Laboratory Animal Science
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Vol 48, No 5
October 1998

# Intraduodenal Inoculation of Adult Rabbits for Evaluating the Immunogenicity of Genetically Attenuated *Vibrio cholerae* Strains

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Cholera is a major diarrheal disease in several regions of the world where the causative agents *Vibrio cholerae* strains O1 and 0139 remain endemic. Efforts are underway to develop vaccines of greater efficacy to prevent the disease and control its spread.

Research on new cholera vaccines has focused on oral formulations that stimulate the mucosal immune system, thereby mimicking natural infection (1). Genetically attenuated *V. cholerae* strains, administered orally, are at present the most promising variety of cholera vaccine (2, 3). Several authors have reported development of attenuated *V. cholerae* El Tor biotype strains, the predominant biotype in the current pandemic. Benítez and co-workers in the National Center for Scientific Research, Havana, Cuba have obtained a group of attenuated El Tor Ogawa and El Tor Inaba, *V. cholerae* strains that have deleted the cholera toxin and other toxin genes (Table 1) (4–6).

Although a large variety of animal models have been used in cholera research, in no single model are all the aspects of the human disease reproduced (7). Various methods have been used to yield information about specific aspects of the disease, including strain virulence and colonization capability and strain immunogenicity. *Vibrio cholerae* can readily colonize and infect infant animals and are used to study microorganism virulence and colonization, but these animals are not effective for immunogenicity and antibody production kinetics assessment. Two models have been useful for evaluation of immunogenicity of attenuated *V. cholera* strains: oral inoculation of adult rabbits (8) and the recently introduced germfree mouse model (9).

We have used intraduodenally inoculated adult rabbits to evaluate the immunogenicity of various genetically attenuated *V. cholerae* strains prior to their use in volunteer clinical studies. A similar model has been successfully used by other authors for evaluation of the immunogenicity of naturally attenuated *V. cholerae* strains (10); however, the details of this method are available only in the Chinese literature.

The animal model is easy to use, and the strains inoculated are capable of stimulating an immune response without the need for neutralization of gastric acid. Despite the fact that this method does not reproduce natural *V. cholerae* infection in humans, it is important for us to know that the attenuated strains are capable of inducing an immune response comparable to that of their wild-type parental strain.

**Table 1.** *Vibrio cholerae* strains

| Strain | Relevant genotype and/or phenotype | Reference |
|---|---|---|
| | wild-type strains | |
| C 7258 | O1 El tor Ogawa, Peru, 1991 | 8 |
| C 6706 | O1 El Tor Inaba, Peru, 1991 | 8 |
| | Attenuated strains | |
| CVD 103 HgR[a] | O1 Classic Inaba ctxA⁻, hlyA::HgR | 9 |
| 81 | ctxA⁻ctxB⁻zot⁻ace⁻orfU⁻cep⁻ mutant from C 7258 | 3 |
| 413 | ctxA⁻ctxB⁻zot⁻ace⁻orfU⁻cep⁻ mutant from C 6706 | 3 |
| 638 | CtxA⁻ctxB⁻zot⁻ace⁻orfU⁻cep⁻ hap::celA mutant from 81 | 4 |
| 1333 | CtxA⁻ctxB⁻zot⁻ace⁻orfU⁻cep⁻ hap::celA mutant from 413 | 4 |

[a]Current cholera oral vaccine strain

Conventional, locally supplied New Zealand White rabbits were used in ligated ileal loop and intraduodenal inoculation models. They weighed 2 to 2.5 kg and were 9 to 11 weeks old when the study began. The experiments and procedures were approved by The Finlay Institute's Committee for the Care and Use of Laboratory Animals. Rabbits were housed in Macrolon rabbit cages (INPUD, Havana, Cuba) and kept in isolators. Temperature (18 ± 2°C), relative humidity (45 ± 5%), and ventilation (15 changes/h) were controlled according to the species requirements. Water and food (special rabbit food purchased by National Center for Production of Laboratory Animals) were supplied ad libitum. Food was withheld from animals before surgery (12 h before intraduodenal administration and 24 h before creation of the ligated ileal loop).

Bacterial strains used are listed in Table 1. Inocula were prepared as follows: stock cultures were stored at -70°C in 10% skim milk with 20% glycerol. To prepare each inoculum, a sample was thawed, inoculated into tryptone soya broth (pH 7.3), and cultured overnight at 37°C. A 250-ml Erlenmeyer flask containing 50 ml of the same medium was inoculated with 2 ml of the overnight culture and was shaken at 200 cycles/min for 6 h at 37°C. Viable bacterial counts were determined on tryptone soya agar by use of the drop-plate method. Inocula were prepared by diluting the bacterial suspensions in fresh tryptone soya broth medium.

The ligated ileal loop model has been successfully used in the evaluation of *V. cholerae* strain pathogenicity and cholera toxin action, with the use of this surgical modification is possible to reach the small bowel colonization and

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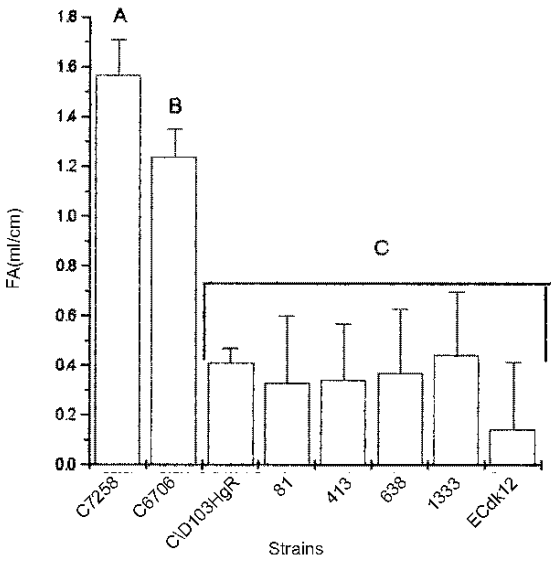


**Figure 1.** Ligated ileal loop results for fluid accumulated (FA) for various *Vibrio cholerae* strains and *Escherichia coli* K12 as negative control. For further details *see* **Materials and Methods**. Each FA value represents the mean of 6 to 10 determinations in three or four animals. Bars depict the SEM. Significant differences between A and B and between A and B and C ($P < 0.05$).

fluid production. Ileal loops were created as described (11). Briefly, rabbits were anesthetized with pentobarbital (25 mg/kg of body weight, intravenously [i.v.]), and the small intestine was withdrawn and ligated approximately 10 cm from the appendix. The intestine was divided into 9 or 10 segments of 5 to 6 cm by ligatures, and $10^8$ colony-forming units (CFU) of live vibrios and *Escherichia coli* K-12 as negative control in 0.5 ml of phosphate-buffered saline were injected. We tested two or three strains (at least two loops/strain) for each animal. After 16 to 18 h, the animals were sacrificed by administration of an overdose of pentobarbital, and loop length and fluid volume were determined. Results are expressed as fluid accumulation (FA, ml/cm).

For the intraduodenal inoculation model, the anesthetic was administered as described previously. The hair on the abdomen was shaved, the skin was disinfected, and after pentobarbital anesthesia (25 mg/kg), a 3-cm laparotomy was performed at the midline and over the navel. The duodenum was localized and extracted, and a 5-ml bacterial suspension containing $10^9$ CFU was injected in the luminal space. The organ was placed back into the abdominal cavity and the wall was sutured. One hour later, atropine (0.1 mg/kg) was administered intramuscularly to each rabbit with the aim of prolonging inhibition of intestinal motility after administration of the anesthetic. Animals were then housed in their cages. Three rabbits were used as control and received phosphate-buffered saline (PBS) instead of viable bacteria.

Blood was collected by puncture of the central artery of the ear, using 23-gauge needles fitted to disposable syringes, on days 0, 7, 14, 21, and 28. Antibacterial antibodies were measured by ELISA with *V. cholerae* Ogawa and Inaba lipopolysaccharides (LPS) as solid-phase antigen in Nunc Maxisorp plates (Nunc, Denmark). The LPS from strains E7946 (El Tor, Ogawa) and N16961 (El Tor, Inaba) were isolated by use of the method of Thomashow and Rittenberg (12). After incubation with PBS-diluted test serum (twofold dilutions), horseradish peroxidase anti-rabbit IgG conjugate (Sigma Chemical Co., St. Louis, Mo.) diluted 1:2,000 was added to the wells. The plates were treated with *o*-phenylenediamine (Sigma Chemical Co.), then were read at an optical density of 492 nm. The antibody titer was considered to be the interpolated dilution of the sample giving an absorbance value of 0.4 above background (13).

**Table 2.** The ELISA IgG lipopolysaccharide antibody titer[a] in rabbits inoculated intraduodenally with attenuated and wild-type *Vibrio cholerae* strains

| | Geometric mean titer[b] (SD) days after inoculation | | | |
|---|---|---|---|---|
| Strain | 7 | 14 | 21 | 28 |
| C 7258 | 1.029 (0.89) | 1.724 (1.65) | 2.689 (0.98) | 3.2 (0.98) |
| C 6706 | 1.312 (1.18) | 2.28 (0.32) | 2.489 (0.32) | 2.483 (0.708) |
| 81 | 1.499 (0.33) | 2.04 (0.45) | 2.83 (0.91) | 3.1 (0.95) |
| 413 | 0.313 (0.54) | 1.853 (0.83) | 2.793 (0.25) | 2.16 (0.56) |
| 638 | ND | 2.17 (1.95) | 2.64 (2.32) | 2.48 (2.17) |
| 1333 | ND | 1.88 (0.93) | 1.761 (0.58) | 1.82 (0.31) |
| CVD 103 HgR | ND | ND | ND | ND |
| Controls | ND | ND | ND | ND |

[a]Interpolated dilution with OD value of 0.4 above background
[b]Each value represents the mean of three animals
ND = Not detectable. No dilution reached OD of 0.4.

Serum vibriocidal antibody titers were measured by use of a microassay on days 0, 14, 21, and 28 (14). Twenty-five microliters of serial twofold dilutions of the test sera in 0.85% saline were placed in the wells of sterile 96-well culture plates. Twenty-five microliters of a $10^7$ CFU/ml culture of *V. cholerae* classical biotype Ogawa serotype VC12 and classical biotype Inaba serotype VC13 strains (used as reference strains under direction of Centers for Disease Control and Prevention, Atlanta, Ga.) with human complement diluted 1:5 was added to the serum dilutions, which were incubated for 1 h at 37°C. Brain-heart infusion broth containing 2% dextrose and 2% bromocresol purple was added to each well, and the plates were incubated for approximately 3 h at the same temperature. In the well where bacterial growth occurs, the medium changes color, from purple to yellow. The vibriocidal antibody titer was calculated as the highest dilution of serum causing complete inhibition of bacterial growth determined by invariability in the medium color, as examined visually.

Statistical analysis was performed, using analysis of variance and the *t* test for comparison of two means, with $P < 0.05$ indicating significant difference.

Animals recovered from surgery within 2 to 3 h without complications. Sutures were removed a week after surgery. During the entire period of the experiment, animals consumed food and water at amounts comparable to those for the negative controls and the rest of the rabbits that were not involved in the experiment.

The amount of fluid accumulated in the ligated ileal loop model is presented in Figure 1. Both parental wild-type strains had significantly higher FA values than those for all attenuated strains, although we found differences be-

Vol 48, No 5
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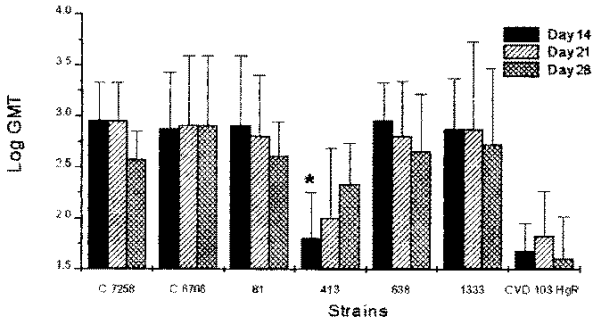


**Figure 2.** Vibriocidal antibody assays after intraduodenal inoculation. *See* text for details. Each value represents the mean of three animals. *Significant ($P < 0.05$) difference from the rest. At day 0, no animal had antibody titer, and control animals did not have titer at any time.

tween C 7258 and C 6706. There were no differences among our attenuated strains or in comparison with the licensed human oral cholera vaccine strain, CVD 103 HgR.

The ELISA LPS antibody titers of rabbits inoculated by the intraduodenal route are shown in Table 2. We did not find significant differences in specific antibody values among all El Tor strains regardless of whether they were wild-type or attenuated. The 638 and 1333 strains that have a second genetic manipulation in addition to the deletion of the "virulence cassette" (Table 1) had different antibody kinetic patterns, with nondetectable titer 7 days after inoculation. The LPS antibodies were not found at any time in animals inoculated with the CVD 103 HgR classical strain.

Similar results were obtained in the vibriocidal titer assay: no significant differences between wild-type and attenuated strains, except in the case of strain 413 at 14 days, when the vibriocidal titer was lower than that for the rest of the strains; and the low vibriocidal titer in animals inoculated with the classical biotype CVD 103 HgR strain. No samples from control animals had vibriocidal antibody titer, similar to the inoculated rabbits on day 0 (Figure 2).

Intestinal colonization of humans by virulent *V. cholerae* O1 and 0139 stimulates substantial lasting immunity against reinfection (15). These observations are the basis for current attempts to develop an effective oral vaccine for cholera by using antigenic products or live attenuated mutants.

Immunogenicity studies of attenuated *V. cholerae* strains in animals could be a useful step prior to evaluation of these candidate live oral vaccine strains in volunteers. No animal model is completely effective, because most animals naturally resist intestinal colonization with *V. cholerae*. However, a variety of nutritional, chemical, antibiotic, and surgical treatments allow adherence and colonization of *V. cholerae* in some animal species.

Transient reduction of peristalsis and careful neutralization of gastric acid by intravenous administration of cimetidine have allowed studies of the immunogenicity of live orally administered *V. cholerae* strains in adult rabbits (8). This study indicated that adult rabbits can be intestinally colonized, with intestinal peristalsis reduction, for several hours, using tincture of opium given intraperitoneally (i.p.). We have tried to obtain the same results using this method but by avoiding opium treatment or by i.p. administration of atropine; however, we did not have good results because we obtained measurable antibody responses only after two doses of orally administered virulent and attenuated *V. cholerae* strains (data not shown).

For obvious economic reasons, an older mouse model would have many advantages for measuring immune response to cholera. Recently a germfree mouse model was successfully developed, but it has two important shortcomings: germfree animals are more expensive than non-germfree animals, and gastrointestinal tract physiology and intestinal immune responses in germfree mice may not be entirely normal (9).

In this study, we used adult rabbits inoculated intraduodenally by use of a simple, quick surgical procedure to evaluate the immunogenicity of living attenuated *V. cholerae* biotype El Tor candidate vaccine strains. Our main objective is to have a method for evaluation of the relative immunogenicity of these strains in comparison with the wild-type parental strains.

Our results indicate that this model is useful for measuring the systemic immune response to these live strains and that it is possible to achieve presumptive colonization of the small bowel and antigenic presentation to the local immune system. There were no significant differences in response, using an ELISA, for LPS antibodies or vibriocidal antibody titer, between different El Tor strains regardless of whether they were wild-type parental or genetically manipulated (except for the unexplained reduced response with 413 strain at 14 days in the second method). An interesting observation is that only strain CDV 103 HgR had undetectable LPS antibody values and low vibriocidal titers determined by ELISA. Despite being the most promising current oral cholera vaccine candidate, this strain is a poor colonizer, higher dosages are required, and the immunity induced is not as solid as that induced by other attenuated strains derived from El Tor biotype (16). Nevertheless, extensive testing has documented the safety of this vaccine, which has recently been licensed in several countries (3, 17, 18). Using the adult rabbit orally inoculated model, Acheson et al. (19) found an immune response against CVD 103 HgR strain 5 weeks after the second dose. In a two-dose regimen scheme, using the intraduodenal inoculation model, we have observed that this strain is capable of eliciting anti-LPS titer 7 days after the second dose (data not shown), while the described systemic immune response elicited by El Tor strains was obtained after a single inoculation.

The ligated ileal loop model has been used extensively to evaluate the virulence of *V. cholerae* and its toxins (5, 20). The low FA values of our attenuated strains, compared with wild-type parental strains, is a confirmation that they have lost the capacity to produce cholera toxin (Figure 1). In addition, we recently reported that these attenuated strains colonize, similar to their parental strains, in the infant mouse model (21).

We conclude that this model of intraduodenal inocu-

lation is useful for evaluating the immunogenicity of *V. cholerae* strains, being the last preclinical study prior to evaluation of strain 638 for reactogenicity and immunogenicity in controlled studies in volunteers in Cuba.

## Acknowledgements

We thank Dr. Jorge Benítez, National Center for Scientific Research, for providing the attenuated *Vibrio cholerae* strains; and Catherine Ribas, Finlay Institute, for revision and correction of the English manuscript.

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