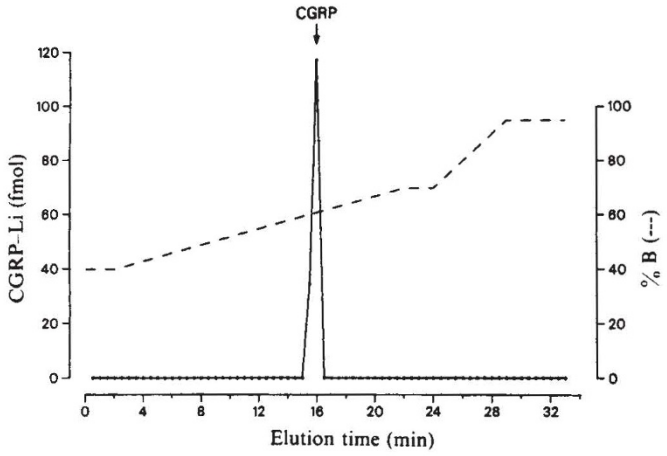

Fig. 3 Reverse-phase HPLC of pooled gel filtration chromatography samples taken from the zone containing >90% of the recoverable CGRP-LI (see text). Solvents for reverse-phase HPLC were 0.1% trifluoroacetic acid (TFA) in deionized water (solution A) and 0.1% TFA in a mixture of 60% acetonitrile/40% deionized water (solution B). Lyophilized sample or synthetic CGRP(1–37) was dissolved in 1.5 ml of solution A and 0.5–1.5 ml was applied to a Vydac C-18 column (0.46×25 cm, particle size 5 μm, pore size 330 Å). Chromatograms were run for 33 min at 20–25 °C in conditions of increasing amounts of solution B (see diagram) using an Altex gradient liquid chromatograph Model 332. Flow rate was 1.5 ml min^{-1} and fractions were collected every 0.5 min into glass tubes containing 5 μl of 10% bovine serum albumin (BSA) in H_2O. Fractions were lyophilized, reconstituted in assay buffer, neutralized with 2 M sodium hydroxide and assayed for CGRP-LI. Blank runs of injections of solution A were performed before each application of sample or synthetic CGRP(1–37). No CGRP-LI was detected in any of these collected fractions. Recovery of CGRP-LI from samples or synthetic CGRP(1–37) across the HPLC column was ~70%. When basal or 59 mM K^+ medium was taken directly for reverse-phase HPLC, using a Vydac or a Waters C-18 column (0.46×30 cm, particle size 10 μm, pore size 120 Å), the media from 50 or 25 dishes was first pooled into an equal volume of 2 M acetic acid at 4 °C containing 12.5 mM EDTA. The acidified medium was then heated at 90 °C for 5 min, cooled to 4 °C and then partially purified over Bond Elut (Analytichem) disposable C-18 cartridges to remove large (>20,000 molecular weight) proteins. Samples were then prepared and applied to the columns as described above. The arrow on the diagram indicates the elution time of synthetic rat CGRP(1–37).

CGRP-LI was attenuated (Fig. 2). Secretion of CGRP-LI by cultured trigeminal ganglion cells resembles calcium-dependent, high potassium-induced secretion of other peptides in different *in vitro* model systems[4,5]. Returning cells that had been stimulated with 59 mM potassium to culture medium for 18–24 h and repeating the experiment the next day demonstrated that cells could typically release significant amounts (70–100%) of CGRP-LI compared with the previous day.

Gel filtration chromatography of CGRP-LI released from trigeminal ganglion cell cultures depolarized with 59 mM potassium medium demonstrated that more than 90% of the total CGRP-LI recovered from the column eluted in the K_d range 0.28–0.41, with the peak at a K_d of 0.33. Synthetic CGRP(1–37) applied to the same column eluted in an almost identical manner (>90% CGRP-LI in K_d range 0.33–0.42, K_d peak at 0.38). In both cases only one peak of immunoreactivity was observed. No calcitonin-like immunoreactivity was detected in the column eluant or in media from cells depolarized with 59 mM potassium. In other experiments, we combined the fractions containing the CGRP-LI peak from gel filtration of medium from high potassium-stimulated cells. The pooled lyophilisate was then subjected to reverse-phase HPLC and only one peak of CGRP-LI was observed, which co-eluted with synthetic CGRP(1–37) (Fig. 3). Media from unstimulated (basal) and stimulated (59 mM potassium) cultured cells were also directly subjected to reverse-phase HPLC (following the procedures outlined in Fig. 3) and an identical profile was observed. Together, these data provide strong evidence that only one molecular form of CGRP-LI is released from our cultured trigeminal ganglion cells and that this is very similar, if not identical, to synthetic rat CGRP(1–37).

In summary, our data provide the first direct evidence of secretion of CGRP-LI from nervous tissue and support the possibility that CGRP may have a role as an extracellular modulator. Secretion appears to depend on the availability of extracellular calcium, as is expected in secretory systems of established physiological significance. Only one molecular form of CGRP-LI, apparently identical to the predicted rat CGRP(1–37), is released. Our observations agree with a previous report describing high levels of CGRP immunoreactivity and CGRP mRNA in trigeminal ganglia[2] and with studies suggesting a tissue specificity in the generation of mRNAs encoding calcitonin or CGRP[1,2]. Furthermore, the data support the possibility that alternative RNA processing mechanisms are a biologically significant means of increasing the diversity of extracellular regulatory molecules.

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Recombinant nontoxinogenic *Vibrio cholerae* strains as attenuated cholera vaccine candidates

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An ideal vacine does not yet exist to prevent cholera, a significant health problem in many less developed countries. *Vibrio cholerae*, the agent of epidemic and endemic cholera, colonizes the small bowel and secretes a potent enterotoxin that consists of a single A subunit, which stimulates adenylate cyclase activity, and five identical B subunits which bind to the ganglioside GM_1 receptor of intestinal mucosal cells[1]. Previous studies in man indicate that toxoid-derived antitoxic immunity by itself is insufficient to provide effective, long-lasting protection against cholera[2–4]. Using recombinant DNA techniques we have now constructed a live, attenuated *V. cholerae* strain by deleting genes encoding the enterotoxin. Restriction enzyme fragments encoding cholera toxin were deleted *in vitro* from cloned vibrio chromosomal DNA and the resulting mutations introduced into the chromosome of a vibrio strain of proven immunogenicity. Recently, Mekalanos and coworkers[5] have reported attenuated *V. cholerae* strains constructed by similar methods. It appears that recombinant DNA techniques offer a promising approach to the development of effective cholera vaccines.

Experimental cholera studies in community volunteers at the Center for Vaccine Development, University of Maryland, have

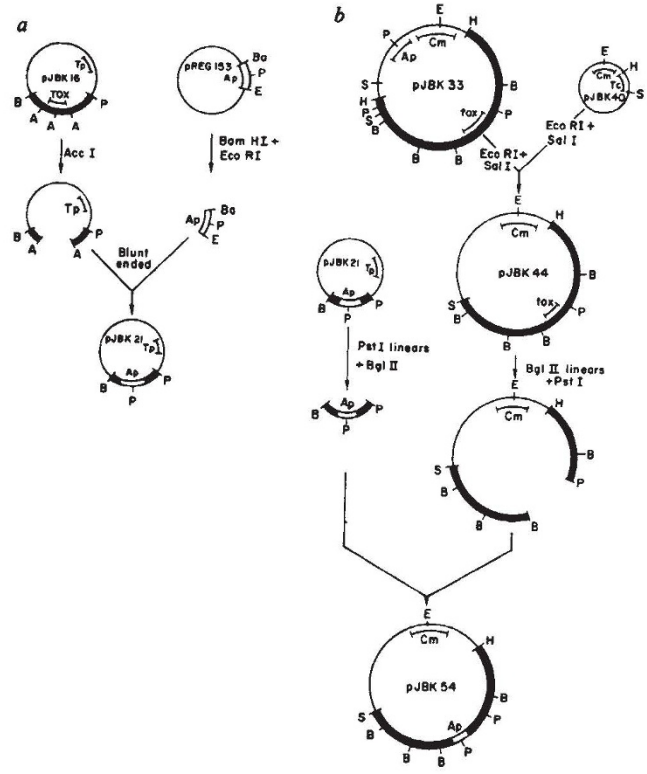

Fig. 1 Deletion of cholera toxin genes from cloned *V. cholerae* chromosomal DNA. *a*, Deletion of toxin genes. A 4 kb *Pst*I–*Bgl*II fragment of the recombinant plasmid pJBK16 (ref. 12) contains genes encoding cholera toxin flanked by *Acc*I sites with a third *Acc*I site in the B subunit structural gene. The two *Acc*I fragments were removed and a fragment from pREG153 encoding β-lactamase was cloned in their place. The heavier lines indicate vibrio DNA sequences. *b*, Addition of flanking *V. cholerae* chromosomal DNA sequences to toxin gene mutation. Plasmid pJBK33 consists of a 20 kb chromosomal *Hin*dIIIDNA fragment from *V. cholerae* N16991 cloned into pBR325 (pJBK33 contains 11 *Acc*I sites and so was unable to be as easily mutated as pJBK16). All but one *Pst*I site was removed by cloning a 19 kb *Eco*RI-*Sal*I fragment into pJBK40 to yield pJBK44. pJBK44 contains a 4.0 kb *Pst*I-*Bgl*II fragment bearing toxin genes as in pJBK16 flanked by approximately 7.0 and 7.5 kb of *V. cholerae* N16961 chromosomal DNA. Partial digestion with *Bgl*II yielded a population of linear molecules each digested at one of four *Bgl*II sites. These linear molecules were then digested to completion with *Pst*I and a 21 kb fragment containing all the sequences of pJBK44 except for the 4.0 kb *Pst*I–*Bgl*II toxin fragment was isolated. Plasmid pJBK21 DNA was partially digested with *Pst*I and then cut to completion with *Bgl*II to generate a 3.8 kb *Pst*I–*Bgl*II fragment containing the β-lactamase genes cloned in place of the cholera toxin genes. The purified fragments from pJBK44 and pJBK21 were then ligated to yield pJBK54, which contains a 19 kb *Hin*dIII–*Sal*I fragment of *V. cholerae* chromosomal DNA with β-lactamase cloned in place of the deleted cholera toxin genes.
Methods: *a*, pJBK16 (Tp^r) was digested to completion with the restriction endonuclease *Acc*I (BRL) and the resulting fragments separated by gel electrophoresis through 0.7% agarose. The largest fragment contained the plasmid vector and DNA sequences flanking, but not including the genes encoding cholera toxin. This fragment was electroeluted from the gel and extracted with phenol and ether. The ends were made blunt ended by filling in with the Klenow fragment of DNA polymerase I (BRL) according to the manufacturer's instructions. A fragment containing genes encoding β-lactamase and therefore resistance to ampicillin was obtained from plasmid pREG153 (received from R. E. Gill) by digesting with *Bam*HI and *Eco*RI (BRL) and purifying by agarose gel electrophoresis. The ends of this fragment were filled in with Klenow fragment, mixed with the purified, blunt-ended fragment from pJBK16 and ligated overnight using T4 DNA ligase (BRL) at 15 °C. The ligation mixture was then transformed into *Escherichia coli* HB101 and plated on Mueller Hinton agar (Difco) containing 50 μg ml^{-1} trimethoprim and 100 μg ml^{-1} ampicillin. Plasmids from the transformants were extracted by the method of Birnboim and Doly[17] and digested by a variety of restriction enzymes, including *Pst*I, *Bgl*II, *Eco*RI and *Bam*HI. One clone which gave the expected restriction pattern was designated pJBK21. *b*, pJBK33 DNA was digested to completion with *Eco*RI and *Sal*I (BRL) and mixed with pJBK40 DNA similarly digested with *Eco*RI and *Sal*I. pJBK40 is an ampicillin sensitive derivative of pBR325 which lacks a *Pst*I site. It was constructed by digesting pBR325 with *Pst*I and transforming linear molecules into *E. coli* strain HB101. Recircularization within the *E. coli* cell resulted in loss of Ap resistance and the *Pst*I site. Digested pJBK33 (Ap^r, Cm^r) and pJBK40 (Cm^r, Tc^r) DNA was ligated overnight at 15 °C with T4 ligase and transformed into HB101. Plasmid content of Cm^r, Tc^s, Ap^s colonies was analysed and one clone which gave the expected restriction pattern with only a single *Pst* site was designated pJBK44. Linear molecules of pJBK44 in which only one of the four *Bgl*II sites was cut were obtained by digesting pJBK44 with *Bgl*II enzyme in the presence of 0.1 mg ml^{-1} ethidium bromide[18]. After linearization, the ethidium bromide was removed by extraction with water saturated *n*-butanol and the DNA precipitated with ethanol. The *Bgl*II linears were then digested to completion with *Pst*I and the resulting fragments separated by gel electrophoresis through 0.3% agarose. The separated fragments were eluted, purified and analysed by digestion with *Bgl*II to identify the desired fragment, that is, a 21 kb fragment with all the DNA of pJBK44 except for the 4 kb *Pst*I–*Bgl*II fragment containing the toxin genes. Plasmid pJBK21 was treated similarly by linearization with *Pst*I in ethidium bromide followed by *Bgl*II digestion and separation by agarose gel electrophoresis. The desired fragment, a 3.8 kb *Bgl*II–*Pst*I fragment containing β-lactamase with an internal *Pst*I site, was mixed with the purified fragment from pJBK44, ligated and transformed into HB101. Plasmid DNA from Cm^r, Ap^r transformants was analysed and one clone which gave the expected restriction endonuclease pattern was designated pJBK54. This plasmid was identical to pJBK44 except for the substitution of the 3.8 kb *Pst*I–*Bgl*II fragment encoding β-lactamase from pJBK21 for the 4.0 kb *Pst*I–*Bgl*II fragment encoding cholera toxin from pJBK33.

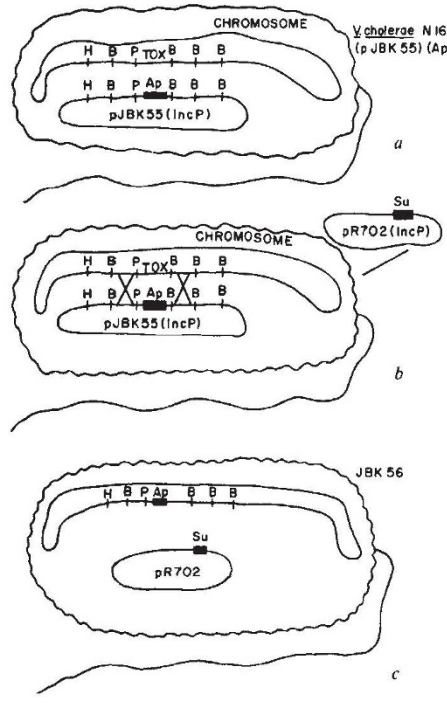

Fig. 2 Introduction of cholera toxin gene deletion into the chromosome of enterotoxigenic *V. cholerae* N16961. *a*, The plasmid pJBK55 was constructed by digesting pJBK54 (Ap^r, Cm^r) (Fig. 1*b*) and pRK 290 (Tc^r)[14] with *Eco*RI, ligating and selecting Ap^r, Tc^r, Cm^r transformants. pJBK55 was mobilized from *E. coli* HB101 into *V. cholerae* N16961 with the aid of a conjugative 'helper' plasmid pRK2013 (ref. 15). *b*, Selection of *V. cholerae* cells in which the toxin deletion mutation had recombined into the chromosome. Plasmid pR702 (Su^r, Sm^r, Tc^r, Km^r) (ref. 16) belongs to the Inc P group and was mated into *V. cholerae* N16961 (pJBK55). PB^r, Ap^r and Su^r colonies were selected, screened for Cm sensitivity and tested for toxinogenicity by GM_1 enzyme-linked immunosorbent assay (ELISA). *c*, One nontoxinogenic mutant, designated *V. cholerae* JBK56, was selected and confirmed as nontoxinogenic by GM_1 ELISA, Y-1 adrenal cell assay and DNA hybridization using a labelled cholera toxin gene probe.
Methods: Restriction enzyme digestion, ligation and plasmid extraction methods are as given in the legend for Fig. 1. *a*, Conjugal mating between *E. coli* HB101 (pJBK55) and *V. cholerae* N16961 was carried out by mixing 1 ml of mid-log cultures of donor and recipient grown statically at 37 °C in L-broth. The mixed cultures were filtered through 0.4 μm filters (Nuclepore) which were then placed on L-agar plates for approximately 4 h at 37 °C. At the end of the mating period, bacteria on the filter were resuspended in 1 ml L-broth and plated on L-agar containing 100 μg ml^{-1} ampicillin and 50 U per ml polymyxin B (Aerosporin; Burroughs Wellcome). (*V. cholerae* El Tor, unlike *E. coli*, is naturally resistant to 50 U per ml polymyxin B.) Plasmid content of Ap^r *V. cholerae* transconjugants was examined to confirm the presence of pJBK55. *b*, Conjugal mating between *E. coli* J53 (pR702) and *V. cholerae* N16961 (pJBK55) was performed as described above with selection on L-agar with 100 g ml^{-1} ampicillin and 100 μg ml^{-1} sulphonamide. *c*, Toxinogenicity testing by the Y-1 adrenal cell assay and GM_1 ELISA was performed as previous described[19,20]. DNA hybridization studies were performed using a colony hybridization technique[21] and labelled cholera toxin gene sequences. A cholera toxin gene probe was prepared by labelling a 1,200 base pair (bp) *Acc*I fragment from pCVD002 (ref. 13) by nick translation[22] using (^{32}P-dATP (800 Ci $mmol^{-1}$; NEN) and DNA polymerase I (NEN). Approximately 10^6 c.p.m. of labelled DNA was hybridized to the nitrocellulose filter in a solution consisting of 50% formamide, 5× SSC (SSC; 0.15 M NaCl plus 0.015 M sodium citrate), 1× Denhardt solution (0.02% Ficoll, molecular weight (MW) 400,000; 0.02% polyvinyl pyrrolidone, MW 360,000; 0.02% bovine serum albumin), 0.1% SDS and 1 mM EDTA at 37 °C for 18 h. After washing in 5×SSC–0.1% SDS at 65 °C for 1 h, the blots were dried and exposed to Kodak XR-5 film for autoradiography.

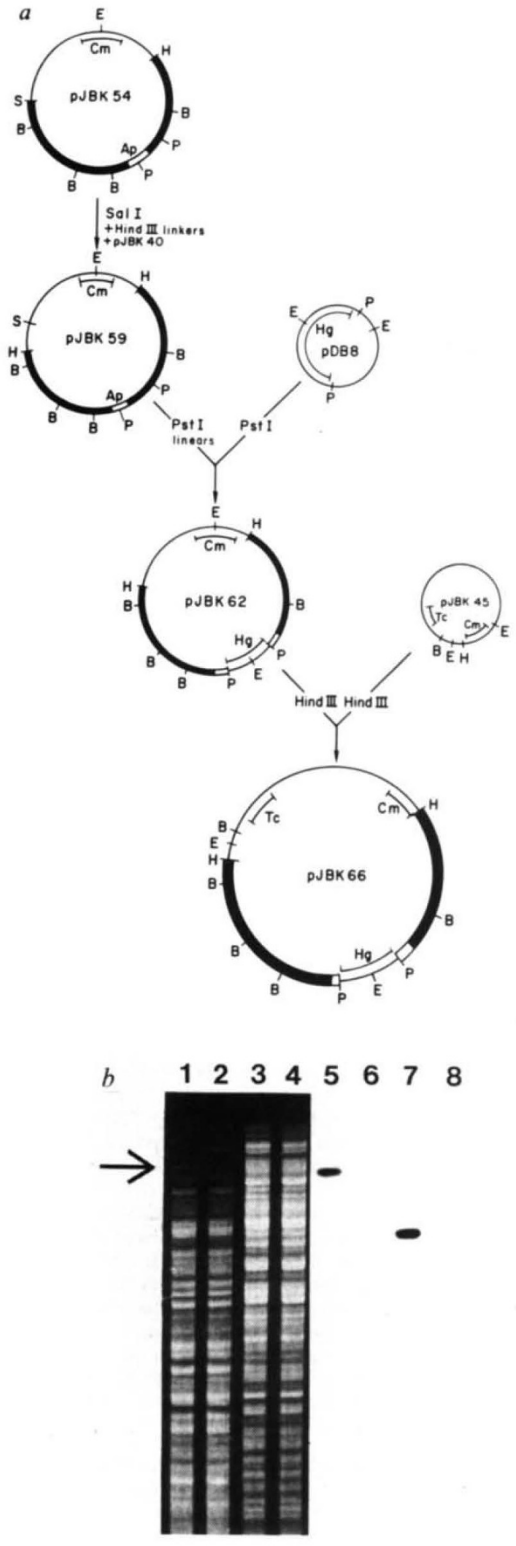

Fig. 3 Construction of mercury resistant, ampicillin sensitive non-toxinogenic *V. cholerae*. *a*, Construction of *V. cholerae* JBK70 (Hg^r). A second *Hin*dIII restriction site was added to pJBK54 by digesting pJBK54 with *Sal*I, filling in the ends with Klenow fragment and ligating *Hin*dIII linkers (BRL) to the filled in *Sal*I site. The ligated mixture was digested with *Hin*dIII and the large fragment containing the mutated vibrio DNA and the β-lactamase was purified by agarose gel electrophoresis. The purified fragment was ligated to *Hin*dIII digested pJBK40, transformed into *E. coli* HB101 and Ap^r, Cm^r clones were analysed for the presence of two *Hin*dIII sites flanking the vibrio DNA. The resulting plasmid, pJBK59, was partially digested with *Pst*I in the presence of ethidium bromide to yield linear molecules. These were mixed with *Pst*I-digested pDB8 (received from A. Summers), which contains the mercury resistance genes from R100 on a 7.5 kb *Pst*I fragment. Overnight ligations were transformed and Cm^r, Hg^r (50 M $HgCl_2$), Ap^s clones were analysed yielding pJBK62. The *Hin*dIII fragment from pJBK62 containing vibrio DNA and the mercury resistance genes inserted into the β-lactamase *Pst*I site was purified and cloned into pJBK45 to yield pJBK66. pJBK45 is a derivative of pRK290 (ref. 15) which contains a 4 kb *Eco*RI fragment containing chloramphenicol resistance gene and a *Hin*dIII site from the plasmid vector pKT212 (ref. 23). This plasmid was mobilized into *V. cholerae* JBK56 which had been spontaneously cured of pR702 and the homologous recombination process repeated (Fig. 2). The resulting nontoxinogenic Hg^r Ap^s *V. cholerae* strain was subsequently spontaneously cured of pR702 and was designated JBK70. *b*, Change in *V. cholerae* N16961 chromosomal restriction fragments due to homologous recombination of deleted toxin gene region shown by agarose gel electrophoresis and Southern blot analysis of restriction endonuclease fragments of *V. cholerae* strains N16961 and JBK70. Lanes 1, 5: N16961, *Hin*dIII; lanes 2, 6: JBK 70, *Hin*dIII; lanes 3, 7: N16961, *Pst*I; lanes 4, 8: JBK70, *Pst*I. The nontoxinogenic derivative, pJBK70, shows no homology with the cholera toxin probe, as shown in lanes 6 and 8. In lane 2, the total chromosomal digest of JBK70 appears unaltered from the parent strain (lane 1) except for the change in molecular weight of the largest *Hin*dIII fragment of N16961, which contains the toxin gene (indicated by arrow). As would be expected, the largest *Hin*dIII fragment shows an increase of approximately 7 kb due to the insertion of the mercury resistance gene (lane 2).

Methods: Restriction enzyme digestion, ligation and plasmid extraction methods are as given in the legend for Fig. 1. Chromosomal DNA was extracted from toxinogenic *V. cholerae* strain N16961 and the nontoxinogenic mutant JBK70 by the method of Brenner *et al.*[24]. Approximately 1 μg of DNA was digested with restriction endonuclease *Hin*dIII or *Pst*I (BRL) and restriction fragments were separated on a 0.7% agarose gel, transferred to nitrocellulose paper by the method of Southern[25] and hybridized to a ^{32}P-labelled cholera toxin gene probe as described in the legend to Fig. 2.

demonstrated that, as in nature, protective immunity follows infection with pathogenic *V. cholerae* and these studies have identified toxinogenic, immunogenic strains for attenuation as candidate cholera vaccines[6–10]. One such strain, N16961 (El Tor Inaba)[8], extensively studied in volunteers, was attenuated by specific deletions of restriction fragments containing the genes encoding cholera toxin. The fragments encoding enterotoxin were deleted *in vitro* from the recombinant plasmid pJBK16[11,12], and replaced with a fragment encoding β-lactamase and therefore resistance to ampicillin (Fig. 1a). Additional flanking vibrio chromosomal DNA was then added to ensure the homologous recombination of this deletion into the chromosome (Fig. 1*b*). The resulting plasmid, pJBK54, contained the β-lactamase in place of the toxin genes flanked by approximately 7 and 9 kilobases (kb) of vibrio DNA.

The construct was then introduced into the chromosome of N16961, by site-directed mutagenesis[13]. The mutated vibrio DNA in pJBK54 was cloned into the incompatibility group P plasmid pRK290[14] to produce pJBK55 which was then introduced into *V. cholerae* N16961 (Fig. 2*a*). In a small percentage of cells, the plasmid vibrio DNA sequences flanking the β-lactamase genes recombined with the homologous chromosomal sequences flanking the toxin genes. Homologous cross-over occurred, resulting in displacement of the toxin genes by the β-lactamase genes (Fig. 2*b*). This event was detected in a population of *V. cholerae* containing pJBK55 by introduction of a second P-group plasmid, pR702 (ref. 15), which is incompatible, that is, cannot be stably maintained with derivatives of pRK290. Resulting ampicillin and sulphonamide resistant cells represent those cells in which pR702 (Su^r, Sm^r, Tc^r, Km^r) is stably maintained extrachromosomally, the ampicillin resistance is integrated into the chromosome and the toxin genes and the pRK290 replicon are lost (Fig. 2*c*). One such mutant, JBK56, was found to lack the genes encoding cholera toxin. Because of possible clinical objections to a vaccine resistant to a widely used antibiotic, a second mutant, JBK70, was constructed which substitutes mercury resistance for ampicillin resistance (Fig. 3).

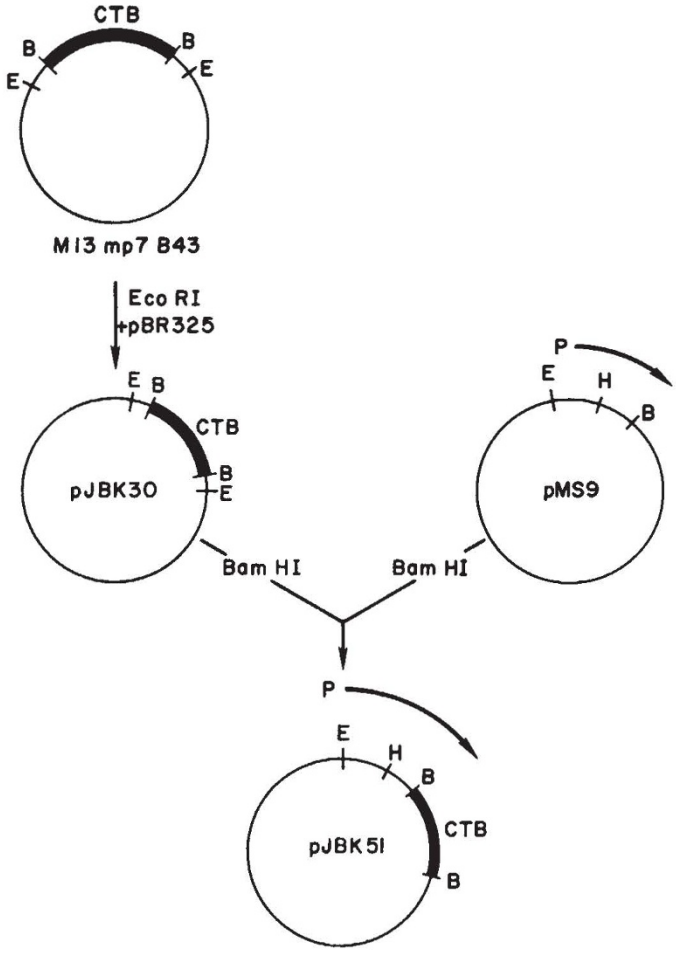

Fig. 4 Construction of *V. cholerae* JBK70 (pJBK51), an A^-B^+ derivative. During DNA sequencing studies of cholera toxin[13] a 1.3 kb *Hpa*II fragment containing the A_2 and B, but not the A_1 subunit genes, was cloned into the M13 mp7 vector, resulting in M13 mp7 B43. The placement in the M13 vector positioned *Bam*HI and *Eco*RI sites adjacent to this fragment which was subsequently cloned into pBR325, yielding pJBK30. The *Bam*HI fragment from pJBK30 was then cloned into pMS9, which contains the trp promoter from *Serratia marcescens*. This vector consists of a pBR32R vector plus 94 bp of *S. marcescens* DNA containing the promoter, but no attenuator region or ribosome binding site (ref. 26 and C. Yanofsky, personal communication). A putative ribosome binding site for the cholera toxin B subunit is found in the structural gene for the A_2 subunit[13] and when the *Bam*HI fragment from pJBK30 was cloned into the *Bam*HI site of pMS9, ~50% of the resulting clones produced B subunit (as measured by GM_1 ELISA), reflecting the orientation of the insert. The resulting plasmid, pJBK51, was mobilized into *V. cholerae* JBK70 by pRK2013 to produce an attenuated *V. cholerae* strain producing B subunit only.

Volunteer studies indicate that an antibacterial response is essential for immunity[4], so immunization with the non-toxinogenic strain may be sufficient for protection against pathogenic toxinogenic *V. cholerae*. Inhibition of the initial colonization of the vibrios along the mucosa of the small bowel is apparently the key immune mechanism in the protection that follows natural infection. Nevertheless, as antibodies to cholera toxin work synergistically in animal models to enhance immunity, a plasmid has been constructed which produces only the nontoxic B subunit (Fig. 4). This plasmid has been introduced into *V. cholerae* JBK70, after which it was found to produce B antigen but not holotoxin. A previously studied A^-B^+ *V. cholerae* strain, Texas Star-SR, attenuated by multiple rounds of nitrosoguanidine mutagenesis[16], stimulated infrequent and meagre antitoxin responses that appeared to be unrelated to successful protection against challenge with pathogenic *V. cholerae*[9]. Attenuated vaccine strains derived by such methods, however, suffer from a number of disadvantages: (1) the precise genetic mutation is unknown so the theoretical risk of reversion to toxinogenicity remains; (2) nitrosoguanidine may induce other unrecognized mutations affecting other antigens that contribute to immunity. The method we have described is free of these disadvantages as it eliminates the possibility of reversion to toxinogenicity, due to the complete deletion of the toxin genes, while it leaves unaffected all other antigens important for immunity. Clinical studies are underway at the Center for Vaccine Development to assess the safety and efficacy in man of the attenuated *V. cholerae* strain JBK70.

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Scrapie infectious agent is virus-like in size and susceptibility to inactivation

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The virions of all known viruses are composed of small amounts of genomic nucleic acid enveloped by proteins and other macromolecules. The aetiological agents of scrapie disease and the other subacute spongiform virus encephalopathies (SSVE), a group of slow, fatal degenerative diseases of the central nervous system, are, based on their resistance to sterilization and on indirect measurements suggesting subviral size, thought to have non-viral structures (see refs 1–3 for reviews). The kinetic studies reported here demonstrate that scrapie's resistance to many inactivants is limited to small subpopulations of the total infectivity, the majority population being highly sensitive to inactivation. Moreover, control inactivations of conven-