A RECOMBINANT LIVE ORAL CHOLERA VACCINE

James B. Kaper, Hank Lockman, Mary M. Baldini, and Myron M. Levine
Center for Vaccine Development, University of Maryland School of Medicine, 10 S. Pine Street, Baltimore, Maryland 21201

A pathogenic strain of *Vibrio cholerae* was attenuated by deletion of DNA sequences encoding the A_1 subunit of the cholera enterotoxin. A restriction endonuclease fragment encoding the A_1, but not the A_2 or B sequences was deleted *in vitro* from cloned cholera toxin genes. The mutation was then recombined into the chromosome of *V. cholerae* Ogawa 395, a pathogenic strain that confers complete immunity to subsequent infection following an initial clinical infection. The resulting strain, which produces the immunogenic but nontoxic B subunit of cholera toxin and is unaltered in other critical antigens, represents a promising candidate for an attenuated live oral cholera vaccine.

Despite nearly a century of effort, a satisfactory cholera vaccine is not yet a reality. Current parenteral vaccines offer only moderate protection of brief duration against infection with pathogenic *Vibrio cholerae* O1, the agent of epidemic and endemic cholera. Experimental cholera studies in community volunteers at the Center for Vaccine Development have demonstrated that, as in nature, protective immunity follows infection with pathogenic *V. cholerae* and suggest that a live, attenuated *V. cholerae* strain could serve as an effective oral vaccine to mimic infection-derived immunity[1–5]. In development of other vaccines, the techniques of recombinant DNA have been exploited to yield large quantities of protective antigens[6,7]. However, with cholera it is not known exactly which antigens of *V. cholerae* confer protective immunity; therefore, an alternative approach is required for a cholera vaccine. We have employed recombinant DNA techniques to construct an attenuated *V. cholerae* strain that holds promise as a live oral vaccine. The method of attenuation involves the specific removal of genes encoding known toxic moieties, i.e., cholera enterotoxin, while preserving genes encoding all other antigens such as lipopolysaccharide, outer membrane proteins, colonization factors, etc., that are likely to be involved in immunity[8].

V. cholerae colonizes the small bowel and secretes a potent enterotoxin—cholera toxin—that leads to the profuse watery diarrhea characterstic of the disease. Cholera toxin consists of a single A subunit, which stimulates adenylate cyclase activity, and five identical B subunits, which bind to the GM_1 ganglioside receptor of intestinal mucosal cells. Although cholera toxin is very potent, observations derived from studies in man indicate that toxoid-derived antitoxic immunity alone is insufficient to provide effective, long-lasting protection against cholera[1,9,10]. We have previously reported the cloning and sequencing of genes encoding cholera toxin[11]. Based on the sequence data, we have now employed restriction endonucleases to delete the specific sequences encoding the toxic A subunit, while retaining expression of the immunogenic but non-toxic B subunit. This mutation created *in vitro* was recombined into the chromosome of a virulent *V. cholerae* strain (Ogawa 395) previously demonstrated to confer solid immunity in volunteers[2–4]. The recombinational event results in the replacement of the proficient toxin genes in *V. cholerae* 395 with the deletion mutation constructed *in vitro* without affecting other antigens important for immunity.

RESULTS

Vibrio cholerae strain 395 contains two copies of the cholera enterotoxin genes on its chromosome[12,13]. Both copies were cloned individually into the vector pBR325; the organization of the toxin genes is shown in Figure 1. Both the A and B genes are transcribed from a single promoter located proximal to the A gene. DNA sequence data suggested the existence of separate ribosomal binding sites for both the A and B genes, allowing for independent translation of the B subunit[11,14,15]. The A subunit is synthesized initially as a single polypeptide that is proteolytically nicked to yield the peptides A_1 and A_2

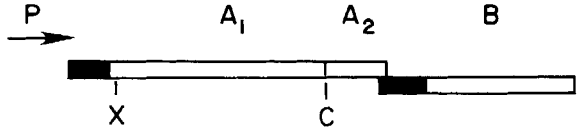

FIGURE 1 Organization of cholera enterotoxin genes showing the relative positions of the cholera toxin promoter (P), A_1, A_2, and B structural genes. Arrow indicates direction of transcription. The A subunit cistron consists of sequences encoding a putative 18 amino acid-residue leader peptide (solid bar), a 194 residue A_1 peptide, and a 46 residue A_2 peptide[11,15]. The B subunit cistron consists of sequences encoding a putative 21 amino acid residue leader peptide (solid bar) and a 103 residue B peptide. The termination codon of the A subunit overlaps the initiation codon of the B subunit by four base pairs. Xba I (X) and Cla I (C) sites are located in the initial sequences encoding the A_1 and A_2 peptides, respectively.

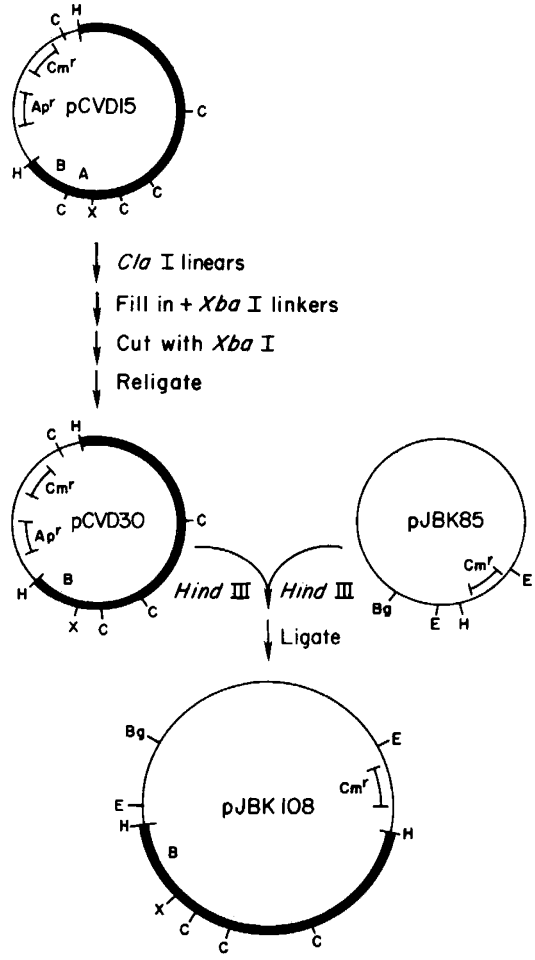

FIGURE 2 Deletion of the 550 bp Xba I-Cla I fragment encoding the A_1 peptide from cloned cholera toxin genes. The heavy lines indicate cloned *V. cholerae* chromosomal DNA and the light lines are vector DNA. pCVD15 was constructed by cloning a 12 kb Hind III (H) containing cholera toxin genes from *V. cholerae* 395 into pBR325. Restriction endonuclease sites (X = Xba I, C = Cla I, E = Eco RI, Bg = Bgl II) and fragments encoding the toxin subunits (A and B) are shown. The 550 bp Xba I-Cla I fragment encoding the A_1 peptide was deleted by linearization with Cla I, addition of Xba I linkers followed by Xba I digestion, and religation. The Hind III fragment from pCVD30 was then cloned into pJBK85 for mobilization into *V. cholerae*.

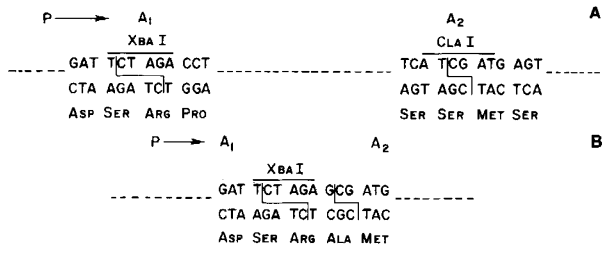

FIGURE 3 A. DNA sequence and predicted amino acid residues surrounding the Xba I and Cla I sites in unmutated cholera toxin operon. The arginine residue is residue number 11 of the A_1 peptide and the methionine residue is the first residue of the A_2 peptide. The exact sites of restriction enzyme cleavage are indicated. B. Predicted sequences after deletion of the 550 bp Xba I-Cla I fragment, filling in the Cla I end with DNA polymerase I, and ligation of a synthetic Xba I linker.

found in the mature toxin. The ADP ribosylating, viz. toxic, activity resides solely in the A_1 peptide while the A_2 peptide serves to noncovalently join the active and binding (B) subunits[16,17]. The DNA sequence of the toxin gene revealed structural features and restriction endonuclease sites that provided the rationale for the *in vitro* construction of deletion mutants.

The recombinant plasmid pCVD15 contains one of the toxin gene copies from strain 395 (Fig. 2). An Xba I site is contained within the DNA sequence encoding residues 10 and 11 of the A_1 subunit, and a Cla I site spans the codons of the last residue of the A_1 and the first residue of A_2 (Fig. 3A). The 550 bp Xba I-Cla I fragment was deleted from pCVD15 and the Cla I site was modified by the addition of synthetic Xba I linkers (Fig. 2). Following religation, the resulting molecule, designated pCVD30, should contain the sequences shown in Figure 3B. The net result of this mutation is the deletion of 183 of the 194 amino acid residues of the A_1 peptide, leaving only the first 11 residues of A_1 and all residues of the A_2 and B peptides. Thus, active holotoxin is not produced and 94% of the A_1 cistron is completely deleted. Deletion of the A_1 gene with preservation of the B gene was confirmed by DNA hybridization using cloned A_1 and B genes as probes. These results correlated with a negative Y-1 adrenal cell assay for holotoxin and a positive GM_1 ELISA for B subunit. The Hind III fragment containing this deletion was then cloned into pJBK85 for mobilization into *V. cholerae* (Fig. 2).

The deletion mutation constructed *in vitro* was then introduced into the chromosome of *V. cholerae* 395 by a site-directed mutagenesis procedure[18]. In this procedure, the cloned genes mutated *in vitro* are mobilized on a plasmid into the wild type strain where they replicate extrachromosomally. At a low frequency, the sequences flanking the plasmid-borne mutated genes recombine with the homologous sequences flanking the proficient genes in the chromosome. The recombinational crossover event results in the displacement of the chromosomal toxin gene sequences by the mutated toxin genes. To introduce the toxin deletion borne on the plasmid pJBK108 into the chromosome of *V. cholerae* 395, an insertion mutation was first constructed as shown in Figure 4. Plasmid pJBK107 consists of the Hind III fragment of pCVD15 containing the cloned cholera toxin genes and flanking sequences cloned into the incompatibility group P (Inc P) plasmid vector pJBK85. The cholera toxin genes of pJBK107 have been insertionally inactivated by cloning a tetracycline resistance gene into the Xba I site of the A_1 subunit (Fig. 4). This plasmid was mobilized into *V. cholerae* 395 using the conjugative plasmid pRK2013. In a small percentage of cells, homologous crossover occurred (Fig. 4A), resulting in the displacement of the chromosomal A_1 sequences by the A_1 sequences containing the tetracycline resistance gene (Fig. 4B). This event was detected in a population of *V. cholerae* 395 strains containing pJBK107 by introduction of a second Inc P group plasmid pR751, which is incompatible, i.e., cannot be stably maintained with other Inc P group plasmids such as pJBK107. Resulting tetracycline- and trimethoprim-resistant cells represent those cells in which pR751 (determining trimethoprim resistance) is stably maintained extrachromosomally, the tetracycline resistance genes are integrated into the chromosomal toxin genes, and the pJBK107 replicon is lost (Fig. 4B). One such isolate was designated *V. cholerae* JBK113. The tetracycline resistance genes in *V. cholerae* JBK113 provided us with a selectable marker to detect subsequent recombinational events resulting in deletion of the A_1 sequence. The toxin gene deletion borne on plasmid

pJBK108 (Fig. 2) was then mobilized into *V. cholerae* JBK113 (which was first spontaneously cured of pR751). Homologous recombination of the deleted toxin gene sequences into the chromosome of *V. cholerae* JBK113 occurred (Fig. 4C) and resulted in the loss of the Xba I-Cla I A_1 fragment and the tetracycline resistance gene. One mutant resulting from this recombination was designated *V. cholerae* CVD101; this strain lacks holotoxin (by Y-1 adrenal cell assay) but produces B subunit (by ELISA), and is completely deleted of the 550 base pair Xba I-Cla I A_1 fragment. Both chromosomal toxin gene copies were deleted, as indicated by the complete lack of DNA homology with the A_1 probe (Fig. 5). DNA hybridization studies with labeled B subunit probe indicate that multiple recombinational events occurred since two copies of the B subunit gene remain (Fig. 5).

DISCUSSION

V. cholerae CVD101 holds great promise as an attenuated strain for use as a live oral cholera vaccine. Such a vaccine offers advantages over other cholera vaccines currently in use or being evaluated. It should be highly efficacious in preventing cholera since the parental strain (395) from which it was derived conferred long-lasting immunity in 100% of volunteers who were infected with it[3]. The vaccine strain retains all features of the parental strain except the production of holotoxin and should stimulate both antibacterial and antitoxic immunity, since B subunit is still produced. Because the vaccine would be administered orally and would consist of a lyophilized bacterial culture, it should be very inexpensive and practical to administer in the developing countries where cholera occurs. Attenuated cholera vaccine strains evaluated previously in humans have offered promise but have not been completely satisfactory. One major disadvantage of attenuated strains evaluated previously in humans has been the method of attenuation employed[19]. Use of nonspecific mutagenic agents such as nitrosoguanidine is not satisfactory because of the possibility of inducing unrecognized mutations affecting other antigens that may contribute to immunity. Furthermore, it is impossible to predict the exact genetic lesion with such methods, so the theoretical risk of reversion to toxigenicity remains. The method we have described using techniques of recombinant DNA is free of these disadvantages and represents the beginning of a new generation of cholera vaccines. This approach eliminates the possibility of reversion to toxinogenicity, due to the nearly complete deletion of the DNA sequences encoding the A_1 toxin subunit, while it leaves unaffected all other antigens important for immunity. Such an approach was also used by Mekalanos et al.[15], who recently reported the construction of attenuated *V. cholerae* strains similar to CVD101 but these have not been tested in clinical trials. The attenuated *Vibrio cholerae* strain CVD101 is currently being evaluated in human volunteer studies at the Center for Vaccine Development to assess the safety and efficacy of this strain in man.

EXPERIMENTAL PROTOCOL

Bacterial Strains and Plasmids Employed. *Vibro cholerae* strain 395 is of the classical biotype, Ogawa serotype, and has been characterized extensively in volunteer studies[2–4]. *E. coli* strain HB101 (F^-, *hsd*S20 [r^-_B, m^-_B], *rec*A13, *ara*-14, *pro*A2, *lac*Y1, *gal*K2, *rps*L20 [Sm^r], *xyl*-5, *mtl*-1, *sup*E44) was used for all cloning procedures and matings with *V. cholerae*. pJBK85 (J. B. Kaper, unpublished) is a chloramphenicol-resistant, tetracycline-sensitive derivative of the broad host range, non-conjugative incompatibility group P plasmid pRK290[20]. pR751 is a broad host range, conjugative plasmid of the P incompati-

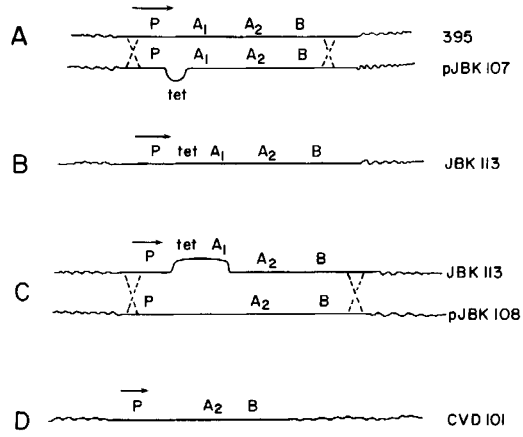

FIGURE 4 Introduction of cholera toxin gene deletion into the chromosome of *V. cholerae* 395. **A.** Recombinational crossover event (indicated by dashed lines) resulting in insertion of tetracycline resistance genes (*tet*) into a chromosomal gene copy of cholera toxin A_1 subunit. The top line depicts chromosomal toxin region of *V. cholerae* 395 and the lower line depicts the mutated, plasmid-borne toxin sequences. Plasmid pJBK107 contains cloned cholera toxin promoter (P), A_1, A_2, and B sequences with a fragment encoding tetracycline resistance cloned into the Xba I site of the A_1 sequence. Arrows indicate direction of transcription. **B.** The recombinational crossover event depicted in part A results in *V. cholerae* strain JBK113 containing tetracycline resistance genes inserted in chromosomal A_1 sequences. **C.** Recombinational crossover event resulting in displacement of tetracycline resistance gene by cholera toxin deletion mutation. Plasmid pJBK108 contains the cholera toxin promoter, A_2, and B subunit sequences with nearly all of the A_1 sequence deleted (Fig. 2). pJBK108 was mobilized into *V. cholerae* JBK113 using the conjugative plasmid pRK2013, and tetracycline-sensitive *V. cholerae* cells were selected. **D.** *V. cholerae* CVD101, resulting from recombinational crossover event depicted in part C is tetracycline-sensitive, produces B subunit antigen but not holotoxin, and is deleted for the 550 bp Xba I-Cla IA_1 fragment.

FIGURE 5 Southern blot analysis of *V. cholerae* DNA extracted from strains 395 and CVD101. Chromosomal DNA was digested with Hind III, separated by agarose gel electrophoresis, transferred to nitrocellulose paper and hybridized with ^{32}P-labeled probes specific for A_1 (lanes 1 and 2) or B subunits (lanes 3 and 4). Lanes 1 and 3 are *V. cholerae* 395 and lanes 2 and 4 are *V. cholerae* CVD101.

bility group that encodes trimethoprim resistance[18]. pRK2013 is a conjugative kanamycin-resistant plasmid capable of mobilizing pRK290 and derivatives[20].

Media and Growth Conditions. All culture were grown at 37°C in L broth and plated on L-agar[21]. When required, L-agar was supplemented with chloramphenicol at 20 μg/ml, tetracycline at 5 μg/ml, trimethoprim at 50 μg/ml, ampicillin at 100 μg/ml or polymyxin B at 5 units/ml. Tetracycline-sensitive *V. cholerae* were enriched from a population of tetracycline-resistant and -sensitive cells by growth in L-broth in the presence of the bacteristatic tetracycline (2 μg/ml) and the bactericidal antibiotics ampicillin (50 μg/ml) and d-cycloserine (50 μg/ml). Donor and recipient strains for *V. cholerae* and *E. coli* matings were grown statically at 37° until early exponential phase, then mixed and filtered through 0.4 μm filters (Nuclepore). The filters were placed on L-agar plates, incubated at 37° for 2 hr to overnight, then resuspended and plated on L-agar containing appropriate antibiotics. Counterselection for the vibrio recipient was achieved by the use of polymyxin B at 5 units/ml.

Recombinant DNA techniques. Chromosomal DNA from *V. cholerae* was prepared by the method of Brenner et al.[22] Plasmid DNA was extracted by the method of Birnboim and Doly[23] and purified by cesium chloride-ethidium bromide equilibrium density gradient centrifugation as detailed by Maniatis et al.[24] Restriction endonucleases, T4 ligase, DNA polymerases, and calf intestinal alkaline phosphatase were purchased from Bethesda Research Laboratories and used as suggested by the manufacturer. The two chromosomal gene copies encoding cholera toxin in *V. cholerae* 395 were cloned by separating chromosomal Hind III restriction fragments by preparative 0.7% agarose gel electrophoresis. Fragments demonstrating homology with cloned cholera toxin gene probes were electroeluted, successively extracted with phenol, chloroform, and ether, and ethanol precipitated. Purified fragments were mixed with the vector pBR325[25], which had been cleaved with Hind III and treated with alkaline phosphatase. The DNA mixture was incubated overnight at 15°C in a solution containing 50 mM Tris HCl, pH 7.5, 50mM NaCl, 10mM $MgCl_2$, 0.5mM ATP, 10mM dithiothreitol and 1 unit T4 DNA ligase. Transformation of this ligation mixture into *E. coli* HB101 yielded clones which were screened by colony hybridization using a cholera toxin gene probe.

The 550 bp Xba I-Cla I fragment encoding the A_1 peptide of cholera toxin was deleted *in vitro* by the following method: pCVD15 consists of a 12 kb Hind III vibrio fragment cloned into pBR325 and contains one copy of the cholera enterotoxin operon. Linear molecules of pCVD15 in which one of the five Cla I sites was cut were obtained by partial digestion with Cla I. Digestion conditions were determined empirically by varying the time, temperature, and concentration of enzyme. Following linerization, the single stranded Cla I ends were filled in with DNA polymerase I (Klenow fragment). Synthetic octameric Xba I linkers (Collaborative Research) were then ligated onto the filled in Cla I sites, and the ligation mixture was digested with Xba I to trim the linkers. Digestion with Xba I and religation served to delete one or more Xba I-Cla I fragments per molecule, depending upon which Cla I site was cut in linearization and subsequently converted to an Xba I site. The ligation mixture was transformed into *E. coli* HB101 and the resulting clones were probed with the ^{32}P-labeled 550 bp Xba I-Cla I fragment to detect deletions of the fragment and mapped with restriction endonucleases to determine the extent of the deletion.

A fragment containing tetracycline resistance genes flanked by Xba I sites was cloned by digesting pBR322[25] with Eco RI and Ava I and isolating the resulting 1424 bp fragment encoding tetracycline resistance. The single stranded ends were filled in with Klenow fragment and synthetic Xba I linkers ligated onto the filled-in ends. Following digestion with Xba I, the fragment was cloned into the unique Xba I site of pCVD2[11], resulting in a tetracycline resistant clone designated pCVD18.

DNA Hybridization. Chromosomal DNA (1 μg) was digested with Hind III and fragments separated by gel electrophoresis in 0.7% agarose. After electrophoresis and photography, the gels were denatured in 0.2M NaOH-0.6M NaCl for 45 min, then neutralized in 1M Tris-0.6M NaCl, pH 7.5 for 45 min, and the DNA fragments were transferred to nitrocellulose filters (Schleicher & Schuell) by the technique of Southern[27]. After overnight transfer, the filters were baked at 30°C for 2 h *in vacuo* and stored until hybridization. Colony hybridizations were performed by inoculating cultures onto sterile nitrocellulose filters placed on L-agar. After overnight growth, filters were placed onto Whatman No. 3 filter paper saturated with 0.5M NaOH for 10 min to lyse the cells and denature the DNA. The filters were then transferred to three successive filters saturated with 1M Tris, pH 7.0, for 1 min each and finally to a filter saturated with 1M Tris-1.15M NaCl, pH 7.0, for 10 min. They were air dried, and baked *in vacuo* at 80°C for 2 h, and stored until hybridization.

Probes specific for cholera toxin A and B subunit genes were prepared by digesting pCVD2[11] with Xba I and Cla I to yield a 550 bp fragment specific for A_1 and with AccI to yield a 650 bp fragment containing B sequences. These fragments were isolated from 5% polyacrylamide gel slices by electroelution and labeled with α^{32}P-dATP (800 Ci/mmole, New England Nuclear) by nick translation[28]. Hybridizations were performed by preincubation of the filters for at least 3 h at 37°C in a solution of 50% formamide-5X SSC (1X SSC = 0.15M NaCl plus 0.015M sodium citrate)-1 Denhardt solution (0.02% Ficoll, mw 400,000; 0.02% polyvinylpyrrolidone, molecular weight 360,000; 0.02% bovine serum albumin)-0.1% sodium dodecyl sulfate (SDS)-1mM EDTA. After preincubation, the filters were transferred to 10 ml of fresh solution (described above) containing about 2×10^6 cpm of ^{32}P-labeled DNA and 100 μg of denatured calf thymus DNA per ml sheared by sonication. Hybridization was carried out at 37°C for 18 h, after which the filters were rinsed in 5X SSC-0.1% SDS, then washed for 1 h in fresh 5X SSC-0.1% SDS at 65°C. After a final rinse in 2SSC at ambient temperature, the filters were air dried, placed in a cassette with Kodak X-Omat R film and Du Pont Cronex Lightning Plus intensifying screens for 24 h at −70°C, and then developed.

Toxin Assays. Bacterial cultures were assayed for cholera toxin by growing in casamino acids yeast extract broth[29] at 37°C for 18 hr with aeration. Culture supernatants were collected by centrifrigation and assayed for holotoxin by the Y-1 adrenal cell assay[30] and for B subunit antigen by a ganglioside-dependent, enzyme-linked immunosorbent assay[29].

Acknowledgements

The skilled technical assistance of Susan Trinker and Yu Lim is gratefully acknowledged. This investigation was supported by grant R22AI19716 and contract NO1AI12666 from the National Institute of Allergy and Infectious Diseases.

Received 3 February 1984; accepted 10 February 1984.

References

1. Levine, M. M., Nalin, D. R., Craig, J. P., Hoover, D., Bergquist, E. J., Waterman, D., Holley, H. P., Hornick, R. B., Pierce, N. P., and Libonati, J. P. 1979. Immunity to cholera in man: relative role of antibacterial versus antitoxic immunity. Trans. Roy. Soc. Trop. Med. Hyg. **73:**3–9.
2. Levine, M. M. 1980. Immunity to cholera as evaluated volunteers, pp. 195–203. *In* Cholera and Related Diarrhoeas. O. Ouchterlony and J. Holmgren (eds.), Karger, Basel.
3. Levine, M. M., Black, R. E., Clements, M. L., Cisneros, L., Nalin, D. R., and Young, C. R. 1981. Duration of infection-derived immunity to cholera. J. Infect. Dis. **143:**818–820.
4. Levine, M. M., Black, R. E., Clements, M. L., Nalin, D. R., Cisneros, L., and Finkelstein, R. A. 1981. Volunteer studies in development of vaccines against cholera and enterotoxigenic *Escherichia coli:* a review, pp. 449–459. *In* Acute Enteric Infections in Children: New Prospects for Treatment and Prevention. T. Holme, J. Holmgren, M. H. Merson, and R. Mollby (eds.), Elsevier, Amsterdam.
5. Glass, R. I., Becker, S., Huq, M. I., Stoll, B. J., Khan, M. U., Merson, M. H., Lee, J. V., and Black, R. E. 1982. Endemic cholera in rural Bangladesh, 1966–1980. Am. J. Epidemiol. **116:**959–970.
6. Kleid, D. G., Yansura, D., Small, B., Dowbenko, D., Moore, D. M., Grubman, M. J., McKercher, P. D., Morgan, D. O., Robertson, B. H., and Bachrach, H. L. 1981. Cloned viral protein vaccine for foot-and-mouth disease: responses in cattle and swine. Science **214:**1125–1129.
7. Bachrach, H. L. 1982. Recombinant DNA technology for the preparation of subunit vaccines. J. Am. Vet. Med. Assoc. **181:**992–999.
8. Levine, M. M., Kaper, J. B., Black, R. E., and Clements, M. L. 1983. New knowledge on pathogenesis of bacterial enteric infections as applied to vaccine development. Microbiol. Rev. **47:**510–550.
9. Curlin, G., Levine, R., Azia, K. M. A., Mizanur Rahman, A. C. M., and Verway, W. F. 1975. Field trial of cholera toxoid, pp. 314–329. Proc. 11th Joint Conference on Cholera, Washington, D.C.
10. Noriki, H. 1976. Evaluation of toxoid field trials in the Philippines, pp. 302–310. Proc. 12th Joint Conference on Cholera, Tokyo.
11. Lockman, H. and Kaper, J. B. 1983. Nucleotide sequence analysis of the A_2 and B subunits of *Vibrio cholerae* enterotoxin. J. Biol. Chem. **258:**13722–13726.
12. Kaper, J. B., Moseley, S. L., and Falkow, S. 1981. Molecular characterization of environmental and nontoxinogenic strains of *Vibrio cholerae*. Infect. Immun. **32:**661–667.
13. Mekalanos, J. J. 1983. Duplication and amplification of toxin genes in *Vibrio cholerae*. Cell **35:**253–263.
14. Gennaro, M. L. and Greenway, P. J. 1983. Nucleotide sequences within the cholera toxin operon. Nucl. Acids. Res. **11:**3855–3861.
15. Mekalanos, J. J., Swartz, D. J., Pearson, G. D. N., Harford, N., Groyne, F., and de Wilde, M. 1983. Cholera toxin genes: nucleotide sequence, deletion analysis, and vaccine development. Nature **306:**551–557.
16. Gill, D. M. 1976. The arrangement of subunits in cholera toxin. Biochemistry **15:**1242–1248.
17. Gill, D. M. and Meren, R. 1978. ADP-ribosylation of membrane proteins catalyzed by cholera toxin: basis of the activation of adenylate cyclase. Proc. Natl. Acad. Sci. U.S.A. **75:**3050–3054.
18. Ruvkun, G. R. and Ausubel, F. M. 1981. A general method for site-directed mutagenesis in prokaryotes. Nature **289:**85–88.
19. Levine, M. M., Black, R. E., Clements, M. L., Young, C. R., Lanata, C., Sears, S., Honda, T., and Finkelstein, R. 1983. Texas Star-SR: attenuated *Vibrio cholerae* oral vaccine candidate. Develop. Biol. Stand. **53:**59–65.
20. Ditta, G., Stanfield, S., Corbin, D., and Helinski, D. R. 1980. Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. U.S.A. **77:**7347–7351.
21. Miller, J. M. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
22. Brenner, D. J., Fanning, G. R., Johnson K. E., Citarella, R. V., and Falkow S. 1969. Polynucleotide sequence relationships among members of *Enterobacteriaceae*. J. Bacteriol. **98:**637–650.
23. Birnboim, H. C. and Doly, J. 1979. A rapid alkaline extraction procedure for screening recombinant DNA. Nucl. Acids. Res. **7:**1513–1523.
24. Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular cloning. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
25. Bolivar, F. 1978. Construction and characterization of new cloning vehicles. III. Derivatives of plasmid pBR322 carrying unique Eco RI sites for selection of Eco RI generated recombinant DNA molecules. Gene **4:**121–136.
26. Bolivar, F., Rodriguez, R. L., Green, P. J., Betlach, M. C., Heynecker, H. L., Boyer, H. W., Crosa, J. M., and Falkow, S. 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2:**95–113.
27. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
28. Maniatis, T., Jeffrey, A., and Kleid, D. G. 1975. Nucleotide sequence of the rightward operator of phage lambda. Proc. Natl. Acad. Sci. U.S.A. **72:**1184–1188.
29. Sack, D. A. and Sack, R. B. 1975. Test for enterotoxigenic *Escherichia coli* using Y1 adrenal cells in miniculture. Infect. Immun. **11:**334–336.
30. Sack, D. A., Huda, S., Neogi, P. K. B., Daniel, R. R., and Spira, W. M. 1980. Microtiter ganglioside enzyme-linked immunosorbent assay for *Vibrio* and *Escherichia coli* heat-labile enterotoxins and antitoxin. J. Clin. Microbiol. **11:**35–40.

ENZYME MEMBRANE IMMUNOASSAY (EMIA)

J. C. Braman, R. J. Broeze, D. W. Bowden, A. Myles, T. R. Fulton, M. Rising, J. Thurston, F. X. Cole, and G. F. Vovis

Department of Molecular Biology and Immunology, Collaborative Research, Inc., Lexington, MA 02173

Homogeneous enzyme membrane immunoassays (EMIAs) have been developed for thyroxine (T_4) and human immunoglobulin G (IgG). Liposomes tagged with T_4 and containing alkaline phosphatase were used as a model system for optimization of (a) liposome membrane attack in the presence of T_4 antiserum and guinea pig complement and (b) subsequent measurement of unmasked enzyme activity. Using a one-incubation-step format, the concentration of T_4 in human serum samples was measured. Alkaline phosphatase activity was inversely proportional to the concentration of T_4 in the sample. The T_4 values obtained by EMIA correlated well with values obtained by radioimmunoassay (r = 0.96). Likewise, there was an excellent correlation between IgG values obtained by EMIA and radialimmunodiffusion or nephelometry (r = 0.99).

Liposomes are spherical, membranous vesicles often prepared from phospholipids, sterols, and charged amphiphiles. Depending on the method of preparation, these vesicles are either multilamellar or unilamellar and have a variety of sizes. Because liposomes can be engineered to mimic the properties of biological membranes, they have been used as a model system to study the mechanism of complement-mediated immune cytolysis[1]. For example, Haxby et al.[2] demonstrated that liposomes prepared from sheep erythrocyte membranes and containing glucose as a marker could be damaged immunospecifically in the presence of rabbit anti-sheep erythrocyte serum and guinea pig complement. The extent and rate of glucose loss from these damaged liposomes was found to be dependent upon the amount of Forssman hapten incorporated into the vesicles, the concentration of