*Proc. Natl. Acad. Sci. USA*
Vol. 76, No. 4, pp. 2052–2056, April 1979
Microbiology

# Selection and characteristics of a *Vibrio cholerae* mutant lacking the A (ADP-ribosylating) portion of the cholera enterotoxin

(cholera vaccine candidate/diarrhea/intestinal immunity/halo technique/choleragen)

TAKESHI HONDA* AND RICHARD A. FINKELSTEIN†

Department of Microbiology, University of Texas Southwestern Medical School, Dallas, Texas 75235

*Communicated by Maclyn McCarty, February 2, 1979*

ABSTRACT **After mutagenesis with nitrosoguanidine and selection by immuno-halo techniques, an avirulent mutant, designated Texas Star-SR, which produces no detectable A (active; ADP-ribosylating) region of the cholera enterotoxin (choleragen) but produces the B region (choleragenoid) in amounts similar to the hypertoxinogenic wild-type parent *Vibrio cholerae* (biotype El Tor serotype Ogawa), has been isolated. The mutant retains the colonizing ability, motility, prototrophy, and serologic characteristics of the parent. In relevant intestinal experimental models, it has been shown to be avirulent and to induce protection against challenge with virulent cholera vibrios. The mutant appears to be suitable for further evaluation in volunteers as a candidate living enteric vaccine against cholera and related enterotoxic enteropathies.**

Cholera is the prototype of an increasing number of newly recognized diarrheal diseases, enterotoxic enteropathies, which depend on soluble enterotoxins, some of which are related to the cholera enterotoxin (choleragen), both immunologically and by mode of action (1). Perhaps because of its intestinal locus, conventional cholera vaccines composed of killed bacteria, administered parenterally, are relatively ineffective in preventing cholera (2). However, the disease itself is an immunizing process (3). Further, a perorally administered hypotoxinogenic *Vibrio cholerae* mutant, M13 (4), was found to be avirulent for human beings and to induce substantial immunity against subsequent challenge with virulent cholera vibrios (5, 6). Because of its limited production of toxin antigen, M13 would offer little, if any, protection against other enterotoxic enteropathies. Because it also appeared to be unstable (5, 6), further evaluation of M13 as a vaccine was contra-indicated.

Choleragen is a polymeric protein consisting of noncovalently associated subunits (7), of which the A region is responsible for activation of adenylate cyclase and the B region (choleragenoid), which has been shown to be highly immunogenic, is responsible for binding of the holotoxin to host cell membrane receptors, thereby facilitating entry of the A region (8–11). Thus an $A^-B^+$ *V. cholerae* mutant could be predicted to be avirulent and capable of stimulating local immunity against cholera and the immunologically related enterotoxins that share B-related antigens. The potential value of such a mutant was postulated earlier (12). The isolation and characterization of such an $A^-B^+$ mutant, which appears to be stable, is described in this report.

## METHODS

***V. cholerae* Strains.** Two hypertoxinogenic strains of *V. cholerae* were used. The first, *V. cholerae* 569B, a classical vibrio of the Inaba serotype, has been widely used by us and by others for production of choleragen and in genetic studies (4, 13–16). The other was 3083, an El Tor biotype Ogawa serotype, isolated by one of us (R.A.F.) from a patient in Vietnam in 1964 (17), which has been shown to be highly adherent in infant rabbits (18). All strains and mutants were maintained by lyophilization and by freezing (−70°C) in trypticase soy broth containing 20% glycerol. Mutagenesis was essentially as described (4, 13).

**Halo Technique.** To demonstrate halos of antigen–antibody precipitate around surface colonies, larger amounts of antisera are required than were used previously (4) to demonstrate halos around subsurface colonies. Anticholera A and anticholera B were produced in goats immunized with subunits derived from pure choleragen subjected to gel filtration under dissociating conditions (19) as described (20). These sera were added separately, at concentrations of 10% and 2.5%, respectively, to molten meat extract agar at 45–50°C. Mutagenized suspensions of bacteria were diluted in sterile 1% peptone saline to provide approximately 100–300 colonies per 90-mm petri dish containing approximately 15 ml of the serum agar. After surface inoculation, plates were incubated at 30°C for 24–48 hr. Colonies that elaborated substantial amounts of specific antigen were differentiated by halos of immune precipitate surrounding the colonies. In some instances, when smaller amounts of antigen were produced, the immune precipitate, if detectable, was revealed underneath the colony after the bacterial growth was scraped away.

**Detection and Quantitation of Antigens.** Presence or absence of antigen was determined by Ouchterlony tests using culture supernatants concentrated by ultrafiltration on PM10 membranes (Amicon, Lexington, MA) (20). Antigen was quantified in parallel with purified choleragen by radial immunodiffusion by a modified Mancini test (12) with equine anticholeragenoid (21). Tests for biologic activity in Y-1 adrenal cells were essentially as described (22), the end point being the final dilution or smallest amount of sample causing rounding of more than 50% of the cells.

**Animal Studies.** Virulence and ability to colonize were evaluated in infant rabbits, 7–10 days old, inoculated intraintestinally with 0.5 ml of appropriately diluted bacterial suspensions. The choleragenic score was determined as described (12). Rabbit ileal loop tests were performed essentially according to modification of De's technique (23). Rabbits were killed at 18–20 hr, at which time the ratio of fluid to loop length (g/cm) was determined, and portions of the small bowel were weighed and homogenized to determine the viable adherent *V. cholerae* by dilution plating. The rabbit skin tests were essentially as described by Craig (24). Virulence, colonizing ability, and ability to stimulate immunity were evaluated in locally obtained chinchillas, 1–2 years old, by the techniques of Blachman *et al.* (25, 26). Assays for activation of adenylate cyclase in pigeon erythrocyte lysates (27) were performed on

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

* On leave from Research Institute for Microbial Diseases, Osaka University, Osaka 565, Japan.
† To whom reprint requests should be addressed.

Table 1. Tests of virulence and stability of Texas Star mutants in infant rabbits

| Inoculum | No. of exps. | Choleragenic score* ≤1 | >1≤2 | >2≤6 | >6–10 | Total |
|---|---|---|---|---|---|---|
| Texas Star† | 9 | 117 | 14 | 8 | 0 | 139 |
| Texas Star-SR† | 7 | 54 | 2 | 0 | 0 | 56 |
| Total | 16 | 171 | 16 | 8 | 0 | 195 |
| Wild-type parent‡ | 5 | 2 | 3 | 3 | 10 | 18 |
| Wild-type parent + Texas Star-SR§ | 5 | 11 | 6 | 5 | 20 | 42 |
| Total | 10 | 13 | 9 | 8 | 30 | 60 |

* As described (12). Values are number of animals with indicated choleragenic score.
† Inocula $10^8$–$10^{10}$ viable vibrios.
‡ Inocula 140–320 viable vibrios. This is the approximate $LD_{50}$; higher inocula invariably caused lethal diarrhea (choleragenic score = 10) within 24 hr.
§ Inocula $7.5 \times 10^2$–$1.8 \times 10^{10}$ (34 of 42 animals received Texas Star-SR in numbers ranging from $10^7$ to $1.8 \times 10^{10}$).

coded preparations by D. M. Gill, to whom we are indebted for those results.

**Purification of Antigen.** Antigen was isolated from fermentor-grown cultures after adsorption and elution from $Al(OH)_3$ as described (28), with the exception that cultures of mutant strains were incubated for 48 hr. Disc and sodium dodecyl sulfate gel electrophoresis were essentially as described (7). Protein was determined by the technique of Bradford (29).

## RESULTS

**Isolation of $A^-B^+$ Mutant from *V. cholerae* 3083.** Initial efforts directed toward isolation of an $A^-B^+$ mutant from strain 569B, during which more than 60,000 mutagenized colonies were examined, were unsuccessful. Many were apparently hypotoxinogenic, like M13, and a smaller number appeared to be $A^+B^-$. Some putative $A^-B^+$ mutants either failed to colonize or caused overt diarrhea (reverted) in infant rabbits.

In contrast, after mutagenesis of strain 3083 with nitrosoguanidine, which killed approximately 98.7% of the vibrios, screening of approximately 3000 colonies yielded 8 colonies that failed to produce halos on anticholera A agar. Of these, five produced halos on anticholera B medium. These five putative $A^-B^+$ mutants were further evaluated in Ouchterlony tests with 20 times concentrates of culture supernatant, and three were found to produce some A antigen when 20 times concentrated culture supernatants were tested against anti-A serum in Ouchterlony tests. Two gave equivocal reactions.

Table 2. Comparison of activities of preparations from *V. cholerae* strains

| Strain or preparation* | Assay system: Y-1 cells† | Rabbit skin‡ | Pigeon RBC lysate§ | Pigeon RBC lysate¶ | Mancini‖ |
|---|---|---|---|---|---|
| Texas Star-SR | | | | | |
| 1 | $5^{-3}$ | 0 | $<10^{-6}$ | <0.2 pg | 114 |
| 2 | $5^{-2}$ | 0 | $<10^{-6}$ | <0.2 pg | 85 |
| 3 | $5^{-2}$ | 0 | $<10^{-6}$ | <0.2 pg | 98 |
| Wild-type parent | $5^{-9}$ | $10^{-6}$ | 20 | 7 μg | 112 |
| 569B | $5^{-8}$ | $10^{-7}$ | 1 | 0.3 μg | 91 |
| M13 | $5^{-4}$ | $10^{-3}$ | $2 \times 10^{-4}$ | 70 pg | <10 |
| Syncase medium | $5^{-2}$ | $10^{-2}$ | $<10^{-6}$ | <0.2 pg | <10 |
| Choleragen (100 μg/ml) | $5^{-9}$ | $10^{-7}$ | ND | ND | 100 |

* Twenty-five-fold concentrates (on PM-10 membranes) of Syncase medium culture supernatants.
† Highest dilution of preparation that caused positive reaction (≥50% rounding of adrenal cells) when 25 μl was added to 225 μl in the tissue culture well.
‡ Highest dilution of preparation that caused typical reaction (>4 mm diameter of bluing); 0 = no bluing detected although some edema was observed at $\leq 10^{-2}$.
§ Results from M. Gill. Potency relative to preparation from 569B set at 1.0. RBC, red blood cell; ND, not determined.
¶ Estimate of cholera toxin per ml of culture filtrate, M. Gill (personal communication).
‖ Microgram of cholera toxin antigen per ml of 25 times concentrate in comparison with standard curve of purified choleragen. <10 = undetectable.

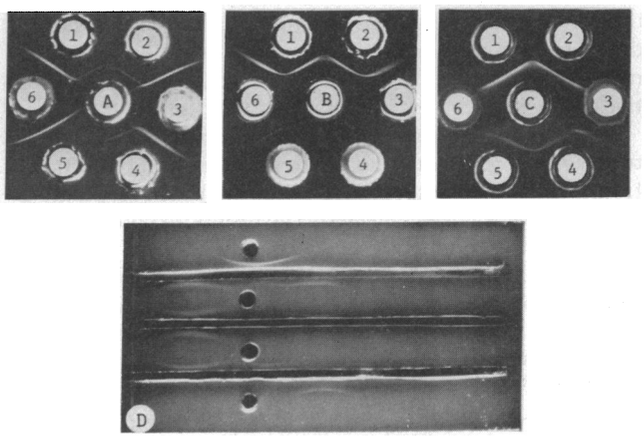


FIG. 1. Immunologic reactions of preparations from Texas Star-SR. (*A*) Goat anticholera A (center well); 1, choleragen (5 μg); 2, supernate of wild-type parent (25 times concentrated, 50 μl); 3, goat anticholera B; 4, choleragen; 5, supernate of Texas Star-SR (25 times concentrated, 50 μl) (note absence of specific precipitation with goat anticholera A); 6, goat anticholera B. (*B*) Supernate of Texas Star-SR (center well); 1, goat anticholera B; 2, goat anticholera B; 3, purified choleragenoid (5 μg); 4, goat anticholera A; 5, goat anticholera A; 6, choleragen (5 μg). [Precipitation between 4 and 5 and center well is a nonspecific reaction, found also in base-line sera, with a factor in crude culture supernatants, which crosses (i.e., is unrelated to) the anti-A–choleragen band (wells 5 and 6).] (*C*) Choleragen (center well); 1, goat anticholera B; 2, goat anticholera B; 3, lysate of cells from 25 ml of wild-type parent culture suspended in 2 ml (50 μl); 4, goat anticholera A; 5, goat anticholera A; 6, lysate of cells from 25 ml of Texas Star-SR culture suspended in 2 ml (50 μl). (*D*) Immunoelectrophoresis of Texas Star-SR supernatant (anode to right). Sample wells, top to bottom: choleragenoid (5 μg); Texas Star-SR 25 times concentrated supernatant plus added choleragen; Texas Star-SR 25 times concentrated supernatant; choleragen (5 μg). Upper and lower troughs contain goat anticholera B. Center trough, goat anticholera A. (Precipitin arcs left of sample wells are nonspecific reactions.)

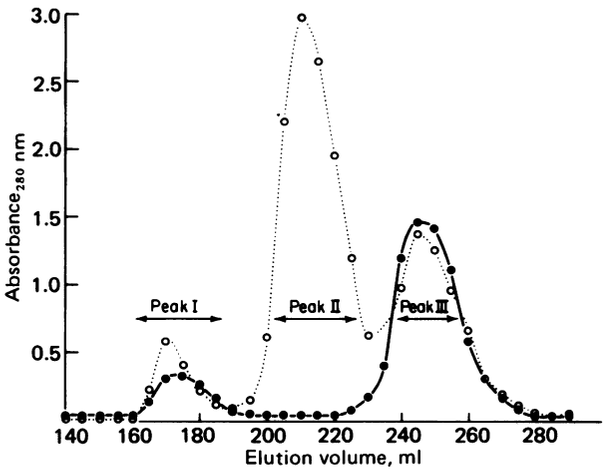


FIG. 2. Gel filtration of concentrated $Al(OH)_3$ eluate of culture supernatants on Sephadex G-75 (2.5 × 90 cm). Note the absence of the second major (choleragen) peak in the preparation from Texas Star-SR. Half the volume of culture supernatant was used with the Texas Star-SR preparation; thus the amount of choleragenoid it elaborated was equivalent to that elaborated by the wild-type parent, which appears in both the choleragen and choleragenoid peaks (peaks II and III). Peak I (in both instances) contains irrelevant large molecular weight products, including, largely, lipopolysaccharide. o · · · o, 3083; ● — ●, Texas Star-SR.

One of the latter mutants was subjected to a second-step mutagenesis, and approximately 1% of the survivors (24/2041) appeared to be $A^-$ on initial screening.‡ Of these, five were found to be $B^+$, and of these, only one gave no evidence of A antigen production in Ouchterlony tests. Parallel assays revealed that 23 of the 24 putative $A^-$ second-step mutants had varying degrees of activity in Y-1 adrenal cells: only the A antigen-negative strain, hereafter designated "Texas Star," had essentially no activity.

After nine serial passages in infant rabbits (Table 1), to introduce another genetic marker to facilitate its differentiation from wild type, a spontaneous streptomycin-resistant (20 μg/ml) clone was then selected. The results to follow were derived with this mutant, designated Texas Star-SR.

**Tests on Culture Supernatants of Texas Star-SR.** Results of tests of supernatants from Texas Star-SR are summarized in Table 2 in comparison with wild-type parent, strain 569B, and M13, the previously described (4) hypotoxinogenic mutant. Although amounts of B-region antigen detectable in Mancini tests were similar to those of wild type, no toxin-like activity could be detected in Y-1 adrenal cells, rabbit skin tests, or pigeon erythrocyte lysates.

In Ouchterlony tests (Fig. 1 *A* and *B*), no evidence of A antigen could be detected in concentrated crude culture supernatants of Texas Star-SR, whereas A reactivity, merging with the B band, was clearly evident with purified choleragen and with an equivalent concentrated culture supernatant from the wild-type parent. Likewise, in immunoelectrophoresis, no choleragen precipitin arc could be demonstrated (Fig. 1*D*) in supernatant from Texas Star although precipitation was evident in the choleragenoid region. In contrast to the wild-type parent or strain 569B, polyacrylamide gel electrophoresis of supernatants of Texas Star-SR revealed the presence of a prominent stained band in the choleragenoid region but none in the choleragen region (results not shown).

‡ It is not clear why the putative $A^-B^+$ mutant isolated after the first-step mutagenesis gave such a high proportion of $A^+$ colonies after the second-step mutagenesis, higher than one would expect from back mutation to $A^+$ by the mutagen used (unless $A^+$ has "survival" advantage), but it should be mentioned that the criteria of A negativity were more strict in the second mutagenesis; i.e., in each instance, the colonies were scraped to determine whether any precipitate could be seen under the colonies. Thus, the first mutant may have produced slight amounts of A, not detectable as a halo around the colonies in the first assay and barely detectable in the Ouchterlony test.

Table 3. Neutralization of adrenal cell activity of choleragen and *E. coli* LT by antiserum against purified mutant protein from Texas Star-SR

| Antiserum | Toxin preparation* Choleragen | LT |
|---|---|---|
| Anti-Texas Star-SR mutant protein | 5,120 | 1280 |
| Goat anticholera B | 10,240 | 5120 |

Values are reciprocals of highest serum dilution showing complete neutralization of biological activity. Base-line sera were inactive at 1:2.

* Used at a level of approximately 8 adrenal cell rounding doses. Heat-labile enterotoxin (LT) was from a cell-free supernatant (21).

With lysed cells, no toxin-related antigens were demonstrable in freeze-thaw lysates of Texas Star-SR (Fig. 1*C*), whereas A antigen (but not B) was readily demonstrable in the wild type.

**Purification of Texas Star-SR Toxin-Related Protein.** The choleragenoid-related protein was purified from fermentor-grown cultures of Texas Star-SR by the same procedures we have used previously for isolation of choleragen and choleragenoid from *V. cholerae* 569B (28, 30). Fractionation of Texas Star-SR culture supernatants on Sephadex G-75 revealed a major choleragenoid peak (Fig. 2, peak III) that was equivalent to that of twice the volume of wild-type culture supernatants. Significantly, the choleragen-containing peak (Fig. 2, peak II), most prominent in wild-type culture supernatants, was absent. During subsequent purification steps (on Agarose Bio-Gel A5m, Sephadex G-75, and polyacrylamide P-150), the Texas Star-SR protein behaved identically to choleragenoid (data not shown). Likewise, in sodium dodecyl sulfate gel electrophoresis, unheated as well as heated purified protein preparations migrated identically to choleragenoid. Antiserum raised in rabbits against the purified Texas Star-SR protein gave only a single Ouchterlony precipitin band when tested against concentrated culture supernatant. This precipitin band and the bands produced with purified Texas Star-SR protein, choleragen, and choleragenoid all gave reactions of identity (data not shown). The antiserum neutralized the activity, in adrenal cells, of choleragen and heat-labile enterotoxin (LT) from *Escherichia coli* (20) to high and nearly equivalent titers (Table 3).

**Virulence Tests of Texas Star and Texas Star-SR Mutants.** The virulence of the Texas Star mutants was evaluated by a total of 16 serial passages of $10^8$–$10^{10}$ living vibrios in a total of 195 infant rabbits (Table 1). For the serial passages, the inocula were composed of mixtures of the progeny of multiple colonies isolated from the baby rabbits killed at 48 hr. During early passages, some evidence of fluid accumulation (choleragenic score >2<6) was observed in a few animals, but this was in marked contrast to the results with smaller inocula of the wild type, for which an $LD_{50}$ was approximately $10^2$ viable organisms (Table 1). Smaller inocula ($10^2$) of the Texas Star and Texas Star-SR mutants multiplied in the baby rabbits to levels of approximately $10^7$ in small intestinal tissue without causing any symptomatology. Scanning electron microscopy (18) revealed the mutant vibrios with typical morphology adhering to the epithelium of the small bowel in the baby rabbits.

Although the Texas Star-SR mutant appeared to be stably

Table 4. Adult rabbit ileal loop responses to Texas Star-SR and wild-type parent

| Rabbit | Wild type | | | | Texas Star-SR | | | |
|---|---|---|---|---|---|---|---|---|
| | $10^{7}$* | $10^{5}$ | $10^{3}$ | $10^{1}$ | $10^{9}$ | $10^{7}$ | $10^{5}$ | $10^{3}$ |
| 1 | 1.5 | 0.5 | 0 ($1.5 \times 10^{8}$) | 0 | 0 | 0 | 0 | 0 ($9.8 \times 10^{6}$) |
| 2 | 1.2 | 1.1 | 1.1 ($1.1 \times 10^{9}$) | 1.1 | 0.3 | 0 | 0.4 | 0 ($1.1 \times 10^{6}$) |
| 3 | 1.6 | 1.5 | 1.3 ($8.3 \times 10^{8}$) | 0 | 0.1 | 0.2 | 0 | 0 ($2.6 \times 10^{7}$) |
| 4 | 1.5 | 1.2 | 0.3 ($4.4 \times 10^{8}$) | 0 | 0 | 0 | 0 | 0 ($1.7 \times 10^{8}$) |
| 5 | 1.5 | 1.1 | 0.7 ($7.0 \times 10^{8}$) | 0 | 0.1 | 0.3 | 0 | 0 ($1.2 \times 10^{8}$) |
| Mean | 1.5 | 1.1 | 0.7 | 0.2 | 0.1 | 0.1 | 0.1 | 0 |
| 6† | 0.4 | 0.3 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7† | 0.5 | 1.1 | 0.2 | 0 | 0 | 0 | 0 | 0 |
| Mean† | 0.5 | 0.7 | 0.1 | 0 | 0 | 0 | 0 | 0 |

Values given are fluid/length ratios (g/cm). Values in parentheses are the no. of viable cells recovered from the loop per g of loop tissue.

* Number of viable cells inoculated.

† Rabbits 6 and 7 were older rabbits and less responsive.

avirulent in infant rabbits and none of more than 800 colonies isolated from infant rabbits during these experiments were found to be toxigenic for adrenal cells, it is possible that a "revertant" to virulence or toxinogenicity could not express itself in the presence of overwhelming populations of Texas Star-SR. To evaluate this possibility, we mixed small inocula of wild type with large inocula of Texas Star-SR and inoculated them intraintestinally in baby rabbits. The results (Table 1) were identical to those obtained with the wild-type inoculum alone. Thus we conclude that had a revertant occurred, it could have expressed itself.

Texas Star-SR was also found to be avirulent in adult rabbit ileal loops, although it multiplied from small inocula to levels comparable to those of the wild type (Table 4).

**Induction by Texas Star-SR of Protective Immunity against Virulent *V. cholerae* in Chinchillas.** After preliminary tests revealed that Texas Star-SR ($8 \times 10^{9}$), inoculated intraintestinally, did not elicit fluid accumulation in chinchillas whereas the parent wild-type strain ($6.8 \times 10^{8}$) did, the ability of the mutant to stimulate resistance to subsequent challenge with wild-type was evaluated. The results (Fig. 3) indicated that prior inoculation with Texas Star-SR induced significant resistance to challenge with virulent *V. cholerae*. The immunized animals were protected against both colonization and fluid production by the wild type.

## DISCUSSION

Using immunologic screening techniques after mutagenesis with nitrosoguanidine, we have isolated a mutant strain of *V. cholerae* biotype El Tor serotype Ogawa that lacks the ability to produce a functional or antigenic equivalent of the A (active, ADP-ribosylating) region of the cholera enterotoxin (choleragen) but that produces the B (binding or choleragenoid) region in amounts similar to its wild-type parent. The mutant protein appears to be identical with authentic choleragenoid in all tests performed, including its ability to precipitate with $G_{M1}$ ganglioside (Supelco, Inc., Bellefonte, PA) in Ouchterlony tests (data not shown). Although, like its parent and other freshly

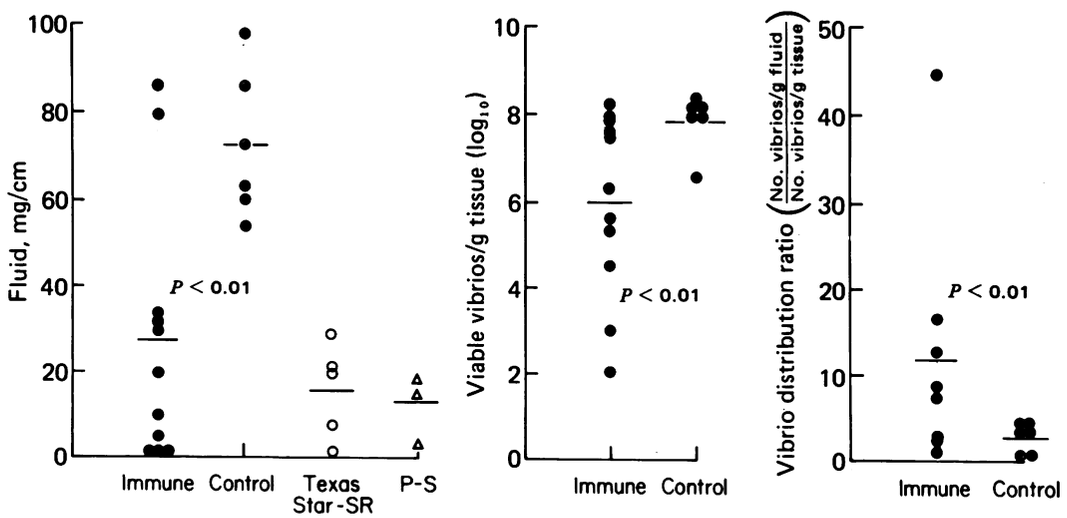


FIG. 3. Placebo-treated [inoculated intraintestinally with 1% peptone/saline (P-S)] and immunized (inoculated intraintestinally with $4.6 \times 10^{9}$ viable Texas Star-SR) chinchillas were challenged, intraintestinally, with $6.8 \times 10^{8}$ viable wild-type parent vibrios 20 days after inoculation. In the control group, one animal was dead and two were moribund 24 hr after challenge, whereas in the immunized animals, one animal was moribund and the others were outwardly healthy. All surviving animals were killed at that time. Additional controls (*Left*) included unimmunized animals challenged with $8 \times 10^{9}$ viable Texas Star-SR or with P-S.

isolated cholera vibrios (31), the mutant is prototrophic, it grows more slowly than its parent in defined and complex media. This raises interesting questions, to be resolved by further study, about the role of the A protein in the physiology of the cholera vibrios themselves. Previously, it had been presumed that the cholera enterotoxin serves to aid the dissemination of cholera vibrios in nature by causing diarrhea in their sole host, human beings and thus confers survival advantage (32). However, because of the known propensity of nitrosoguanidine to cause multiple genetic alterations, it is possible that a lesion other than the loss of the A-region protein is responsible for its slow growth. Nevertheless, the mutant retains the ability to colonize from small inocula in experimental animal models and it retains the motility and the somatic antigenic character of its parent (as demonstrated in agglutination tests with group- and type-specific antisera; data not shown).

The present study establishes that Texas Star-SR induces effective immunity to experimental cholera in a relevant intestinal model system although the mechanism(s) remain to be determined. If, as we suspect, there is an antitoxic component due to stimulation of local immune mechanisms by choleragenoid elaborated by the mutant, then it is not unlikely that the mutant, used as a living enteric vaccine, may offer some degree of protection against those enterotoxic enteropathies—far more important worldwide than cholera as causes of morbidity and mortality—which depend on enterotoxins that are immunologically related to the cholera enterotoxin through the B (choleragenoid) region. That there is also a specific antivibrio component may be inferred from the effects noted on colonization in immunized animals, which resemble those reported earlier by Freter (33). The reported (34) synergy between the antibacterial and antitoxic components of cholera immunity, the fact that the disease itself is an effective immunizing process, and the observation that hypotoxinogenic M13 induced protection in volunteers which was not quite as solid as that of the disease itself, all suggest that Texas Star-SR may be expected to harmlessly simulate infection with virulent vibrios and induce substantial immunity against cholera. It is hoped that studies in volunteers will support these contentions.

**Note Added in Proof.** The Texas Star-SR mutant was given to Charlotte D. Parker (University of Texas, Austin), who independently passed the strain sequentially, using both direct gut-to-gut passages and vibrios cultured after each passage, in parallel, 10 times in groups of five infant mice inoculated orally with approximately $10^7$ viable vibrios. In each of the passages, the fluid accumulation ratios were within normal limits (ref. 35; C. D. Parker, personal communication).

We thank D. Michael Gill for providing the pigeon erythrocyte lysate data and David A. Hart and Leon Eidels for helpful suggestions. This study was supported in part by Public Health Service Grant AI-08877 (under the United States–Japan Cooperative Medical Science Program) from the National Institute of Allergy and Infectious Diseases.

1. Finkelstein, R. A. (1976) in *Mechanisms in Bacterial Toxinology*, ed. Bernheimer, A. (Wiley, New York), pp. 53–84.
2. Finkelstein, R. A. (1975) *Curr. Top. Microbiol. Immunol.* **69,** 137–196.
3. Cash, R. A., Music, S. I., Libonati, J. P., Craig, J. P., Pierce, N. F. & Hornick, R. B. (1974) *J. Infect. Dis.* **130,** 325–333.
4. Finkelstein, R. A., Vasil, M. L. & Holmes, R. K. (1974) *J. Infect. Dis.* **129,** 117–123.
5. Woodward, W. E., Gilman, R. H., Hornick, R. B., Libonati, J. P. & Cash, R. A. (1975) in *Proceedings of the Eleventh Joint Conference on Cholera, New Orleans* (U.S. Public Health Service, National Institutes of Health, Washington, DC), pp. 330–335.
6. Woodward, W. E., Gilman, R. H., Hornick, R. B., Libonati, J. P. & Cash, R. A. (1976) in *Developments in Biological Standardization*, eds. Regamey, R. H., Hennessen, W., Hulse, E. C. & Perkins, F. T. (Karger, Basel), Vol. 33, pp. 108–112.
7. Finkelstein, R. A., Boesman, M., Neoh, S. H., LaRue, M. K. & Delaney, R. (1974) *J. Immunol.* **113,** 145–150.
8. Gill, D. M. (1977) in *Advances in Cyclic Nucleotide Research*, eds. Greengard, P. & Robison, G. A. (Raven, New York), Vol. 8, pp. 85–118.
9. Gill, D. M. & Meren, R. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 3050–3054.
10. Gill, D. M. (1978) in *Bacterial Toxins and Cell Membranes*, eds. Jeljaszewicz, J. & Wadstrom, T. (Academic, London), pp. 291–332.
11. Knoop, F. C. (1978) *Can. J. Microbiol.* **24,** 915–921.
12. Finkelstein, R. A. & LoSpalluto, J. J. (1969) *J. Exp. Med.* **130,** 185–202.
13. Holmes, R. K., Vasil, M. L. & Finkelstein, R. A. (1975) *J. Clin. Invest.* **55,** 551–560.
14. Vasil, M. L., Holmes, R. K. & Finkelstein, R. A. (1976) *Infect. Immun.* **9,** 195–197.
15. Ruch, F. E., Jr., Murphy, J. R., Graf, L. H. & Field, M. (1978) *J. Infect. Dis.* **137,** 747–755.
16. Mekalanos, J. J., Collier, R. J. & Romig, W. R. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 941–945.
17. Finkelstein, R. A., Sobocinski, P. Z., Atthasampunna, P. & Charunmethee, P. (1966) *J. Immunol.* **97,** 25–33.
18. Nelson, E. T., Clements, J. D. & Finkelstein, R. A. (1976) *Infect. Immun.* **14,** 527–547.
19. Klapper, D. G., Finkelstein, R. A. & Capra, J. D. (1976) *Immunochemistry* **13,** 605–611.
20. Cements, J. D. & Finkelstein, R. A. (1978) *Infect. Immun.* **21,** 1036–1039.
21. Finkelstein, R. A. (1970) *Infect. Immun.* **2,** 691–697.
22. Sack, D. A. & Sack, R. B. (1975) *Infect. Immun.* **11,** 334–336.
23. Kasai, G. J. & Burrows, W. (1966) *J. Infect. Dis.* **116,** 606–614.
24. Craig, J. P. (1971) in *Microbial Toxins*, eds. Kadis, S., Montie, T. C. & Ajl, S. J. (Academic, New York), Vol. IIA, pp. 189–254.
25. Blachman, U., Goss, S. J. & Pickett, M. J. (1974) *J. Infect. Dis.* **129,** 376–384.
26. Blachman, U., Graboff, S. R., Haag, G. E., Gottfeld, E. & Pickett, M. J. (1974) *Infect. Immun.* **10,** 1098–1104.
27. Gill, D. M. & King, C. A. (1975) *J. Biol. Chem.* **250,** 6424–6432.
28. Finkelstein, R. A., Fujita, K. & LoSpalluto, J. J. (1971) *J. Immunol.* **107,** 1043–1051.
29. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
30. Finkelstein, R. A. & LoSpalluto, J. J. (1970) *J. Infect. Dis., Suppl.,* **121,** S63–S72.
31. Finkelstein, R. A. (1955) Dissertation (Univ. of Texas, Austin, TX).
32. Finkelstein, R. A. (1973) *CRC Crit. Rev. Microbiol.* **2,** 553–623.
33. Freter, R. (1972) *Infect. Immun.* **6,** 134–141.
34. Svennerholm, A.-M. & Holmgren, J. (1976) *Infect. Immun.* **13,** 735–740.
35. Baselski, V. S. Medina, R. A. & Parker, C. D. (1978) *Infect. Immun.* **22,** 435–440.