Proc. Natl. Acad. Sci. USA
Vol. 75, No. 2, pp. 941–945, February 1978
Genetics

Affinity filters, a new approach to the isolation of *tox* mutants of *Vibrio cholerae*

(cholera toxin/gangliosides/immunoassay/hypertoxinogenic mutants)

JOHN J. MEKALANOS*, R. J. COLLIER*†, AND W. R. ROMIG*†

* Department of Bacteriology and the † Molecular Biology Institute, University of California, Los Angeles, California 90024

Communicated by A. M. Pappenheimer, Jr., November 21, 1977

ABSTRACT **We have devised a novel plate assay method for detecting mutants of *Vibrio cholerae* altered in the production of cholera toxin (*tox* mutants). Colonies replicated from a master plate are grown on the surface of a cellulose filter disc to which ganglioside–albumin conjugates have been attached. Toxin secreted by the colonies is tightly bound to the ganglioside filters. After removal of the cells by washing, the bound toxin may be detected by treating the filters with radioactively labeled antibodies against either whole toxin or one of its constituent polypeptide chains, followed by autoradiography. Colonies producing significantly greater or lesser amounts of toxin than the parental type are easily recognized and can be shown in liquid culture to have the corresponding hypertoxinogenic or hypotoxinogenic phenotype. This method, termed "the ganglioside filter assay," is applicable to screening large numbers of colonies and should facilitate isolation of various specific classes of mutants in cholera toxin production. In modified form the method will be applicable to various systems in which mutants of secreted proteins are sought.**

Isolation of bacterial mutants defective in the production of protein toxins (*tox* mutants) has proven to be laborious, primarily because toxin production usually confers no known selective advantage for bacterial growth or survival *in vitro*. Generally, large numbers of clones from mutagenized populations of bacteria must be tested for production of toxic material or specific antigen. Screening for these properties has been greatly facilitated in some instances by assay methods performed either directly on colonies growing in agar [e.g., immunoprecipitin assays (1)] or in microtiter plates [e.g., toxicity or immunological assays (2)] but there is a great need for improved methods in this area.

Cholera toxin (choleragen), a multimeric protein of molecular weight about 84,000, consists of a B protomer containing five noncovalently linked chains (molecular weight, 11,590) perhaps in the form of a ring, and an A protomer containing two peptides, A_1 (molecular weight, 24,000) and A_2 (molecular weight, 9700) linked by a single disulfide bridge (3, 4). The B protomer has been shown to have a high affinity for the oligosaccharide moiety of ganglioside GM_1, and there is evidence to indicate that this ganglioside may serve as the cell surface receptor for cholera toxin (5). Free B protomer, which has been termed "choleragenoid," is often found in culture fluids in addition to whole toxin and has been shown to block competitively the action of cholera toxin on intact cells (5). The A_1 chain promotes the stimulation of adenylate cyclase activity in erythrocyte ghosts and certain other systems by a NAD-dependent reaction (6). A similar reaction in intestinal epithelium is believed to be responsible for the massive loss of fluid into the intestinal lumen, which gives rise to the major symptoms of clinical cholera (7). The A_2 subunit is required for reassembly of choleragen from its subunits and may serve to hold the active A_1 chain onto the B protomer (4).

Isolation of *tox* mutants of *Vibrio cholerae* is important in the study of the genetics and biochemistry of cholera toxin and offers a promising route to the development of a live oral vaccine against cholera. Here we describe one of several *affinity filter* methods recently developed in this laboratory to facilitate isolation and characterization of mutants in toxin production. Among the mutants so far isolated are some that produce increased levels of cholera toxin (hypertoxinogenic) and others that produce decreased levels (hypotoxinogenic). Affinity filter methods should be applicable to the isolation of other more-specific classes of *tox* mutants of *V. cholerae* and also to other systems in which mutants in the production of secreted proteins are sought.

MATERIALS AND METHODS

Bacterial Strains and Growth. Two derivatives of *V. cholerae* 569B Inaba (strain RV5 in our collection) were used to isolate *tox* mutants. Strain RV501 *spc*^{R} is a prototroph that spontaneously mutated to spectinomycin resistance. Strain RV502 (*his str*^{R}) is a histidine auxotroph isolated by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG, Sigma Chemical, St. Louis, MO) as described (8) and subsequently selected for spontaneous resistance to streptomycin. *tox* mutants derived from these two strains are identified by their strain number, followed by the appropriate symbol describing their mutant phenotypes. Hypertoxinogenic mutants are designated *Htx* and hypotoxinogenic mutants, *Ltx*. The mutants in each of these classes were serially numbered in the order of isolation. Strains were maintained as described (8). CYE medium (9) was used to measure toxin production both in broth culture and on agar. Ultrafiltration with Amicon UM-10 membranes was used to concentrate the macromolecular fraction from culture supernatants.

Ganglioside Filters. Crude gangliosides (Sigma, type III) were coupled to bovine serum albumin (Sigma) by reaction with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (Bio-Rad Laboratories, Richmond, CA) at room temperature in water at pH 4.7 (10). A 2:1 molar ratio of gangliosides to protein was used, and the reaction was terminated after 4 hr by precipitation with ammonium sulfate (90% saturation), followed by dialysis against 0.1 M sodium acetate buffer, pH 5.0. Whatman no. 1 cellulose filters (9 cm) were soaked in a solution containing the protein–ganglioside conjugate (1 mg/ml), 0.4% glutaraldehyde (Eastman–Kodak, Rochester, NY), and unsubstituted serum albumin (30 mg/ml) in 0.1 M sodium acetate buffer, pH

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

Abbreviations: *tox* mutant, bacterial mutant defective in the production of protein toxins; *Htx*, hypertoxinogenic mutant; *Ltx*, hypotoxinogenic mutant.

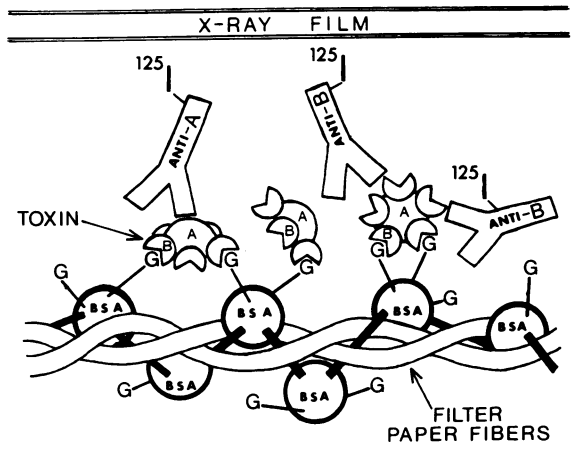

FIG. 1. Schematic representation of the ganglioside filter assay. Toxin secreted by colonies binds to gangliosides (G) attached to albumin (BSA) crosslinked into the filter with glutaraldehyde. ^{125}I-Labeled IgG directed against either the A subunit (A) or the B subunit (B) binds the toxin and produces an exposure on x-ray film after autoradiography.

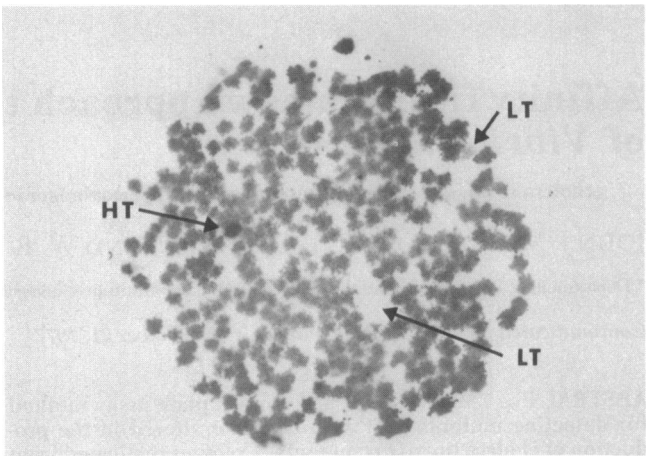

FIG. 2. The ganglioside filter assay as used in *tox* mutant isolation. Shown is the pattern of exposures on x-ray film obtained after autoradiography of a ganglioside filter used in a typical screening of mutagenized clones of strain RV501. Arrows indicate exposures corresponding to *Htx* (HT) and two *Ltx* (LT) mutant clones.

5.0. After the filters were dried at room temperature, unreacted groups were blocked by incubation with 0.25 M ethanolamine in acetate buffer, pH 5.0. The filters were washed with 10 mM sodium phosphate, pH 7.4, containing 0.85% NaCl and were then dried and sterilized by ultraviolet irradiation. Autoradiography was performed by using Dupont Cronex-5 film, and radioactivity was measured by liquid scintillation in a Beckman LS-100C counter.

Antisera. Antisera to cholera toxin and its B and A_1 chains were prepared as described (9). The A_1 chain was purified from the toxin by the procedure of Lai *et al.* (11). IgG from the immune sera was prepared by precipitation with ammonium sulfate and chromatography on DEAE-cellulose (Whatman DE-52) (12). Labeling of IgG was by the lactoperoxidase procedure of David (13) with carrier-free Na^{125}I (New England Nuclear, Boston, MA) (final specific activity of the product, 0.5–1.0 mCi/mg of protein).

Bacterial Growth and Toxin Production *In Vivo*. Radial immunodiffusion was used to measure the concentration of toxin antigen in CYE culture supernatants. Toxin production was monitored *in vivo* with the ligated intestinal loop model in adult rabbits (14). Strains were diluted to approximately 10^7 colony forming units per ml in brain heart infusion broth (Difco, Detroit, MI), and 0.5 ml was injected into each 5-cm test loop. A total of 18–20 strains could be tested per animal. Strains were arranged in alternating antibiotic resistance, and each test loop was separated from the next by a 2-cm spacer loop to minimize cross contamination. After 18 hr, the response was measured and recorded as ml of fluid accumulated per cm of loop. The maximal response elicited by 10 μg of purified cholera toxin was about 1.7 ml/cm. Cell densities attained in each loop were measured by quantitative plating of the homogenized loop and its contents on agar plates with the approprite antibiotic (spectinomycin or streptomycin, at 100 μg/ml).

RESULTS

The Ganglioside Filter Assay. The high affinity of cholera toxin for ganglioside GM_1 provides a potential means of identifying toxin released locally onto a two-dimensional surface. We have fabricated a ganglioside-containing, two-dimensional, solid-state support and have shown that cholera toxin released from isolated colonies of *V. cholerae* growing on the support does in fact attach. Bound toxin can be detected by autoradiography after treatment of the support with radioactively labeled IgG against cholera toxin or one of its constituent polypeptide chains. The method has been termed "the ganglioside filter assay."

To prepare the ganglioside-containing support, we first conjugate a mixed populaton of gangliosides to bovine serum albumin by using a water-soluble carbodiimide reagent. The predominant linkage formed is presumably between the carboxyl groups of sialic acid residues of the gangliosides and free amino groups of the serum albumin. Next, the ganglioside–albumin conjugate is mixed with glutaraldehyde as a crosslinking agent and excess unconjugated albumin. Whatman no. 1 cellulose filter discs (9 cm) are dipped in the mixture and allowed to dry. Unreacted glutaraldehyde groups on the filters are blocked with ethanolamine, and the filters are washed, dried, and sterilized by ultraviolet irradiation. The resulting "ganglioside filter" (Fig. 1) contains a network of covalently crosslinked albumin and ganglioside–albumin molecules that cannot be eluted from the filter by washing with 0.5 M NaOH or 1% dodecyl sulfate. Such filters are strong even when wet and can withstand extensive manipulation.

In routine use, a ganglioside filter is placed on the surface of a CYE agar plate, and 200–400 colonies are transferred from a master plate onto the moist filter by velvet replica plating. After incubation at 30° for 16 hr, the filter is removed from the agar plate and washed free from colonial debris with phosphate-buffered saline. It is then soaked for 1 hr at 37° in phosphate-buffered saline containing bovine serum albumin (0.1%) and ^{125}I-labeled IgG against whole toxin or one of its subunits; next, it is washed on a Buchner funnel with 100 ml of phosphate-buffered saline to remove unbound antibody. Finally, the filter is taped to a glass plate and dried under a heat lamp. Autoradiography with x-ray film for 18–36 hr reveals an exposure that corresponds to the toxinogenic colonies on the master plate.

Isolation and Characterization of *tox* Mutants. When a mutagenized population of cells is screened by the ganglioside filter assay, colonies producing greater than average intensities of exposure and those producing lower than average intensities are readily distinguished from the majority population (Fig. 2). Additional flexibility is provided in the screening by the choice of labeled antibody available. For illustrative purposes, we prepared a master plate with a mixture of the wild-type

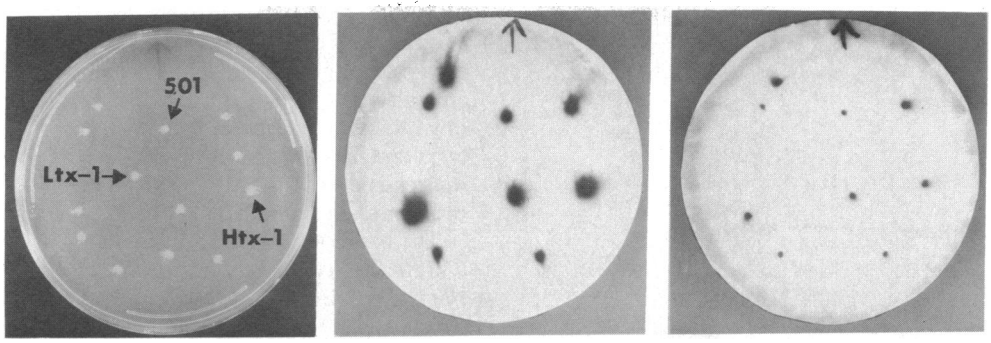

FIG. 3. Demonstration of the ganglioside filter assay on isolated wild-type, *Htx*, and *Ltx* colonies. A master plate (*Left*) with isolated colonies of three strains (501, *Htx*-1, and *Ltx*-1) was replicated to two ganglioside filters, one (*Middle*) treated with ^{125}I-labeled anti-B chain IgG and one (*Right*) treated with ^{125}I-labeled anti-A chain IgG. The exposures on x-ray film obtained after autoradiography correspond to either the wild-type toxinogenic colonies (501) or *Htx* colonies (*Htx*-1) on the regrown master plate (*Left*). *Ltx* colonies (*Ltx*-1) produced no images on either filter.

strain (501) and *Htx*-1 and *Ltx*-1 mutants and treated the corresponding ganglioside filters with ^{125}I-labeled IgG against either the B chain (Fig. 3 *middle*) or the A_1 chain (Fig. 3 *right*) of cholera toxin. The images obtained with labeled anti-A_1 IgG are generally less intense than those with anti-B chain IgG because of the relatively weak antigenic properties of the A_1 chain. However, the contrast is generally sufficient for meaningful comparisons with analogous images produced with filters treated with anti-B chain antibodies. We routinely retest putative mutants by direct inoculation from the master plate to a ganglioside filter on agar medium, followed by processing of the filter in the usual manner. More than 60 isolates may be retested per filter by this method.

The intensity of exposure produced by a colony in the ganglioside filter assay has been found to be representative of its capacity to produce cholera toxin in liquid culture. Various isolates and their parental strains were grown in CYE broth for 18 hr at 30°, at which time all cultures had attained similar densities (3–5 × 10^9 colony forming units per ml). The concentration of toxin antigen, as measured by a radial immunodiffusion assay, correlated well with the intensity of exposure produced in the ganglioside filter assay (Fig. 4). Moreover, when the radioactive spots for various clones were cut out from a retest filter and assayed for radioactivity, nearly quantitative estimates of toxinogenicity in liquid culture were obtained (Table 1). Purified toxin produced by all *Htx* mutants tested appeared to be identical to the toxin produced by the parental strain with respect to its isoelectric point and specific toxicity in the rabbit skin test.

Table 1. Correlation of filter-bound radioactivity in the ganglioside filter assay with toxin production in liquid culture

Strain	Toxin production,* μg/ml	Ganglioside filter assay, cpm†
RV501	10	5,189
RV501 *Htx*-1	35	12,133
RV501 *Ltx*-1	<0.1	1,056
RV501 *Ltx*-2	<0.1	917
RV501 *Ltx*-3	<0.1	760
RV502	7	4,513
RV502 *Htx*-2	45	13,120
RV502 *Ltx*-4	<0.1	1,224

* Toxin antigen produced in CYE broth cultures as measured by radial immunodiffusion with purified toxin as the standard.

† Radioactivity (^{125}I) associated with excised sections of the ganglioside filter used to produce the exposure presented in Fig. 4. Background for an uninoculated section of the filter was about 1000 cpm; this value has not been subtracted from the numbers given.

In all the *Htx* mutants tested thus far, both the A and B chains of the toxin were produced in increased concentrations, as judged by electrophoresis of concentrated culture fluids on dodecyl sulfate/polyacrylamide gels (Fig. 5). Similarly, all *Ltx* mutants have shown a decrease or loss of both these chains. These observations are consistent with other published data and the concept of coordinate control of synthesis of the A and B chains (15).

The capacities of several *tox* mutants to multiply and to induce a secretory response *in vivo* were measured with the ligated intestinal loop model in adult rabbits. Mutants *Ltx-1* and *Ltx-2* failed to produce a secretory response (<0.01 ml/cm) during serial passage through three rabbits, although they did multiply in the rabbit intestine to the same extent as the parental strains and *Htx* mutants tested. Cell densities attained 18 hr after inoculation were approximately 10^9 colony-forming units/cm of intestine. Two other hypotoxinogenic strains (*Ltx*-3

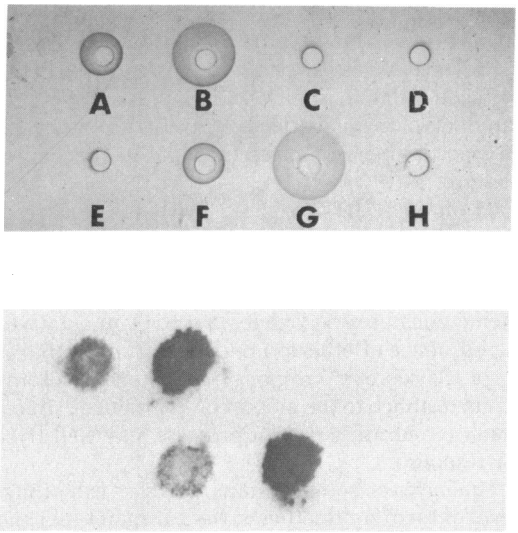

FIG. 4. Correlation of toxinogenicity in liquid culture with intensity of radioactivity produced by various clones in the ganglioside filter assay. Eight strains (A, 501; B, *Htx*-1; C, *Ltx*-1; D, *Ltx*-2; E, *Ltx*-3; F, 502; G, *Htx*-2; H, *Ltx*-4) were tested for toxin antigen produced in broth culture by radial immunodiffusion (*Upper*). The same strains were inoculated onto the surface of a ganglioside filter in the same pattern as above, grown at 30° for 16 hr, and processed with ^{125}I anti-B chain IgG. The intensities of radioactivity obtained on x-ray film (*Lower*) may be compared with the diameters of the precipitin rings formed in the radial immunodiffusion assay.

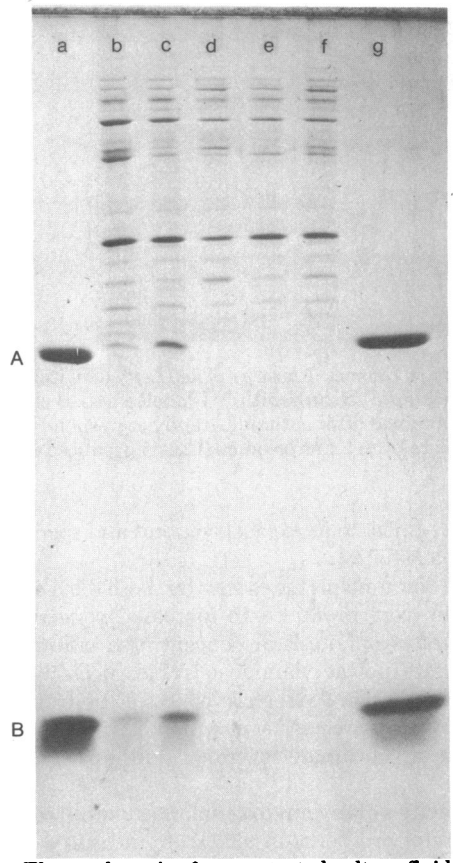

FIG. 5. Electrophoresis of concentrated culture fluids on a slab polyacrylamide gel in the presence of sodium dodecyl sulfate. Culture supernatants of wild-type and mutant strains of RV501 were concentrated 4-fold by ultrafiltration before electrophoresis. Lanes: (a and g) purified cholera toxin; (b) RV501; (c) *Htx*-1; (d) *Ltx*-1; (e) *Ltx*-2; (f) *Ltx*-3. A, position of the A subunit of cholera toxin; B, position of the B subunit of cholera toxin.

and *Ltx*-4) reverted to the toxinogenic state during serial passage (e.g., mutant *Ltx*-3 elicited secretory responses of 0.02, 0.15, and 0.51 ml/cm on serial passage through the three rabbits). The reversion was shown by the ganglioside filter assay to involve accumulation of cells with the capacity to produce normal amounts of toxin. A selective advantage for toxinogenic clones *in vivo* has been reported (15), but its mechanism remains obscure.

One *Htx* mutant (*Htx*-1) elicited nearly maximal responses in the ileal loop assay (1.6 ml/cm), but another (*Htx*-2) elicited only a mild response (0.9 ml/cm) although both multiplied in the gut effectively. The latter case might be explained either by an attenuation of toxinogenicity *in vivo* by unknown factors or by the influence of other properties of *V. chorlerae* on the intensity of the secretory response. The capacity of an individual strain to attach to the mucosa or to produce extracellular factors such as neuraminidase or protease may well affect the secretory response.

The frequencies of both *Ltx* mutants (those exhibiting <1% of the level of toxin production of the parental type) and *Htx* mutants (those exhibiting at least 200% of the toxin production of the parental type) were 0.1–0.2%. The frequency for *Ltx* mutants agrees well with the results of Ruch *et al.* (2), obtained by a different screening procedure.

DISCUSSION

The ganglioside filter assay is one of several affinity filter methods being developed in this laboratory. The general affinity filter method combines the power of affinity chromatography with the ease of manipulation possible with a two-dimensional matrix (filters) containing large numbers of microscale samples. Sequential steps are greatly simplified, inasmuch as large numbers of samples may be treated batchwise once they have been attached to the solid-state support. Also, the geometry of the filters allows the highly sensitive technique of autoradiography to be applied.

The basic requirements of the affinity filter method are: (*i*) a high-affinity ligand that may be covalently attached to albumin, or directly to a two-dimensional solid-state support, without loss of capacity to bind the protein in question; and (*ii*) labeled antibodies capable of attaching to the ligand-bound protein. The ligand requirements are met in the case of cholera toxin and choleragenoid by ganglioside GM_1, which can be attached to albumin via the carboxyl group of its sialic acid moiety without loss of affinity for the cholera toxin B chain.

A potentially more versatile filter-bound ligand is IgG against the protein in question. We have found (unpublished data) that anticholeragenoid IgG meets the ligand requirements set forth above; when covalently attached to albumin-containing filters, it binds cholera toxin or choleragenoid and still permits detection with labeled anticholeragenoid IgG. This approach should facilitate detection of immunologically crossreacting forms of cholera toxin that lack ganglioside-binding activity. It seems likely that this general method will be applicable to the isolation of mutants in the production of various secreted proteins besides bacterial toxins.

The ganglioside filter assay has enabled us to screen for *tox* mutants in *V. cholerae* with substantially greater ease than was possible with previously described methods (1, 2). Although the time required for a given screening is about 3 days, large numbers of colonies may be screened at the same time (*ca* 20,000 colonies/week per person) and at relatively low expense. A major advantage of the method is that rough quantification of ganglioside-binding toxin antigen is possible simply by inspection. This has permitted us to distinguish and isolate *Htx* as well as *Ltx* mutants of *V. cholerae*.

Mutant M13, a putatively nontoxinogenic mutant of *V. cholerae* 569B isolated by Finkelstein *et al.* (1), has been reported to produce detectable toxic material when assayed by the rabbit skin permeability assay (16). Similarly, although toxin production by some of our *Ltx* mutants is so low as to be undetectable by either the ganglioside filter assay or immunodiffusion, the cell-free culture fluid from these mutants still exhibits detectable toxic activity as measured by the highly sensitive rabbit skin test. It is not clear whether the residual toxic activity (usually 0.1–1% of that observed with the parental strain) is due to traces of *bona fide* cholera toxin or to some unidentified fragment of the toxin.

The ganglioside filter assay has also been used to isolate *Htx* and *Ltx* mutants of RV79, a *V. cholerae* strain of the El tor biotype. This demonstrates that the ganglioside filter assay can be used to screen for *tox* mutants of strains of *V. cholerae* besides the exceptionally toxinogenic "classical" strain, 569B.

The ganglioside filter assay should facilitate isolation of various specific classes of *tox* mutants besides those described here. The use of labeled IgG specific for the B or A chains to treat replicate ganglioside filters should permit us to detect rare mutants producing B but not A chain. Mutants producing only A chain would not be expected to produce images on x-ray film with labeled IgG of either type, but might be detected by further screening of mutants classified initially as nontoxinogenic by the ganglioside filter assay. The use of anti-A or anti-B specific IgG as filter-bound ligand adds another potential screening

parameter, which might be used, for example, to detect mutants producing altered toxins lacking affinity for ganglioside GM_1. Mutations that alter toxicity but do not alter either the toxin's antigenicity or its ganglioside binding of course will not be detected by the ganglioside filter assay.

We hope that the ganglioside filter assay will facilitate the search for a mutant useful for development of a live oral vaccine against cholera. Several classes of mutants might be useful in this regard. A prime candidate would be a mutant lacking an active A chain; the aberrant toxin formed should be nontoxic but retain immunogenicity by virtue of the B chain produced. Another interesting possibility would be a stable *Ltx* mutant. Such a mutant might provide antigenic stimulation and even adjuvant activity (17) while producing only mild and transient diarrhea. Inasmuch as some strains of enteropathogenic *Escherichia coli* produce an enterotoxin that crossreacts antigenically with the B subunit of cholera toxin (18), certain mutants of *V. cholerae* such as those described above, might have potential in controlling enterotoxinogenic *E. coli*-related disease as well as cholera.

We wish to thank Patricia D. Bernard for her assistance in preparing the photographs. This investigation was supported by U.S. Public Health Service Grants AI07877 and AI08024 from the National Institute of Allergy and Infectious Diseases. J.J.M. is a predoctoral trainee supported by U.S. Public Health Service Grant GM07104 from the National Institute of General Medical Sciences.

1. Finkelstein, R. A., Vasil, M. L. & Holmes, R. K. (1974) *J. Infect. Dis.* **129,** 117–123.
2. Ruch, F. E., Murphy, J. R., Graf, L. & Field, M. (1976) *J. Infect Dis.*, in press.
3. Kurosky, A., Markel, D. E., Touchstone, B. & Peterson, J. W. (1976) *J. Infect. Dis.* **133,** S14–S22.
4. Gill, D. M. (1976) *Biochemistry* **15,** 1242–1248.
5. Cuatrecasas, P. (1973) *Biochemistry* **12,** 3577–3581.
6. Gill, D. M. (1976) *J. Infect. Dis.* **133S;** S55–S63.
7. Finkelstein, R. A. (1973) *Crit. Rev. Microbiol.* **2,** 553–623.
8. Parker, C., Gauthier, D., Tate, A. & Romig, W. R. (1971) in *Woods' Hole Symposium on Cholera,* eds. Fukumi, H. & Ohashi, M. (Tokyo, Japan), pp. 24–34.
9. Mekalonos, J. J., Collier, R. J. & Romig, W. R. (1977) *Infect. Immun.* **16,** 789–795.
10. Glazer, A. N., DeLange, R. J. & Sigman, D. S. (1975) *Chemical Modification of Proteins* (American Elsevier Publ. Co., New York), p. 88.
11. Lai, C. Y., Mendez, E. & Chang, D. (1976) *J. Infect. Dis.* **133S,** S23–S30.
12. Sober, H. A., Gutter, F. J. Wycoff, M. M. & Peterson, E. A. (1956) *J. Am. Chem. Soc.* **78,** 756.
13. David, G. S. (1972) *Biochem. Biophys. Res. Commun.* **48,** 464–471.
14. Burrows, W. & Musteikus, G. M. (1966) *J. Infect. Dis.* **116,** 183–190.
15. Holmes, R. K., Vasil, M. L. & Finkelstein, R. A. (1975) *J. Clin. Invest.* **55,** 551–560.
16. Richardson, S. H., Mekenzie, B., Daves, E., Evans, D. J. & Evans, D. G. (1975) in *Kyoto Symposium on Cholera,* eds. Fukumi, H. & Ohashi, M. (Tokyo, Japan), pp. 37–44.
17. Northrup, R. S. & Fauci, A. S. (1972) *J. Infect. Dis.* **125,** 272–273.
18. Gyles, C. L. (1974) *Infect. Immun.* **9,** 564–570.