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Diminished Immunogenicity of a Recombination-Deficient Derivative of *Vibrio cholerae* Vaccine Strain CVD103

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To address potential concerns over the release of genetically engineered live bacterial vaccines, we constructed a recombination-deficient derivative of the *Vibrio cholerae* O1 vaccine strain CVD103 (CVD103RM). Oral immunization of adult volunteers with CVD103RM showed that the *recA* mutation significantly diminished colonization ability and immunogenicity of the vaccine strain.

Vibrio cholerae O1, the etiological agent of cholera, causes severe diarrhea in infected humans following colonization of the small intestine and production of an enterotoxin, cholera toxin. In *V. cholerae* O1, the cholera toxin structural genes (*ctxAB*) are chromosomal. Attenuated live oral vaccines have been derived by constructing defined deletion mutants unable to express the ADP-ribosylating A subunit of cholera toxin but still capable of producing the immunogenic B subunit (11, 14, 22). One such isogenic mutant, CVD103, a derivative of classical Inaba strain 569B, is significantly less reactogenic than are strains tested previously and confers protection against rechallenge with homologous and heterologous virulent *V. cholerae* O1 (13). The theoretical possibility exists that following conjugation or transduction in either the gastrointestinal tract or the environment, the mutated *ctx* gene could regain the deleted sequences by recombination of homologous flanking DNA sequences (15, 17, 27, 30).

The RecA protein plays an important part in homologous recombination in *Escherichia coli* (19, 31, 32). *E. coli recA* mutants have 10^{-3} to 10^{-6} the recombination frequency observed with wild-type strains (5, 7). A RecA protein with seemingly identical functions has been identified in *V. cholerae* O1, and the gene encoding this function has been cloned (5, 9, 28). Thus, *recA* mutations could theoretically have an important function in attenuated vaccine strains, as previously suggested by Goldberg and Mekalanos (5). To address the theoretical potential for reacquisition of proficient *ctx* genes, we constructed a derivative of the attenuated *V. cholerae* O1 strain CVD103 (CVD103RM), which was deficient in its ability to recombine homologous DNA sequences into the chromosome.

V. cholerae O1 chromosomal DNA from strain 569B (18), digested with *Eco*RI, was cloned into pBR322 and a clone containing the *recA* gene was selected by complementation in *E. coli* strain HB101 (*recA*). The resulting plasmid, pCVD839, contained an approximately 7-kilobase *Eco*RI DNA fragment which conferred resistance to methyl methanesulfonate (5) (Fig. 1). A frameshift mutation was constructed in the *recA* gene by the ligation of an 8-base-pair synthetic oligonucleotide containing the restriction site *Kpn*I into pCVD839 digested with *Rsa*I under conditions empirically found to produce plasmid linears (Fig. 1). The insertion mutation in pCVD842 was identified by screening for the loss of resistance to methyl methanesulfonate and was confirmed by restriction analysis.

Following mobilization of pCVD842 into CVD103 with the conjugal plasmid pRK2013 (4) (Fig. 1), plasmid-borne vibrio DNA flanking the inserted synthetic oligonucleotide sequence recombined with homologous chromosomal DNA (frequency of approximately 10^{-4}) to form a recombination-deficient derivative *recA* mutant, CVD103RM. The crossover event was detected by screening for the loss of *V. cholerae* O1 methyl methanesulfonate resistance, with care taken to minimally passage CVD103RM to avoid undesired spontaneous mutations. Hybridization (18, 33) with a *recA* gene probe (consisting of a 3-kilobase *Bgl*II *recA* gene fragment from pCVD839) of *Bgl*II- or *Kpn*I-digested chromosomal DNA or both confirmed the presence of a *Kpn*I site in the chromosomal *recA* gene of CVD103RM (Fig. 2). A spontaneous streptomycin-resistant derivative of CVD103 RM named CVD103RMSR was isolated in order to assist in the future identification and differentiation of the vaccine strain from wild-type *V. cholerae* O1.

To assess the effect of the *recA* mutation on CVD103RM, community volunteers were immunized orally with CVD-103RM and CVD103RMSR (Table 1). Volunteer studies were carried out under legal quarantine in the 32-bed isolation ward maintained by the Center for Vaccine Development in the University of Maryland Hospital (Baltimore, Md.) and were approved by the Human Volunteers Research Committee of the University of Maryland at Baltimore. The methods of medical screening, volunteer care, informed consent, stool cultures, and duodenal gelatin string cultures are described elsewhere (10, 13, 14, 23). The immunizing dose was administered orally following neutralization of gastric acid as previously described (10, 13, 14, 23). The reciprocal vibriocidal titer (2) is defined as the highest dilution of serum that completely inhibits growth of *V. cholerae* O1. An antitoxin unit is defined as the net optical density at 405 nm of sera tested at a 1:50 dilution by enzyme-linked immunosorbent assay against cholera toxin (16). Seroconversion is defined as a fourfold increase in vibriocidal titer or as a rise in antitoxin units 0.2 units above the prechallenge control.

The results of the volunteer studies showed that both CVD103RM and CVD103RMSR colonized volunteers less frequently and elicited lower peak serum vibriocidal and antitoxin levels than had been previously observed with CVD103 (Table 1). Of the 15 volunteers immunized, none

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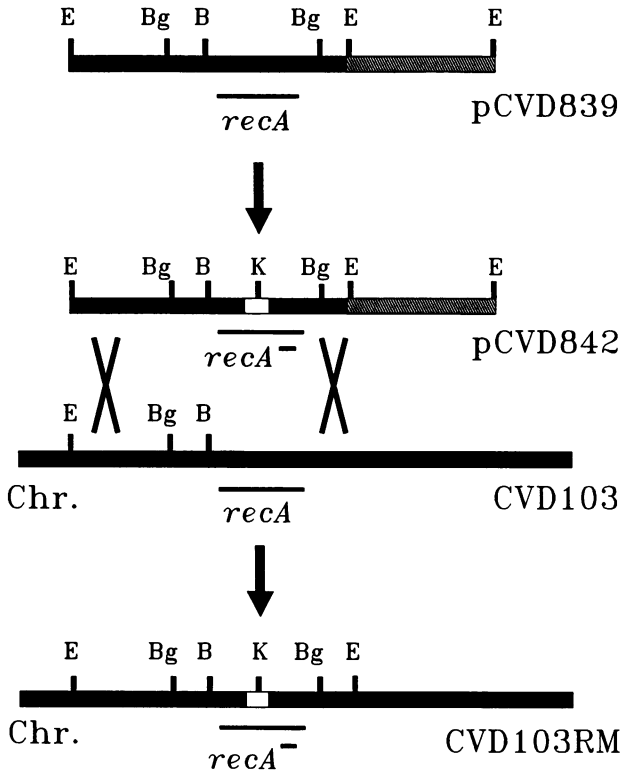

FIG. 1. Cloning and mutation of *recA* gene from 569B and introduction of mutated *recA* gene into CVD103. Hatched boxes indicate vector DNA. E, *Eco*RI; B, *Bam*HI; K, *Kpn*I; Bg, *Bgl*II. Chr., Chromosome.

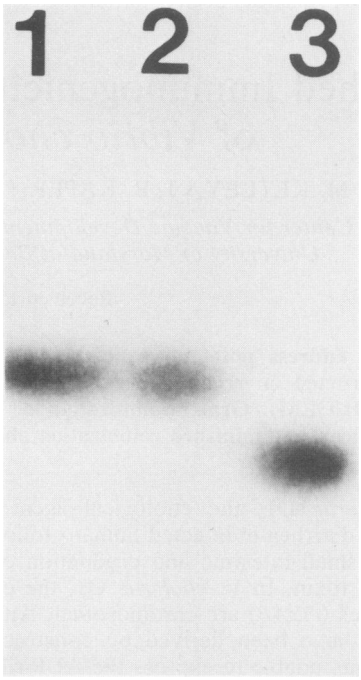

FIG. 2. Chromosomal DNA from CVD103 and CVD103RM restriction enzyme digested and probed with a *recA* gene probe to demonstrate the crossover event incorporating a *Kpn*I site into the *recA* gene. Lane 1, 569B restricted with *Bgl*II and *Kpn*I; lane 2, CVD103RM restricted with *Bgl*II alone; lane 3, CVD103RM restricted with *Bgl*II and *Kpn*I. The second expected labeled fragment (approx. 0.5 kilobases) in lane 3 was not retained on this gel.

experienced vomiting or other objective adverse reactions. Given that vibriocidal antibody levels correlate with protection (24–26; M. M. Levine and N. F. Pierce, *in* D. Barua and W. B. Greenough III, ed., *Cholera*, in press) and in view of the poor antibacterial and antitoxic immune response elicited in volunteers immunized with CVD103RM and CVD103RMSR, an experimental challenge study to assess the protective efficacy was not carried out for ethical reasons.

This study suggests that *recA* derivatives of live bacterial vaccine strains, such as CVD103, that require efficient gastrointestinal colonization for stimulation of protective immunity may be significantly reduced in their ability to protect. The reason for the reduced colonization by the *recA* mutant is not clear, but some hypotheses can be proposed. RecA is a crucial component of cellular recombination-repair mechanisms (3, 19, 31, 32). In the highly competitive gastrointestinal niche, a *V. cholerae* O1 *recA* strain may be at a disadvantage because of an accumulation of deleterious mutations. Indeed, comparisons of in vitro growth kinetics of CVD103 and CVD103RM showed a reduced growth rate in the mutant (a slightly reduced growth rate and extended lag phase were repeatedly observed with CVD103RM; J. M. Ketley, unpublished data). In addition, *recA* mutants of *E. coli* are more sensitive to deoxycholate than are *recA*$^+$ strains (12). *V. cholerae* CVD103RM is more sensitive than CVD103 to this compound (J. B. Kaper, unpublished data), and it is possible that CVD103RM is inhibited by intestinal bile salts.

A direct effect of the *recA* mutation on *V. cholerae* colonization factors may be possible. Colonization of the human intestine by *V. cholerae* O1 is incompletely understood; one factor, the toxin coregulated pilus (TCP) (34), is clearly involved in colonization (10), but several other fimbriae (8) and accessory colonization factors (29) could potentially play a role in this process. In *Serratia marcescens*, a variety of extracellular proteins, such as nuclease, chitinase, and lipase, require a functional *recA* gene for expression (1). *V. cholerae* could conceivably require *recA* for expression of surface colonization factors. Finally, *recA*-dependent in vivo amplification of the *ctx* genetic element during passage in rabbits has been demonstrated for *V. cholerae* O1 (6, 20). In the El Tor biotype, amplification requires one or more copies of the transposable RS1 genetic element flanking the *ctx* genes (20). (Amplification of *ctx* has not been reported in the classical biotype, which contains RS1 only on one side of *ctx* [21].) It is possible that undescribed RS1-like genetic elements duplicated in a *recA*-dependent manner may contain genes which affect colonization. In this case, a *recA* mutant could be at a disadvantage in the intestine. Both 569B and CVD103RM have been shown to produce toxin coregulated pilus (8; R. H. Hall, unpublished data) but it has not been reported whether these genes are associated with an amplifiable genetic element.

An alternative mutation to assist in addressing concerns about environmental release would be an assayable marker to distinguish the live attenuated vaccine strain from wild-type *V. cholerae* O1. As the use of an antibiotic resistance marker would be inadvisable, we have introduced a mercury resistance operon (*mer*) into the unexpressed *hlyA* gene of CVD103. The resulting strain, CVD103-HgR, has been tested in closed environments in adult volunteers at the Center for Vaccine Development in Baltimore (13) and at the

TABLE 1. Immunization of adult volunteers with CVD103RM and CVD103RMSR

Strain	Immunizing dose (CFU)	Positive stool culture[a] (%)	Positive duodenal culture (%)	No. of subjects seroconverted/total (geometric mean peak titer)[b]	
				Vibriocidal	Antitoxin
CVD103[c]	1×10^8	40/46* (87)	23/46 (50)	44/46 (1,260)	43/46 (1.06)
CVD103RM	1.9×10^8	3/8** (38)	3/8 (38)	7/8 (380)	7/8 (0.67)
CVD103RMSR	1.1×10^8	4/7*** (57)	1/5 (20)	7/7 (706)	6/7 (0.57)

[a] * versus **, $P = 0.003$; * versus ** + ***, $P = 0.0059$ (Fisher's exact test, two tails).
[b] The differences in the before and after changes in vibriocidal and antitoxin titers between volunteers vaccinated with CVD103 and those vaccinated with CVD103RM or CVD103RMSR are statistically different with P values of 0.04 and 0.0001, respectively (Kruskal-Wallis test).
[c] Volunteer challenge data from CVD103 were obtained during previous studies (13).

Vaccine Trial Center (Mahidol University, Bangkok, Thailand [23]) and has undergone authorized open trials at the Center for Vaccine Development and in Thailand (Thai component, Armed Forces Research Institute of Medical Sciences, Bangkok, and Vaccine Trial Center, Mahidol University). Given the negative effect of the *recA* mutation on *V. cholerae* CVD103, the *mer* marker has proven to be more useful than the *recA* mutation for addressing some of the concerns about environmental release of a live vaccine strain. However, it is conceivable that with future vaccine strains that exhibit a higher propensity for intestinal colonization, *recA* mutants may yet play a role in live oral *V. cholerae* vaccines.

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LITERATURE CITED

1. **Ball, T. K., C. R. Wasmuth, S. C. Braunagel, and M. J. Benedik.** 1990. Expression of *Serratia marcescens* extracellular proteins requires *recA*. J. Bacteriol. **172**:342–349.
2. **Clements, M. L., M. M. Levine, C. R. Young, R. E. Black, Y.-L. Lim, R. M. Robins-Browne.** 1982. Magnitude, kinetics and duration of vibriocidal antibody response in North Americans after ingestion of *Vibrio cholerae*. J. Infect. Dis. **145**:465–473.
3. **Cox, M. M., and I. R. Lehman.** 1987. Enzymes of general recombination. Annu. Rev. Biochem. **56**:229–262.
4. **Figurski, D., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. USA **76**:1648–1652.
5. **Goldberg, I., and J. J. Mekalanos.** 1986. Cloning of the *Vibrio cholerae recA* gene and construction of a *Vibrio cholerae recA* mutant. J. Bacteriol. **165**:715–722.
6. **Goldberg, I., and J. J. Mekalanos.** 1986. Effect of a *recA* mutation on cholera toxin gene amplification and deletion events. J. Bacteriol. **165**:723–731.
7. **Guyer, M. S., and A. J. Clark.** 1976. *cis*-Dominant, transfer-deficient mutants of the *Escherichia coli* K-12 F sex factor. J. Bacteriol. **125**:233–247.
8. **Hall, R. H., P. A. Vial, J. B. Kaper, J. J. Mekalanos, and M. M. Levine.** 1988. Morphological studies on fimbriae expressed by *Vibrio cholerae* O1. Microb. Pathog. **4**:257–265.
9. **Hamood, A. N., G. S. Pettis, C. D. Parker, and M. A. McIntosh.** 1986. Isolation and characterization of the *Vibrio cholerae recA* gene. J. Bacteriol. **167**:375–378.
10. **Herrington, D. A., R. H. Hall, G. Losonski, J. J. Mekalanos, R. K. Taylor, and M. M. Levine.** 1988. Toxin, toxin-coregulated pili, and the *toxR* regulon are essential for *Vibrio cholerae* pathogenesis in humans. J. Exp. Med. **168**:1487–1492.
11. **Kaper, J. B., H. Lockman, M. M. Baldini, and M. M. Levine.** 1984. Recombinant live oral cholera vaccine. Bio/Technology **2**:345–349.
12. **Lechevallier, M. W., A. K. Camper, S. C. Broadaway, J. M. Henson, and G. A. McFeters.** 1987. Sensitivity of genetically engineered organisms to selective media. Appl. Environ. Microbiol. **53**:606–609.
13. **Levine, M. M., J. B. Kaper, D. Herrington, J. Ketley, G. Losonski, C. O. Tacket, B. Tall, and S. Cryz.** 1989. Safety, immunogenicity, and efficacy of recombinant live oral cholera vaccines, CVD103 and CVD103-HgR. Lancet **ii**:467–470.
14. **Levine, M. M., J. B. Kaper, D. Herrington, G. Losonsky, J. G. Morris, M. L. Clements, R. E. Black, B. Tall, and R. Hall.** 1988. Volunteer studies of deletion mutants of *Vibrio cholerae* O1 prepared by recombinant techniques. Infect. Immun. **56**:161–167.
15. **Levine, M. M., J. B. Kaper, H. Lockman, R. E. Black, M. L. Clements, and S. Falkow.** 1983. Recombinant DNA risk assessment studies in humans: efficacy of poorly mobilizable plasmids in biologic containment. J. Infect. Dis. **148**:699–709.
16. **Levine, M. M., C. R. Young, R. E. Black, Y. Takeda, and R. A. Finkelstein.** 1985. Enzyme-linked immunosorbent assay to measure antibodies to purified heat-labile enterotoxins from human and porcine strains of *Escherichia coli* and to cholera toxin: application in serodiagnosis and seroepidemiology. J. Clin. Microbiol. **21**:174–179.
17. **Levy, S. B., and B. M. Marshall.** 1988. Genetic transfer in the natural environment, p. 61–76. *In* M. Sussman, C. H. Collins, F. A. Skinner, and D. E. Stewart-Tull (ed.), The release of genetically engineered microorganisms. Academic Press, Inc. (London), Ltd., London.
18. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
19. **McEntee, K., G. M. Weinstock, and I. R. Lehman.** 1979. Initiation of general recombination catalyzed *in vitro* by the *recA* protein of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **76**:2615–2619.
20. **Mekalanos, J. J.** 1983. Duplication and amplification of toxin genes in *Vibrio cholerae*. Cell **35**:253–263.
21. **Mekalanos, J. J., V. L. Miller, R. K. Taylor, I. Goldberg, and G. D. N. Pearson.** 1988. Regulation of cholera toxin synthesis, p. 247–258. *In* S. Kuwahara and N. F. Pierce (ed.), Advances in research on cholera and related diarrheas. KTK Scientific Publishers, Tokyo.
22. **Mekalanos, J. J., D. J. Swartz, G. D. N. Pearson, N. Harford, F. Groyne, and M. de Wilde.** 1983. Cholera toxin genes: nucleotide sequence, deletion analysis, and vaccine development. Nature (London) **306**:551–557.
23. **Migasena, S., P. Pitisuttitham, B. Prayurahong, P. Suntharasamai, W. Supanaranond, V. Desakorn, U. Vongsthongsri, B. Tall, J. Ketley, G. Losonsky, S. Cryz, J. B. Kaper, and M. M. Levine.** 1989. Preliminary assessment of the safety and immunogenicity of live oral cholera vaccine strain CVD103-HgR in healthy Thai adults. Infect. Immun. **57**:3261–3264.
24. **Mosley, W. H.** 1969. The role of immunity in cholera. A review of epidemiological and serological studies. Tex. Rep. Biol. Med. **27**(Suppl. 1):227–244.
25. **Mosley, W. H., W. M. McCormack, A. Ahmed, A. K. M. E. Chowdhury, and R. K. Barui.** 1969. Report of the 1966–67

cholera vaccine field trial in rural East Pakistan. 1. Study design and results of the first year of observation. Bull. W.H.O. **40**:177–185.

26. **Mosley, W. H., W. M. McCormack, A. Ahmed, A. K. M. E. Chowdhury, and R. K. Barui.** 1969. Report of the 1966–67 cholera vaccine field trial in rural East Pakistan. 2. Results of the serological surveys in the study population—the relationship of case rate to antibody titre and an estimate of the inapparent infection rate with *Vibrio cholerae*. Bull. W.H.O. **40**:187–97.
27. **O'Morchoe, S. B., O. Ogunseitan, G. S. Sayler, and R. V. Miller.** 1988. Conjugal transfer of R68.45 and FP5 between *Pseudomonas aeruginosa* strains in a freshwater environment. Appl. Environ. Microbiol. **54**:1923–1929.
28. **Paul, K., S. K. Ghosh, and J. Das.** 1986. Cloning and expression in *Escherichia coli* of a recA-like gene from *Vibrio cholerae*. Mol. Gen. Genet. **203**:58–63.
29. **Peterson, K. M., and J. J. Mekalanos.** 1988. Characterization of the *Vibrio cholerae* ToxR regulon: identification of novel genes involved in intestinal colonization. Infect. Immun. **56**:2822–2829.
30. **Saye, D. J., O. Ogunseitan, G. S. Sayler, and R. V. Miller.** 1987. Potential for transduction of plasmids in a natural freshwater environment: effect of plasmid donor concentration and a natural microbial community on transduction in *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. **53**:987–995.
31. **Shibata, T., C. DasGupta, R. P. Cunningham, and C. M. Radding.** 1979. Purified *Escherichia coli recA* protein catalyses homologous pairing of superhelical DNA and single-stranded fragments. Proc. Natl. Acad. Sci. USA **76**:1638–1642.
32. **Smith, G. R.** 1988. Homologous recombination in procaryotes. Microbiol. Rev. **52**:1–28.
33. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
34. **Taylor, R. K., V. L. Miller, D. B. Furlong, and J. J. Mekalanos.** 1987. Use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. Proc. Natl. Acad. Sci. USA **84**:2833–2837.