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Comparison of Alternative Buffers for Use with a New Live Oral Cholera Vaccine, Peru-15, in Outpatient Volunteers

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During development of Peru-15, a new live oral vaccine for cholera, the role of buffer needed to be evaluated. Generally, oral bacterial vaccines are acid labile and need to be administered by using a formulation which protects them from gastric acid. We compared three different buffers for use with Peru-15, including a standard bicarbonate-ascorbic acid buffer, Alka-Seltzer, and a new electrolyte-rice buffer, CeraVacx. Saline served as the control. Thirty-nine healthy adult volunteers received Peru-15 (10^8 CFU) with one of the three buffers or saline in a double-masked study. The volunteers were monitored for symptoms for 7 days after the dose, serum was tested for antibody responses by vibriocidal antibody and immunoglobulin G antitoxin enzyme-linked immunosorbent assays, and stool samples were tested for excretion of the vaccine strain. Side effects were minimal in all groups. All 30 volunteers who took Peru-15 with a buffer showed significant rises in vibriocidal antibody titer. The magnitude of the rises was higher in the CeraVacx group than in the other two buffer groups. Four of nine volunteers who took the vaccine with saline also showed increased titers, but they were lower than those in any of the three buffer groups. Excretion of the vaccine strain was similar in the buffer groups, but excretion was not associated with the magnitude of the vibriocidal responses. Excretion of Peru-15 was not detected in the saline group. We conclude that buffer does amplify the serological response to Peru-15 and that CeraVacx may provide benefits not provided by other buffers.

Several new oral bacterial vaccines have been developed or are being developed for enteric infections where local intestinal immunity is thought to be the major mediator of protective immunity. These include vaccines against diseases caused by *Salmonella typhi* (11, 12), *Vibrio cholerae* (7, 19), enterotoxigenic *Escherichia coli* (30), *Campylobacter jejuni* (2, 3, 23), *Shigella* spp. (1, 14, 16), and *Helicobacter pylori*. Since the vaccines are generally acid sensitive, they must be formulated to protect the acid-labile antigens (in the case of killed oral vaccines) or the bacteria (in the case of live oral vaccines) from destruction by gastric acid. Gastric acid is known to have a potent protective effect against infection by *V. cholerae* (21, 22), so it would seem that avoidance of the acid is essential for a live oral cholera vaccine. Ty21a typhoid vaccine is formulated as an enteric agent-coated capsule in the United States (11) but is also formulated as a double sachet with buffer salts in one packet and the freeze-dried vaccine in the other (29).

Several buffers have been used with oral bacterial vaccines. These have included sodium bicarbonate solution with live oral cholera vaccine (19), bicarbonate-citric acid solution with killed oral cholera vaccines (7), bicarbonate-ascorbic acid buffer with live oral cholera vaccine and oral typhoid vaccine (9), and a buffer mixture (Samarin) with killed oral cholera and enterotoxigenic *E. coli* vaccines (8, 28). With each of the vaccines, the choice and amount of buffer have been a concern. With a suboptimal amount of buffer or with no buffer, the immunogenicity of the vaccine is diminished (6, 27). However, the buffers used have not always been well tolerated by those taking the vaccine, and there have been suggestions that the buffers caused gastrointestinal symptoms such as bloating, gas, cramps, and diarrhea in at least some persons taking the vaccine (27).

The rationale for the amount of buffer to use has been a calculated amount of buffer salts which will neutralize a maximum amount of acid, and this amount of buffer salt is then dissolved in a reasonable volume of water consistent with the age of the vaccinee. Further, for live vaccines, the buffer should not kill the vaccine bacteria during the time the vaccine is being administered. However, other characteristics of such solutions, such as gastric emptying time, absorption from the intestine, minimization of side effects, and optimization of the recovery of the live oral vaccine from the freeze-dried state, have not been evaluated. Also, in an attempt to standardize conditions of vaccination, participants in many studies were expected to fast for more than 1 h before and after taking the vaccine, although an immune benefit from fasting has not been documented.

Peru-15 is a new live, oral vaccine for cholera derived from a *V. cholerae* El Tor, Inaba strain which produces B subunit but not holotoxin (15). Also, the strain has a *recA* deletion and is nonmotile. When tested previously in outpatients at the Johns Hopkins University Vaccine Testing Unit, it was shown to induce vibriocidal antibodies in 100% of these vaccinees and antitoxin antibodies in over 60% of 32 outpatient volunteers when formulated as a freeze-dried vaccine and administered with the standard buffer (26).

This study was planned to investigate the buffer requirements of this vaccine strain and to compare commercially available buffers for their protective effect. One such buffer which is widely known and available is Alka-Seltzer. This buffer is a mixture of sodium bicarbonate, citric acid, and potassium bicarbonate formulated as an effervescent tablet that appears to be well accepted. An alternative was CeraVacx, a new buffer

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TABLE 1. Buffers being compared

Buffer	Ingredient (g/dose)	Vol (ml)	Osmolality (mmol/liter)
Standard buffer	Sodium bicarbonate (2.5) Ascorbic acid (1.8)	100[a]	513
Alka-Seltzer	Sodium bicarbonate (1.9) Citric acid (1.66) Potassium bicarbonate (0.62)	150	244
CeraVacx	Sodium bicarbonate (2.0) Trisodium citrate (0.5) Rice syrup solids (7)	150	360
Saline	Sodium chloride (1.35)	150	

[a] A volume of 100 ml was used with standard buffer since this was the volume used in the previous protocols.

designed specifically as a vaccine buffer which incorporates a defined form of rice syrup along with sodium bicarbonate and trisodium citrate. These were compared with a sodium bicarbonate-ascorbic acid (standard) buffer which is currently being used with Ty21a and CVD103HgR in Europe and which had been used with Peru-15 in past studies (15, 26). Finally, buffer requirements for Peru-15 were determined by comparing these buffers with saline, which has no buffering capacity.

(Results from this study were presented at the 97th General Meeting of the American Society for Microbiology, Miami Beach, Fla., May 1997.)

MATERIALS AND METHODS

Vaccine and buffers. The vaccine was prepared by Virus Research Institute, Inc., as a freeze-dried preparation containing 10^9 CFU/ml after reconstitution. It was stored at −20°C until immediately before use, when it was reconstituted in 1 ml of the test buffer. The vaccine (0.1 ml) was then added to the test buffer (100 ml of the standard buffer or 150 ml of one of the other buffers) in a plastic cup, and this was offered to the volunteer to drink. Previous studies (26) had used 100 ml of standard buffer; however, the volume of the other buffers was increased to 150 ml in order to lower the osmolarity of these solutions.

The buffers are described in Table 1. Alka-Seltzer was obtained from a local pharmacy. CeraVacx was provided by Cera Products, Inc., Columbia, Md., and the standard buffer and the saline were prepared at the Vaccine Testing Unit with USP reagents.

The buffers were compared in titration experiments (Fig. 1) to determine their ability to neutralize HCl down to pH 5.0.

Recruitment of volunteers. Thirty-nine healthy volunteers between 18 and 50 years old agreed to participate in a double-masked, randomized, four-cell, outpatient study to determine the immunogenicity of Peru-15 oral cholera vaccine when given with one of the buffers or saline. Inclusion criteria included good health, willingness to participate, and completion of 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 as determined by history or by a positive urine human chorionic gonadotropin test 2 days before the study began, travel to an area where cholera is endemic or within 5 years of receipt of cholera vaccine, history of cholera infection, previous participation in a cholera or enterotoxigenic *E. coli* study, inability to pass a written examination on diarrhea and cholera and cholera vaccines, significant abnormality in screening laboratory hematology and chemistry tests, use of antibiotics within 7 days of vaccination, or being a food handler. Volunteers were also excluded if they had close contact with children under age 5 or if they were immunosuppressed or pregnant.

Randomization and vaccination. All volunteers were given a single dose (10^8 lyophilized bacteria) of Peru-15 live oral cholera vaccine (i.e., no one received a placebo) with one of the three buffers or with saline. Assignment of volunteers to the buffer groups was random, using study numbers which were assigned to each volunteer in the order in which they were recruited. The randomization list was prepared prior to the start of the study, and unit doses of dry buffer were prepared in identical containers. At the time of administration, the vaccine was reconstituted in freshly prepared buffer by one of the investigators (J. Shimko) out of sight of the volunteer. She was the only individual at the Vaccine Testing Unit who knew the buffer assignment. A study nurse (K. MacLeod), without knowledge of the buffer assignment, supervised the screening and training of the volunteers and the assessment of side effects. Eating, drinking, and smoking were prohibited for 1 h before and after the vaccine.

Symptom surveillance. For 7 days after receiving the dose of vaccine, the volunteers maintained a daily symptom diary in which they recorded any symptoms they experienced. The data from the symptom diary was reviewed with the study nurse at the time of a clinic visit 7 days after vaccination.

Serology. Serum was collected prior to vaccination and on days 14 and 21 after vaccination. Vibriocidal and antitoxin antibody assays (enzyme-linked immunosorbent assays), using cholera B subunit (Sigma) as the antigen, were carried out in the laboratory of the Vaccine Testing Unit according to a standard procedure (25). A significant antibody response was defined as a fourfold increase for vibriocidal antibody titer and a twofold increase for antitoxin antibody. A high vibriocidal response was one which increased significantly to a titer exceeding 1:1,000. A high antitoxin response was a titer which increased more than fourfold. Serology was carried out in a blinded manner without knowledge of the buffer assignment.

Fecal excretion. Fecal specimens were collected on days 7, 10, 14, and 21 after each dose. From each specimen, two swabs were collected; one was placed in alkaline peptone water, and the other was placed in sterile phosphate-buffered saline. Thiosulfate-citrate-bile salts-sucrose (TCBS) agar was then inoculated with these samples and incubated overnight (after a 6-h incubation in the case of the alkaline peptone water). Colonies suspected to be the vaccine strain on the basis of colony morphology were confirmed by using oxidase reagent and determining agglutination with *V. cholerae* O1 antiserum, and representative colonies were tested in motility agar. Microbiology assays were carried out by using coded specimen numbers in a blinded manner so that the laboratory staff would not know the buffer assignment or the day postvaccination on which the specimen was collected. On the basis of experiments in which normal stools were inoculated with known concentrations of Peru-15, fecal samples, tested in the manner in which the volunteers' samples were tested, were considered positive if the concentration of Peru-15 was $>10^2$ to 10^3 CFU/g.

Analytical plan. The proportions of volunteers with specific symptoms, significant rises in antibody titer or high titers, or positive fecal cultures were compared by two-tailed Fisher's exact tests. Differences in the geometric mean titers (GMTs) of the antibody were examined by Student's *t* test.

The protocol was approved by the Joint Committee on Clinical Investigation at Johns Hopkins University and was done under an Investigational New Drug application from the Food and Drug Administration.

RESULTS

Symptom surveillance. Thirty volunteers received vaccine with one of the buffers (10 in each buffer group), and nine received the vaccine with saline. None of the volunteers called to report an illness during the surveillance period; however, a number of volunteers reported mild symptoms in their diaries, as shown in Table 2. Symptoms reported by the volunteers included headache, muscle aches, chilly feeling, light-headed feeling, drowsiness, cough, runny nose, and conjunctivitis. There were no differences in symptoms between the different buffer groups.

Serology. All 30 volunteers (100%) who received Peru-15 with a buffer developed a significant vibriocidal response, and

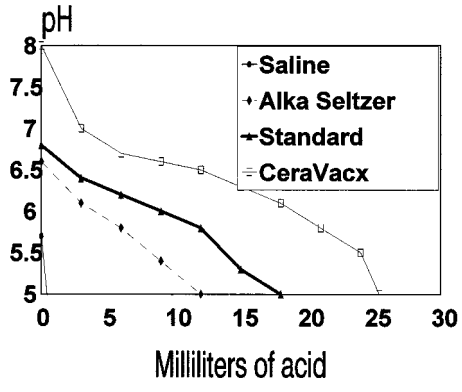

FIG. 1. Acid titration with three buffers. HCl (1 N) was added to one dose of buffer, and the pH was monitored down to pH 5.0.

TABLE 2. Symptoms reported by volunteers receiving Peru-15 cholera vaccine

Parameter	Value for group			
	Standard buffer (n = 10)	Alka-Seltzer (n = 10)	CeraVacx (n = 10)	Saline (n = 9)
Ill feeling				
No. of persons	0	1	2	1
No. of person days	0	3	2	1
Gastrointestinal symptoms				
Diarrhea	0	1	1	0
Nausea	0	2	1	1
Abdominal cramps	1	6	2	2
Gas	3	7	6	2
Decreased appetite	0	2	2	1
Total no. of persons with gastrointestinal symptoms	3	8	7	3
Total no. of persons with nongastrointestinal symptoms[a]	4	6	5	5
No. of persons reporting any symptoms	5	9	7	5

[a] Nongastrointestinal symptoms included headache, cough, fatigue, and conjunctivitis. The differences between these groups were not significant.

4 of 9 volunteers who received it with saline also developed a response (P = 0.0002 for the difference between buffer versus no buffer). The fold increase in vibriocidal responses was highest in those receiving vaccine with CeraVacx (194-fold), followed by those receiving it with Alka-Seltzer (111-fold) and standard buffer (60-fold). The CeraVacx group also showed the greatest increase in GMT, as shown in Fig. 2. Nine of 10 volunteers receiving CeraVacx had a high response, while lower proportions were seen with Alka-Seltzer (6 of 10), the standard buffer (4 of 10), or saline (3 of 9). The proportion with high responses seen with CeraVacx can be considered significant if results for the other two buffer groups are combined (9 of 10 versus 10 of 20; P = 0.048).

Twofold increases in antitoxin were seen in 14 of the 30 volunteers receiving the vaccine with buffer and 1 of 9 who received it without buffer (P = 0.12). The prevaccine GMT was higher for the saline group, which might have affected the results somewhat. Within the buffer groups, the antitoxin fold changes and GMTs were similar (Fig. 3).

Fecal microbiology. Positive fecal cultures were found for 16 of the 30 volunteers (53%) receiving the vaccine with one of the buffers but for none of those who received the vaccine with saline (P = 0.005; Fisher's exact test). For the 10 volunteers given vaccine with standard buffer, four fecal specimens were positive on day 7 and two were positive on day 10. In the Alka-Seltzer group, eight specimens were positive on day 7 and two were positive on day 10. In the CeraVacx group, four were positive on day 7, but later specimens were negative. The recovered strains had the same phenotype as the vaccine given. Within the buffer groups, there was no discernible association between fecal excretion of the vaccine and the magnitude of the vibriocidal response, although those who received saline had negative cultures and also had a lesser serologic response.

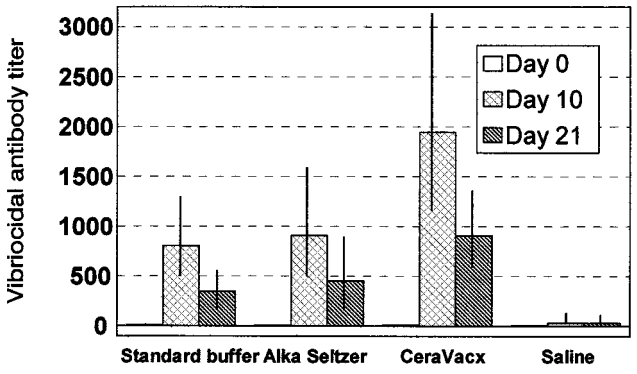

FIG. 2. Geometric mean vibriocidal antibody titers in serum in groups receiving Peru-15 oral cholera vaccine with different buffers (standard errors are indicated).

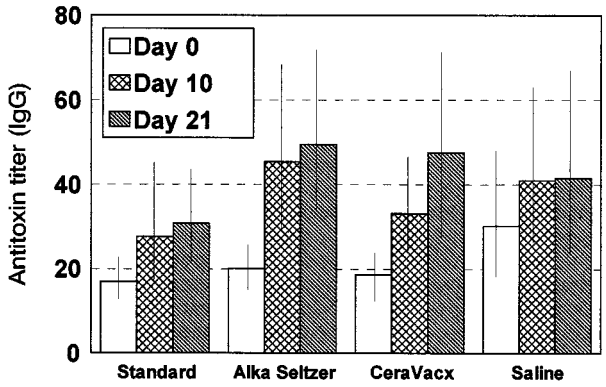

FIG. 3. Geometric mean antitoxin (immunoglobulin G [IgG]) titers in serum in groups receiving Peru-15 oral cholera vaccine with different buffers (standard errors are indicated).

DISCUSSION

In this study, all volunteers received a single dose of the new live, oral cholera vaccine, Peru-15, with different buffers or with no buffer (saline). Among those who received the vaccine with saline, some (four of nine) developed vibriocidal responses, suggesting that brief colonization may have occurred, but overall, the vibriocidal response was less and there was no detectable fecal excretion of the vaccine strain. In contrast, all (100%) of the volunteers who received vaccine with one of the buffer solutions developed a significant vibriocidal response, and many developed high titers (>1:1,000).

Among the groups receiving vaccine with a buffer, the highest vibriocidal response was seen with CeraVacx. In this group, 9 of 10 developed a high titer and the GMT postvaccine was 1:1,940; this was more than twice as high as the GMTs for the other two buffers. The GMT in the CeraVacx group increased 194-fold, compared to 60-fold and 111-fold increases in the standard buffer and the Alka-Seltzer groups, respectively. A previous study with Peru-15 using the same standard buffer and the same lot of vaccine found nearly identical vibriocidal responses (26), adding confidence that this response is consistent for this vaccine strain with the standard buffer. Also consistent with the previous outpatient study, about half of the volunteers who took the vaccine with buffer excreted the vaccine strain in the stool, but as with the previous study, there was no apparent correlation between fecal positivity and the magnitude of the vibriocidal response.

The results of this study demonstrate that the choice of buffer is an important determinant in the magnitude of the vibriocidal response for this live oral cholera vaccine. When the vaccine is taken with saline (i.e., without a buffer), the vibriocidal response is inconsistent and is lower in magnitude. The results also suggest that comparison of different live oral

cholera vaccines in immunogenicity will need to consider the buffer as well as the strain itself.

These vibriocidal responses can also be compared with those of groups of volunteers who developed cholera after challenge with virulent *V. cholerae* El Tor, Inaba (strain N16961). During recent challenge studies at the Vaccine Testing Unit with immunologically naive volunteers using bicarbonate buffer (2 g in 150 ml of water), the rise in vibriocidal antibody GMTs was about 70-fold, similar to the vibriocidal response in volunteers receiving Peru-15 with standard buffer but less than the response with Peru-15 when taken with CeraVacx. In designing a live oral cholera vaccine, it is hoped that the vaccine will stimulate a vibriocidal response which is nearly as high as that which occurs following a virulent disease, but the occurrence of an even higher response to the vaccine has not been considered previously.

The explanation for the better immune response with CeraVacx is not known; however, it may be due to several factors. First, CeraVacx (about 350 mmol per liter) was formulated to be less hypertonic than the standard buffer (>500 mmol per liter). Hypertonic solutions slow gastric emptying and may inhibit the flow of vaccine through the stomach. Hypertonicity by itself does not appear to account for the difference, however, since Alka-Seltzer also has a lower osmolarity. Second, the neutralization curves for the three buffers showed that CeraVacx was able to neutralize more acid than the other buffers while maintaining the pH above 5.0 (Fig. 1). Third, the carbohydrate in CeraVacx likely stimulated more-rapid absorption of the buffer solution once it entered the intestine, like an oral rehydration solution (24). Absorption of water might have concentrated the vaccine, leading to better mucosal contact. By contrast, a hypertonic buffer without substrate would likely draw fluid from the circulation into the intestine, thus diluting the vaccine and limiting mucosal contact. Finally, colonization of this live vibrio might have been enhanced by the carbohydrate nutrient in the buffer. Maltose has been found to up-regulate the expression of colonization factors in *V. cholerae* (17), and this might also have led to improved colonization. Whether the improved immunogenicity with CeraVacx is specific to live oral cholera vaccines or might also occur with other live or killed oral vaccines remains to be tested.

Considerable effort has gone into the discovery and development of adjuvants which could be added to accentuate the immune response to orally administered antigens (5, 10, 13, 18, 31, 32). In fact, cholera toxin appears to be a very effective adjuvant (4, 20). In this study, CeraVacx amplified the immune response to a standard dose of this vaccine, showing that materials other than adjuvants may be useful when maximization of a local immune response is attempted.

Additional studies with improved buffers will be needed to define the most cost-effective dose response. The dose of 10^8 used in this study was based on previous studies using the standard buffer, but the use of an improved buffer may allow for smaller doses (e.g., 10^7 or 10^6). Reasons to consider this smaller dose include the obvious economy of the smaller dose and the possibility that the dose response of expressed antigens (e.g., B subunit) might be different from the dose response to the cell wall antigens, as reflected by the vibriocidal antibody titer.

One rationale for the use of CeraVacx was the expectation that the mild gastrointestinal symptoms would be minimized. In this study, the choice of buffer did not appear to affect the rates of symptoms reported by the volunteers. Similar symptoms occurred among volunteers in the previous outpatient study, including those who received a placebo, suggesting that the symptoms are not specific to the vaccine. None of the symptoms interfered with daily activities, and many may have represented background symptoms which occur normally but are reported when volunteers are encouraged to report any symptoms on a daily basis.

In conclusion, the choice of buffer is an important determinant in the immunogenicity of Peru-15. Further studies are needed to determine if the choice of buffer is also important with other live and killed oral vaccines for this disease and other diseases.

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