Invited Review

Current Status of Cholera and Rise of Novel Mucosal Vaccine

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SUMMARY: Three serious cholera epidemics have threatened the world during the last 10 years. As a countermeasure against such cholera epidemics, three vaccines, CVD 103-HgR, WC/rBS, and Vietnamese WC, showed good performance. CVD 103-HgR is a recombinant attenuated live vaccine for travelers, and its highly safety and protective efficacy have been demonstrated in volunteers in advanced countries. WC/rBS, which consists of heat- and formalin-killed bacteria and cholera toxin B subunit, protects the vaccinees (>5-year-old) from cholera for 6 months. Vietnamese WC, a heat- and formalin-killed vaccine, is inexpensive and effective even for 1 to 5-year-old children. Additionally, irradiated WC vaccines and new serotype (O139) vaccines are being developed. Regarding intestinal immunity, secretory IgA has been mainly examined. In addition, mucosal IgG, as induced by the irradiated WC vaccine, should also be investigated. Development of mucosal adjuvant, such as holotoxin-type mutants of cholera toxin and related Escherichia coli heat-labile enterotoxin, has been actively undertaken. Diverse custom-made vaccines may be one countermeasure for the changing situations in endemic countries or areas and for “barriers” against live vaccines in such areas.

1. Introduction

Since Robert Koch succeeded in the pure culture of Vibrio cholerae (called Komanabazillen at that time) in 1884, cholera epidemics moved from the fifth to the sixth cholera pandemic, then, the seventh cholera pandemic have been occurring from 1961 to date (1) (Fig. 1). The sixth cholera pandemic was caused by V. cholerae with classical biotype, while the causative agent in the seventh pandemic is El Tor biotype (V. cholerae O1 biotype El Tor). V. cholerae O1 includes two serotypes, Ogawa and Inaba, which are used as markers in epidemiological analysis (2, 3).

The cholera toxin (CT) that Robert Koch speculated on in 1884 was purified in 1969 (4). In 1999, it was reported that V. cholerae O1 contains two types of lysogenic phages, and the bacteria exhibit the pathogenicity using these two prophages (5, 6). One is designated CTXφ having the cholera toxin genes (ctxA, ctxB), and the other is designated VPIφ having the TcpA coat protein gene. TcpA constructs TCP pilus (type IV pili), which play a role in the intestines as a colonization factor, and also serve as a receptor for CTXφ phase. The whole V. cholerae O1 genome was determined in 2000 (7). The chromosome was approximately 4 Mbp in length, and had ~3,885-estimated genes. The chromosome has been shown to consist of two circular replicons of approximately 3 and 1 Mbp in size.

Despite such accumulation of knowledge, people’s lives are still threatened by cholera in some areas on the earth. Cholera is a human disease. Although V. cholerae O1 excreted in feces can survive in the environment (e.g., in rivers) (8), infection is controllable if humans are protected by immunity (which can be induced by vaccines) (9).

In the last 15 years, three cholera vaccines have received high evaluations for their properties and effectiveness. These vaccines have been studied in volunteers in the United States (US) and Sweden and in the field in epidemic countries. Two products have become commercially available.

In this manuscript, we summarize the current status of cholera and recent trends in cholera vaccine research.

2. Infection and symptoms

Unlike Helicobacter pylori, V. cholerae O1 can actively move in river water at environmental temperature (10), and does not float away, which causes fecal-oral infection in the contaminated river basin.

The bacteria that are orally ingested colonize in the small intestine (11, 12). In developing countries, the infection rate with H. pylori is high. Chronic gastritis induced by persistent infection of H. pylori causes hypochlorhydria, which makes infection with gastric hydrochloric acid-susceptible V. cholerae O1 easy (13). Persons with type O blood have also been reported to be a high risk group for severe illness (14).

The latent period varies from 6 h to about 3 days, depending on the amount of bacteria ingested. The initial symptom is an unpleasant feeling in the abdomen, and the main symptoms, severe diarrhea and vomiting, develop. Symptoms are sometimes accompanied by muscular pain. Fever is generally absent (15-18).

Usually, the morphology of the intestinal mucosa does not change, and water retention starts in the intestine. This diarrhea consists of a large volume of white turbid watery stool described as rice-washed water, and is excreted in a liquid stream. Five liters of diarrhea is usually excreted within 24 h after onset, and the volume reaches 8-10 liters in a day. Diarrhea may continue for 6 days or longer. Diarrhea stool becomes yellowish, then the color changes to a black-green like that of bilirubin (15-18).

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Diarrhea is categorized by the degree of fluid loss (ratio to body weight) as mild (<5%), moderate (5-10%), and severe diarrhea (>10%). In a dehydrated state, the lips dry, the patient feels thirsty, the skin loses elasticity, the cheek and eyes sink, and the eyeballs are rolled back (Fig. 2). When water loss reaches 10% or more of body weight, the condition becomes serious. In children (especially those under 5 years old), although dehydration is observed, a large volume of watery stool is retained in the intestine, causing marked abdominal extension, and watery stool may not be excreted until a probe is inserted in the rectum.

A characteristic of the watery stools in cholera is a high concentration of sodium ion (Na⁺) (~130 mEq/l) and chlorine ion (Cl⁻) (~100 mEq/l), at the same levels as those in the serum. When CT acts on the target cells, the intracellular cyclic AMP (cAMP) level increases. The increased cAMP is considered to inhibit absorption of Cl⁻ and Na⁺ in epithelial cells of villi, and to facilitate secretion of Cl⁻ (accompanied by outflow of Na⁺ and water) in crypt cells located in the lower region of the villi.

In children, the fecal potassium ion (K⁺) level is high (~30 mEq/l). Abdominal extension is considered to be due to paralytic ileus caused by hypokalemia.

### 3. Epidemiology

Three large scale cholera epidemics occurred during the last 10 years in the world (Fig. 1). In 1991, cholera suddenly occurred in Peru in South America, where an epidemic had not been recorded for 100 years. The epidemic was characterized by over 1,700 cases per day on average; 391,220 per year; and 4,002 patients died (19, 20).

In 1992, V. cholerae belonging to a new serotype (O139) emerged in India (21), and the previous cholera vaccine was not effective; the epidemic spread to neighboring countries, including Thailand and Bangladesh (22, 23). At the beginning, cholera specialists were concerned about "an eighth pandemic" (24), but it is now considered to have been a subtype epidemic in the seventh cholera pandemic.

In 1994, a serious cholera outbreak occurred in Rwandan refugees in Zaire (Goma) in Africa (25). The mortality briefly reached 17% (26).

In 1997 in Japan, cholera occurred in 36 Japanese who had never been abroad and the source of infection became an issue (27).

In 1999, 61 countries were reported by the World Health Organization (WHO) to be contaminated with V. cholerae. The number of patients was 254,310, and 9,175 patients died, showing a mortality rate of 3.6% (28).

### 4. Attenuated live vaccine

A live vaccine, developed by recombinant DNA technology in the US, became available for use by travelers in 1994 (9, 28-30). This vaccine is attenuated V. cholerae O1 classical
biotype Inaba serotype (569B strain), and is called CVD 103-HgR. The attenuated bacteria were prepared by deleting 94% of the cholera toxin A subunit gene (ctxA), conserving the B subunit gene (ctxB), and destroying the hemolysin gene by inserting the mercury resistance gene.

In the US study in volunteers, oral administration of a single dose was possible. The protective effect began 8 days after administration, and continued for 3 months in 95% for classical cholera and 65% for El Tor cholera (28).

The examples from the US study in volunteers are shown in Table 1. An increase in the serum vibriocidal antibody titer, which is used as an index of establishment of immunity against cholera, was observed in 90% or more of subjects vaccinated. When El Tor Inaba strain (10^7 CFU) was challenged 3 months after vaccination, 91% of mild and severe diarrhea (3 liters or more of diarrhea stool) caused by the bacteria challenged was prevented. Shedding of the challenged bacteria into feces (colonizing bacteria) was decreased to 10^4 or lower (29).

A study of CVD 103-HgR vaccine, which has been well examined in volunteers, was performed in the epidemic region (north Jakarta) in Indonesia (31) (Table 1). An increase in the vibriocidal antibody titer was observed in 65%, as expected. Even in children aged 5 years or younger, the titer increased in 78%. However, the 4-year long-term protection was observed only in 14%, showing that the vaccine was not effective against cholera in this region. Studies on the short-term efficacy of a single oral dose and long-term efficacy by two doses are planned.

As a cause of the ineffectiveness in north Jakarta, a "barrier" for live vaccine in children in developing countries is being considered. Actually, to establish immunity with a high defensive index (a high serum vibriocidal antibody titer = a high seroconversion rate) in children in developing countries, vaccination with a large dose of CVD 103-HgR (5 x 10^9 CFU) was necessary. This amount of bacteria corresponds to 10^10 times the experimental value (5 x 10^8 CFU) in North America and Europe (32). Bacterial overgrowth in the intestine is considered to be the "barrier" mechanism (33).

Mixed administration with another attenuated vaccine developed separately (CVD 111) has been investigated (34) (Table 1). CVD 111 is attenuated V. cholerae O1 El Tor biotype, Ogawa serotype (N1617 strain), in which the CT gene was removed and ctxB and mercury resistance gene were developed separately (CVD 111) has been investigated (34) considered to be the "barrier" mechanism (33).

In addition to these vaccines, other attenuated live vaccines are being developed using V. cholerae O1 from Peru (35) and V. cholerae serotype O139 (36).

### 5. Killed vaccine

#### (1) WC/rBS vaccine

A vaccine containing a mixture of heat- and formalin-killed bacteria and the cholera toxin B subunit (CTB) (whole cell/ B subunit, or WC/Bs) was developed by a Swedish group (28, 30, 37, 38) (Table 2). The vaccine contained 2.5 x 10^6 each of heat-killed classical Inaba strain (Cairo 48), heat-killed classical Ogawa strain (Cairo 50), formalin-killed El Tor Inaba strain (Phil 46973), formalin-killed classical Ogawa strain (Cairo 50), and 1 mg of CTB. The B subunit (rBS) obtained from recombinant ctxB has been used in experiments after 1992 (39, 40). WC/Bs and WC/rBS therefore do not differ in the immune response (39).

At the beginning of the BS-WC vaccine study in Bangladesh, three doses of the vaccine were administered 6 weeks apart (37). However, since a similar effect (increases in vibriocidal antibody titer) could be expected by two doses of WC/rBS with a 2-week interval (38), the effect of two doses was investigated in later studies in Peru (41, 42) and other regions.

In field studies in Bangladesh, Columbia, Peru, and Sweden, no adverse effects were observed (30). The protection was observed 1 week after the second vaccination, and a high protection efficacy of 85-90% was obtained for 4-6 months after vaccination.

In the study in Bangladesh (1985-1988) (37, 38), the protection efficacy was 60% at 2 years and 50% at 3 years, and almost all effect disappeared at 5 years after vaccination. In children 5 years old or younger (2-5 years old), the protective effect was weak. The absence of previous immunity was considered to be the cause. The long-term protection was also weak in subjects of blood type O. During 4-6 months after administration, WC/Bs was clearly superior to WC, and the direction of later studies was established.

In a field study of WC/rBS vaccine in Peru initiated in 1993, a marked protective effect was confirmed for the first 4 months or longer after administration (41) (Table 2). However, in another study, the protective effect was not confirmed after the second vaccination (42). Based on these findings, it has been suggested that El Tor Ogawa strain, which is frequently isolated worldwide, should be added to WC/rBS vaccine, and that three doses may be necessary (booster may be necessary after 1 year) (42).

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**Table 1. Results of volunteer and field studies of attenuated live cholera vaccine (CVD 103-HgR)**

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Study period (study place)</th>
<th>Study content (administration)</th>
<th>Results</th>
<th>Reference</th>
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</table>
| CVD103-HgR    | 1999 (USA)                 | Volunteer study for El Tor cholera (single dose) | - Defense index of immunity (vibriocidal antibodies) increased (after 3 months): 91% in the vaccine group vs. 2% in the placebo group.  
- Protection in 91% (after 3 months)  
- The maximal bacterial excretion: 4.9 x 10^8 CFU/g in the vaccine group vs. 1.1 x 10^5 CFU/g in the placebo group. | 29        |
| CVD103-HgR    | 1993-1997 (Indonesia)      | Filed study for El Tor cholera (single dose) | - Defense index of immunity (vibriocidal antibodies) increased (after 10 days): 65% in the vaccine group (78% at 2-4 years old, 60-74% over 5 years old) vs. 2% in the placebo group.  
- Protection in 14% (for 4 years) | 31        |
| Mixture of    | 1999 (US facility in Panama) | Immune response test in volunteers (single dose) | - Defense index of immunity (vibriocidal antibodies) increased: mixed vaccine > single vaccine  
- Adverse event of CVD 111 (diarrhea): 8% | 34        |

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As the controls, heat-killed C13 and EO8 and formalin-killed irradiated WC induced the secretion of not only lgA but also lgG antibodies in the intestinal tract, unlike the heat- or formalin-killed WC (Fig. 3), and the irradiated WC showed a remarkably higher protection against challenge than that of the heat- or formalin-killed WC. Furthermore, the vibriocidal antibodies were detected in intestinal juice of rabbits that received the irradiated WC.

lgG is a vibriocidal antibody, while lgA is not (44). Therefore, mucosal immunity induced by irradiated WC may include the killing of V. cholerae O1, in addition to, for example, the inhibition of the bacterial colonization and neutralization of toxin. The immune response to irradiated WC vaccine may qualitatively differ from that to the previous heat- or formalin-killed WC vaccines. Two forms, irradiated WC and irradiated WC plus adjuvant, are being developed.

6. Mucosal IgG

The main component of mucosal immunity is secretory IgA (45). However, mucosal IgG has been attracting attention in recent years. For example, when Haemophilus influenzae type b oligosaccharide conjugated to diphtheria toxoid vaccine (Hib-DT) in a formulation containing 1α, 25-dihydroxy vitamin D3 (mucosal adjuvant) was subcutaneously injected to mice, anti-diphtheria toxin IgG antibody appeared in feces (46). It has also been shown that the passive administration of IgG1 monoclonal antibody prevented simian-human immunodeficiency virus (SIV) infection in pregnant macaques, and that the neonatal macaques that received IgG1 monoclonal antibody administration were resistant to oral SIV infection (47). This suggests the potential protective effect of IgG on mucosal mother-to-infant HIV infection (48).

7. Mystery of O1 cholera and O139 cholera

In 1992, V. cholerae O139 that produces CT emerged in O1 cholera epidemic region, India (then soon in Bangladesh), overcoming V. cholerae O1, which also produces CT (21,
22). Since most O139 cholera patients were adults, who might have acquired immunity by previous infection with *V. cholerae* O1 and therefore possessed some degree of anti-CT antibody, the infection was expected to cause only slight diarrhea. However, many O139 cholera patients developed severe diarrhea in these regions (49).

It was, therefore, hypothesized that anti-CT immunity is not useful in preventing infection. Indeed, when CVD 103-HgR was administered to rabbits (two doses) and *V. cholerae* O1 (569B strain) or O139 was challenged after 3 weeks, colonization of *V. cholerae* O1 and diarrhea were prevented, but the effects of *V. cholerae* O139 were not prevented (49).

In contrast, in a field study in Bangladesh, WC/BS vaccine provided a short-term protection in approximately 70% against infection with enterotoxigenic *Escherichia coli* (ETEC) (59), which produces heat-labile enterotoxin (LT) similar to CT and induces cholera-like watery diarrhea.

With regard to both of the above cases, experiments must be conducted to investigate whether BS (rBS) in WC/BS (rBS) vaccine acted as antigen (26) or non-specific adjuvant (51).

### 8. Mucosal adjuvant effect of cholera toxin

The mucosal adjuvant effect of CT is non-specific. The actions in mucosa are diverse (52-54), such as the facilitation of passage through the epithelial barrier of antigens, activation of antigen presentation of APC (antigen-presenting cells) including macrophages and dendritic cells, promotion of class switch from IgM-producing to IgA-producing cells, induction of Th2 cells by inhibition of macrophage cytokine IL-12, and activation of macrophages and lymphocytes by macrophage cytokine IL-1β. The toxin exhibits a strong adjuvant effect even by subcutaneous administration (55).

The mucosal adjuvant effect of B subunit is weak. The mucosal adjuvant effect of the holotoxin type mutant toxin (mCT) with a slight residual toxin activity of A subunit is strong, and the mucosal adjuvant effect of CT is more potent (51, 52). This is considered also to be the case for the similar toxin (LT) of ETEC (52). Analysis of the relationship between retrograde transport of the toxin and adjuvant effect is awaited.

The details of the action on CD4-positive cells differ between CT and LT (56). CT inhibits Th1 subset by inducing apoptosis, which results in the induction of Th2 subset, and IL-4, IL-5, and IL-6 cytokines promote the differentiation of B cells to IgA plasma cells, by which IgA production is increased. In contrast, LT induces both Th1 and Th2 subset (57). Both CT and LT enhance the expression of co-stimulatory molecules (B7-2) of APC in Peyer’s patches (56) (Fig. 3).

CT and LT are very toxic. In volunteers, oral administration of 5 μg of CT induced 6 liters of diarrhea (58). However, CTB is very safe, as described above, and oral administration of 1 mg does not induce diarrhea (59). The safety of mCT needs to be examined in volunteers.

### 9. Countermeasures for epidemics

For the control of cholera epidemics, treatment with oral fluid supplement (ORS); supply of clean drinking water; hygienic instruction on sanitation, etc.; and environmental improvements are effective.

Chemotherapy with tetracyclines, new quinolones, and macrolides is also effective for reducing diarrhea or shortening the diarrhea period, and for shortening the bacteria-shedding period, and has been applied in epidemic regions as a supplemental therapy (18). Case-fatality rate (CFR) is controlled to 1% or below if the countermeasures are appropriate (30).

Cholera vaccine (28, 30) may be used in people who move from epidemic areas, and for the control of cholera epidemics that may occur in people (refugees) who move into epidemic areas. It has been proposed that stores of vaccines for two million people should be prepared for emergency use. The
use of WC/rBS vaccine (oral administration twice with a 1-week interval) is considered when an epidemic is expected to occur within 6 months. Vietnamese WC vaccine (oral administration twice with a 1-week interval) has currently been approved only in Vietnam. CVD 103-HgR vaccine (single oral dose) may be available for travelers in advanced countries.

10. Conclusion

In vaccine development, three vaccines have made steady progress during the last 15 years, and irradiated WC vaccine is following. Secretory IgA has been mainly examined in regard to mucosal immunity against bacterial infection. Additional investigation of the role of mucosal IgG is necessary. Diverse custom-made vaccines that can deal with the changing situations in endemic countries or areas might be required in the 21st century for the “barriers” against live vaccines in such areas.

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