Wallerstrom, and U. Westerberg-Berndtsson. 1975. Antitoxic immunity in experimental cholera: protection and serum and local antibody responses in rabbits after enteral and parental immunization. *Infect. Immun.* **12**:1331-1340.
12. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1966. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
13. Mosley, W. H., A. S. Benenson, and R. Barui. 1968. A serological survey for cholera antibodies in rural east Pakistan. 1. The distribution of antibody in the control population of a cholera-vaccine field trial area and the relationship of antibody titre to the pattern of endemic cholera. *Bull. W.H.O.* **38**:327-334.
14. Mosley, W. H., A. S. Benenson, and R. Barui. 1968. A serological survey for cholera antibodies in rural east Pakistan. 2. A comparison of antibody titres in the immunized and control populations of a cholera-vaccine field trial area and the relation of antibody titre to cholera case rate. *Bull. W.H.O.* **38**:335-346.
15. Mosley, W. H., W. E. Woodward, K. M. A. Aziz, A. S. M. Mizanur Rahman, A. K. M. Alauddin Chowdhury, A. Ahmed, and J. C. Feeley. 1970. Epidemiology studies, vaccine trials, and immune response. *J. Infect. Dis.* **121**(Suppl):51-59.
16. Neoh, S. H., and D. Rowley. 1972. Protection of infant mice against cholera by antibodies to three antigens of *Vibrio cholerae*. *J. Infect. Dis.* **126**:41-49.
17. Peterson, J. 1979. Synergistic protection against experimental cholera by immunization with cholera toxoid and vaccine. *Infect. Immun.* **26**:528-533.
18. Rappaport, R. S., W. A. Pierzchala, G. Bonde, T. McCann, and B. A. Rubin. 1976. Development of a purified cholera toxoid. III. Refinements in purification of toxin and methods for the determination of residual somatic antigen. *Infect. Immun.* **14**:687-693.
19. Svennerholm, A., and J. Holmgren. 1976. Synergistic protective effect in rabbits of immunizations with *Vibrio cholerae* lipopolysaccharide and toxin/toxoid. *Infect. Immun.* **13**:735-740.
20. Williams, H. R., Jr., W. F. Verwey, G. D. Schrank, and E. K. Hurry. 1973. An *in vitro* antigen-antibody reaction in relation to an hypothesis of intestinal immunity to cholera, p. 161-173. *In Proceedings of the 9th Joint Cholera Research Conference.* U.S. Department of State, Washington, D.C.
21. Yancey, R. J., D. L. Willis, and L. J. Berry. 1978. Role of motility in experimental cholera in adult rabbits. *Infect. Immun.* **22**:387-392.
22. Yancey, R. J., D. L. Willis, and L. J. Berry. 1979. Flagella-induced immunity against experimental cholera in adult rabbits. *Infect. Immun.* **25**:220-228.
23. Yang, G. C. H., G. D. Schrank, and B. A. Freeman. 1976. Purification of flagella cores of *Vibrio cholerae*. *J. Bacteriol.* **129**:1121-1128.