Evaluation of Peru-15, a New Live Oral Vaccine for Cholera, in Volunteers

David A. Sack, R. Bradley Sack, Janet Shimko, George Gomes, Donna O’Sullivan, Karen Metcalfe, and Dale Spriggs

A new live oral cholera vaccine, Peru-15, was studied for safety, immunogenicity, and excretion in 2 groups of healthy volunteers. Twelve inpatient volunteers received freshly harvested vaccine in doses of either $10^8$ or $10^9$ cfu. Subsequently 50 outpatient volunteers received freeze-dried vaccine in doses of $10^6$ or $10^9$ cfu or placebo in a three-cell, double-masked, placebo-controlled trial. The strain was well tolerated at all dose levels, and it stimulated high levels of vibriocidal antibodies in most inpatient volunteers and in all outpatient volunteers. Although antitoxin responses were less frequent and of lower magnitude than the vibriocidal responses, antitoxin responses were seen in >60% of the outpatient volunteers. About 60% of the volunteers excreted the vaccine in their feces; however, fecal excretion did not correlate with serologic responses. It is concluded that Peru-15 is a safe and immunogenic oral vaccine for cholera.

Cholera remains an important public health problem with cases occurring in nearly all developing countries, including 21 countries in the Western Hemisphere [1]. While improved water and sanitation may eventually control the transmission of *Vibrio cholerae*, development of an effective vaccine could be extremely helpful in limiting morbidity and mortality from the disease. Both killed and live oral vaccines are being developed for cholera [2–5], and each has attractive attributes. Among a group of mutant *V. cholerae* strains [6], one promising live oral vaccine candidate is Peru-15 [7]. Derived from an El Tor Inaba isolate, this new live oral vaccine is similar to the current pandemic strain.

Peru-15 has several defined genetic mutations that contribute to its attenuation. First, wild-type *V. cholerae* C6709 was deleted at what was formerly termed the cholera toxin genetic element [8], recently discovered to encode a filamentous bacteriophage, designated CTX-phage [9]. Next, the cholera toxin B subunit gene, regulated by a *V. cholerae* heat-shock promoter, was inserted into the recA locus, yielding Peru-3. This insertion inactivated recA, causing Peru-3 to be defective in homologous DNA recombination. A nonmotile variant was isolated and designated Peru-15 [7].

Following initial studies by Kenner et al. [7], Peru-15 was tested further at the Vaccine Testing Unit, Johns Hopkins University. Since Peru-15 is a newly developed, live, oral vaccine strain, the first study was conducted in an inpatient unit, using a freshly prepared vaccine. The initial inpatient study was needed for careful monitoring of the volunteers and for decontamination of their stools before disposal. After safety and stability of the vaccine in inpatient volunteers was demonstrated, an outpatient, placebo-controlled study was conducted using a freeze-dried formulation to further evaluate its safety and immunogenicity.

Materials and Methods

**Inpatient study.** Twelve community volunteers, aged 18–50 years, were recruited for the inpatient study using advertisements in a local newspaper. Inclusion criteria included being healthy, being willing to participate, and completing a training session designed to provide sufficient knowledge of the disease and the protocol to give informed consent. Exclusion criteria included the following: chronic illness, immunosuppressive condition, abnormal stool pattern, human immunodeficiency virus antibody positivity, hepatitis B surface antigen positivity, pregnancy by history or by a positive urine human chorionic gonadotropin test 2 days before the study began, travel to a cholera-endemic area within 5 years, receipt of cholera vaccine, history of cholera infection, previous participation in a cholera or enterotoxigenic *Escherichia coli* study, inability to pass the written examination on diarrhea, cholera, and cholera vaccines, significant abnormality in screening laboratory hematologic and chemistry tests, and use of antibiotics within 7 days of vaccination.

The volunteers were admitted to the General Clinical Research Center, Johns Hopkins Hospital, and were randomized to receive either $10^8$ or $10^7$ cfu of the vaccine strain in a double-masked
manner. On the day of admission, the volunteers drank their assigned dose of vaccine with 100 mL of buffer (2.5 g of sodium bicarbonate and 1.65 g of ascorbic acid). Volunteers fasted for 60 min before and after the dose. All volunteers received active vaccine (i.e., none received placebo), but the clinical and laboratory staff were masked as to the dose each volunteer received.

The volunteers were clinically monitored at least twice daily by a study physician, vital signs were taken four times daily, and all stools were examined, graded, and weighed. Stool consistency was graded with a score from 1 to 5 as previously described [5]. Stool grades 1 and 2 were considered normal, while grades 3–5 were considered loose or watery. Intake and output of fluids were measured. Diarrhea was defined as ≥2 stools of a consistency of ≥3 totaling ≥400 g or a single stool with a consistency of ≥3 totaling ≥300 g in 24 h. The first 2 stool specimens of each day from each volunteer were cultured to detect excretion of the vaccine strain, using both quantitative and qualitative methods.

On day 6, all volunteers began taking doxycycline: 200 mg as a first dose, followed by 100 mg twice daily for 3 more days. The volunteers were discharged when their stools were negative for the vaccine strain. During the inpatient stay, volunteers were under enteric (contact) precautions, and all feces were passed into disposable plastic containers for observation by study personnel. After being weighed and sampled, feces were decontaminated by being soaked in disinfectant for 1 h, after which the stool was flushed down the toilet.

Serum samples were collected before vaccination, on day 6 after vaccination (before starting antibiotic), and on days 14 and 28 after vaccination. Stool samples were collected on days 14 and 28 during follow-up visits to evaluate the possibility of continued excretion of the vaccine strain.

**Outpatient study.** Fifty healthy men and women, 18–50 years old, were recruited to participate in the outpatient study, designed to extend the inpatient studies by evaluating the same vaccine strain when prepared in a lyophilized form. Recruitment for the outpatient study was primarily through University bulletins and most volunteers were either students or employees of Johns Hopkins University or Hospital. The same inclusions and exclusions were used for the outpatient study, but since this was the first outpatient study with this strain, potential volunteers were also excluded if they were food handlers or had close contact with children under age 5, immunosuppressed persons, or pregnant women.

Volunteers were randomized to receive either a 10⁹ or 10⁸ cfu dose or a placebo in a double-masked manner. The placebo consisted of freeze-dried cryoprotectant buffer with 1% powdered skim milk, identical to that in which the vaccine strain was prepared. At the time of vaccination the lyophilized bacteria (or placebo) were reconstituted in 1 mL of the buffer administration solution. Then either 1 or 0.1 mL of the vaccine was added to 100 mL of the sodium bicarbonate–ascorbic acid buffer, and this was given to the volunteer to drink. Each volunteer swallowed the dose while being observed by an investigator (J.S.). To protect the masked code, some volunteers were assigned to 1 or 0.1 mL of placebo. Volunteers fasted for at least 1 h before and after immunization.

After immunization, the volunteers kept a daily symptom diary for 7 days, including the number and consistency of their stools. Diarrhea for the outpatient study was defined as ≥3 stools of ≥3 consistency in 24 h. They also submitted fecal specimens on days 3, 7, 10, 14, and 21 after immunization, using tubes of both PBS and alkaline-peptone-water (APW) as transport media for the stool specimen. These media were found to preserve the vaccine strain most effectively during the inpatient study and were superior to Cary Blair transport medium for this purpose. Serum was obtained before and 10 and 21 days after immunization.

**Laboratory procedures.** Freshly harvested bacteria were prepared for the inpatient volunteers following a standard procedure. Briefly, a vial of frozen Peru-15 seed was thawed, 0.1 mL of the thawed culture was spread on a plate of Luria-Bertani agar, and the plate was incubated for 18 h at 30°C. After incubation, the bacteria were harvested with 5 mL of 0.85% saline, and standard dilutions were prepared in saline. The optical density of the suspension was determined and adjusted to produce a stock suspension having a predicted concentration of colony-forming units per milliliter. The vaccine inoculum dose was verified by standard dilution and plate counts on mock vaccine doses that were made in parallel to those administered to the volunteers.

To identify the vaccine strain in the volunteer’s feces during the inpatient study, the first 2 stools passed daily were cultured quantitatively and qualitatively for *V. cholerae* in the Vaccine Testing Unit laboratory. For qualitative cultures, fecal specimens were inoculated in Cary Blair transport medium and transported to the laboratory. There they were plated directly onto TCBS (thiosulfate, citrate, bile salts, sucrose) agar and also inoculated into APW for subsequent plating onto TCBS agar. For quantitative cultures, 10-fold dilutions of stool were prepared in PBS, 100 μL was spread onto TCBS plates, and typical colonies were counted. Plates were incubated at 35°C for 18–24 h. Colonies suspected from the qualitative and quantitative plate cultures to be Peru-15 were confirmed by oxidase and agglutination with *V. cholerae* O1 antisera, and representative colonies were tested for motility using a motility agar plate.

For the outpatient study, the two vials (PBS and APW) were both inoculated onto TCBS media (the APW after 6 h of incubation). Quantitative cultures were not possible with the outpatient specimens because the fecal samples were collected in transport broth. Again, colonies typical of *V. cholerae* were confirmed as Peru-15 using oxidase and agglutination tests and motility agar. By testing normal stools, to which known numbers of Peru-15 were added, these methods yielded positive results if the concentration of bacteria was >5 × 10⁵/g of stool.

Serum specimens were tested for vibriocidal antibody titer using an initial dilution of 1:10 and serial 2-fold dilutions of serum in a standard microtiter plate [10]. The assay used an El Tor Inaba strain (N16961) as the target strain, and the titer was expressed as the reciprocal of the highest dilution yielding “no growth” in the well. IgG antitoxin antibodies were measured by a microtiter ELISA method [10] with 3-fold serial dilutions using 1:10 as the initial dilution. The antitoxin titer shown is the reciprocal of the extrapolated dilution of serum that yielded 0.4 OD in the plate. All serologic assays were carried out in a blinded manner, and the code to the study was not opened until after the laboratory assays were complete.

**Results**

**Inpatient study.** No serious adverse events were noted during the inpatient study. One volunteer experienced a fever to
Table 1. Number of subjects who noted symptoms during the 7 days following immunization with Peru-15 cholera vaccine or placebo in outpatient volunteers.

<table>
<thead>
<tr>
<th>Gastrointestinal symptoms</th>
<th>Upper respiratory symptoms</th>
<th>Headache</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days 0–3</td>
<td>Days 4–7</td>
<td></td>
</tr>
<tr>
<td>Dose = 10^9 cfu</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 16)</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>Dose = 10^8 cfu</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Placebo (n = 18)</td>
<td>6</td>
<td>3</td>
</tr>
</tbody>
</table>

NOTE. None of these differences are significant.

38.7°C the evening following the vaccination, and this was associated with chilly feelings but with no localized symptoms or physical findings. A blood culture and a complete blood count the evening of the fever were negative. The volunteer felt well the next day and remained well throughout the remainder of the study. None of the inpatient volunteers complained of loose stools, and none complained of abdominal cramps, nausea, vomiting, or other intestinal symptoms. On inspection of the stools however, loose stools (grade 3) were observed from 5 of the 12 volunteers, with amounts ranging from 261 to 388 g in 24 h. None of these met a common definition of diarrhea (≥3 loose stools in 24 h). None of the volunteers had a watery stool (grade 4 or 5).

Four of 6 volunteers receiving 10^9 cfu and 2 of 6 receiving 10^8 cfu developed significant (≥4-fold) increases in vibriocidal antibodies; the geometric mean titer (GMT) rose 36- and 5-fold in the 10^9 and 10^8 cfu groups, respectively. Two volunteers in each group had at least a 2-fold rise in serum IgG antitoxin antibodies; the GMT rose 1.7- and 1.2-fold in the 10^8 and 10^7 cfu groups, respectively.

Four of 6 volunteers in the high-dose group and 4 of 6 in the low-dose group excreted the vaccine strain in stool. Those receiving 10^9 cfu tended to have higher concentrations in the stool; all volunteers receiving this dose had peak concentrations >10^7 cfu/g, while 2 of those receiving 10^8 had concentrations <10^6 cfu/g. However, these differences were not significant. Peak concentrations were 10^8 cfu/g in both groups. Seven of the 8 volunteers who excreted vibrios still had positive stool cultures on day 6 when the antibiotic therapy was started. All recovered isolates were nonmotile.

Outpatient study. Fifty volunteers received the vaccine or a placebo in the outpatient study: 16 received 10^9 cfu, 16 received 10^8 cfu, and 18 received placebo. All volunteers completed their symptom diaries, all completed their assigned visits, and all submitted all of their fecal and serum specimens.

Surveillance for adverse events revealed no attributable side effects, though a few mild symptoms were reported by persons who received vaccine as well as placebo as shown in table 1. The gastrointestinal symptoms consisted of mild cramps or abdominal gas, but none of the individuals felt the symptoms to be distressing, nor did they limit usual activities. There were no fevers in the outpatient volunteers.

Serum vibriocidal responses (≥4-fold) were seen in all (100%) of those receiving the vaccine, regardless of the dose, but none of the placebo recipients developed a significant vibriocidal response. The vibriocidal GMT increased in recipients of 10^9 and 10^8 cfu by 73- and 90-fold, respectively, but it was unchanged in the placebo group (table 2). The highest vibriocidal titers were seen on day 10.

IgG serum antitoxin titers increased significantly (≥2-fold) in many (23/32) of the volunteers receiving vaccine, including 11 of 16 in the 10^9 cfu group and 12 of 16 in the 10^8 cfu group. The antitoxin GMTs increased by 4.1- and 4.6-fold for the 10^9 cfu and 10^8 cfu groups, respectively, on days 0, 10, and 21 (table 2). The GMTs increased significantly in both vaccine groups, with the peak titer being seen on day 21, but there was no apparent difference between the vaccine groups in antibody response. None of the placebo recipients had a significant rise in titer, and the GMT did not change in the placebo group.

The vaccine strain was excreted in the feces of 66% (21/32) of the volunteers receiving the vaccine, but it was not isolated from those receiving the placebo (table 3). There was a trend for the vaccine to be excreted longer in those receiving 10^9 cfu, but this was not statistically significant. None of the fecal specimens were positive after day 10.

Examination of the relation between fecal shedding of the vaccine strain and antibody responses revealed no clear relationship. Volunteers with negative fecal cultures had the same frequency and magnitude of vibriocidal and antitoxin responses as those with positive cultures.

Discussion

Peru-15 is a promising new live oral cholera vaccine. When a single dose of a reconstituted freeze-dried formulation of Peru-15 was administered in a manner similar to that appropriate for routine office practice, it was safe and highly immunogenic. The universal seroconversions among the outpatients receiving Peru-15 and the magnitude (fold rises) of the vibriocidal responses were similar to those reported for CVD 103-HgR [11–13], a live oral cholera vaccine licensed in Europe, Canada, and South America. A true comparison would require including the two vaccine strains in the same study. Without a challenge study, one cannot conclude that these serologic responses will be accompanied by high-grade long-lasting protection; however, past experience has shown that the development of vibriocidal antibodies has been accompanied by protection [3], and the past trial with the vaccine using freshly prepared vaccine did suggest protection [7].

The current study evaluated the strain first in inpatient volunteers in a clinical research ward, where the volunteers could be closely observed and the feces could be decontaminated to
Table 2. Serologic responses in outpatient volunteers receiving Peru-15 oral cholera vaccine or placebo.

<table>
<thead>
<tr>
<th>Dose</th>
<th>No. of 4-fold rises Day 0</th>
<th>Geometric mean titer (95% confidence interval) Day 0</th>
<th>Day 10</th>
<th>Day 21</th>
<th>Geometric mean titer (95% confidence interval) Day 0</th>
<th>Day 10</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>109 cfu (n = 16)</td>
<td>16</td>
<td>9.6 (5.5–17)</td>
<td>698 (315–1546)</td>
<td>494 (224–1083)</td>
<td>10</td>
<td>11 (5.7–23)</td>
<td>38 (15–93)</td>
</tr>
<tr>
<td>108 cfu (n = 16)</td>
<td>16</td>
<td>8.4 (5.3–13)</td>
<td>761 (436–1328)</td>
<td>538 (297–975)</td>
<td>12</td>
<td>12 (8.5–17)</td>
<td>33 (17–64)</td>
</tr>
<tr>
<td>Placebo (n = 18)</td>
<td>0</td>
<td>12 (6.8–20)</td>
<td>12 (6.9–21)</td>
<td>11 (6.5–19)</td>
<td>0</td>
<td>8.8 (5.8–13)</td>
<td>8.7 (5.6–13)</td>
</tr>
</tbody>
</table>

Table 3. Fecal shedding of Peru-15 vaccine among outpatient volunteers who received Peru-15 or placebo.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Ever culture-positive*</th>
<th>Stool culture positive on Day 3</th>
<th>Day 7</th>
<th>Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>109 cfu (n = 16)</td>
<td>11</td>
<td>10</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>108 cfu (n = 16)</td>
<td>10</td>
<td>8</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Placebo (n = 18)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Volunteer had at least 1 positive fecal culture.
genes of \( V. \) cholerae (which are chromosomal) can be transferred among strains by way of a filamentous bacteriophage [9]. For Peru-15, however, the possibility of reacquisition of these toxin genes has been effectively eliminated by the nature of the gene deletions engineered into this strain: the recA gene and the att RS1 integration sites [6, 7]. Both of these are required for successful integration and maintenance of the toxin gene cassette.

It is also important to note that bacterial virulence is a complex phenomenon and that there is no a priori reason to assume that reacquisition of one virulence determinant would be sufficient to convert an attenuated strain into a fully virulent organism. For example, a toxigenic \( V. \) cholerae strain that was unable to colonize the small intestine would not be as virulent as a wild-type strain. Peru-15 has other mutations, such as recA deletion and nonmotility, that add to the margin of safety of the strain.

In conclusion, lyophilized Peru-15, when given with buffer, was safe and immunogenic. Further studies are needed to investigate other dose levels and other buffer systems to optimize the administration of the vaccine and to further document protection in volunteers and in the field.

Acknowledgments

We thank the volunteers who took part in these studies; Wendy Luther, Kathy MacLeod, and Carol Agrawal for their technical assistance; and the staff of the General Clinical Research Center, Johns Hopkins Hospital.

References