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 counter-measure 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 years 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 Kommabazillen 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 lysogeneic phages, and the bacteria exhibit the pathogenicity using these two pro-phages (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 pili (type IV pili), which play a role in the intestines as a colonization factor, and also serve as a receptor for CTXΦ phage. The whole *V. cholerae* O1 genome was determined in 2000 (7). The chromosome was approximately 4 Mbp in length, and had Å@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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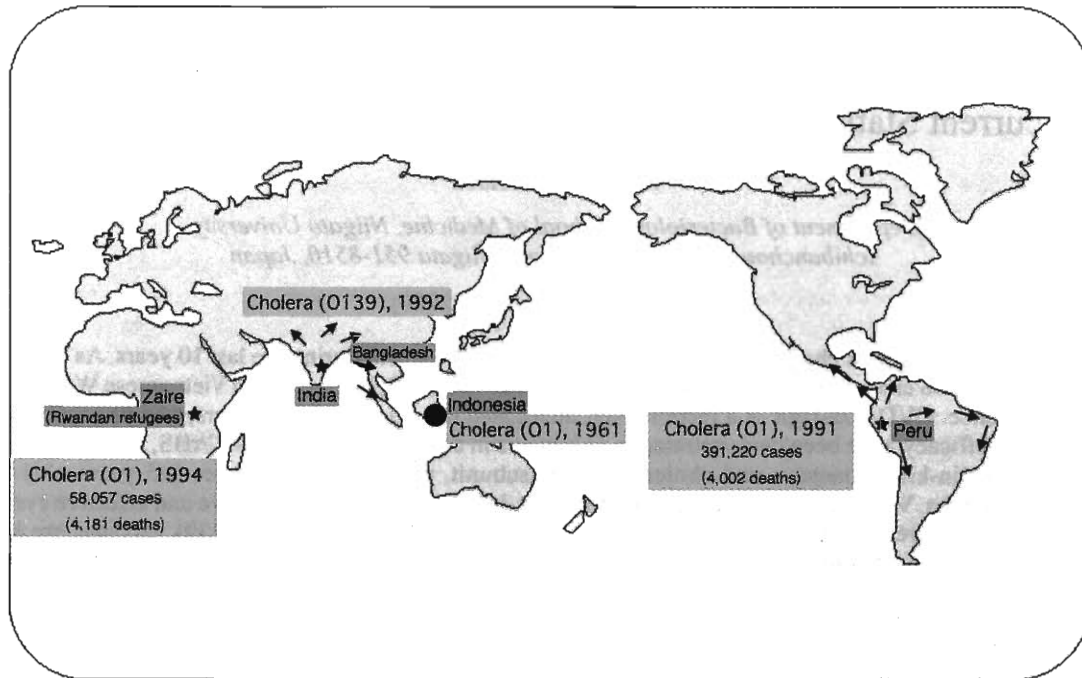
Fig. 1. The seventh cholera pandemic and major cholera epidemics in the last 10 years.

Fig. 2. Facial appearance of O139 cholera patient (supplied by Dr. G.B. Nair, National Institute of Cholera and Enteric Diseases, India).

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 hypopotassemia.

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 (Goa) 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 (10^5 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 field 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 (5×10^9 CFU) was necessary. This amount of bacteria corresponds to 10 times the experimental value (5×10^8 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 (N16117 strain), in which the CT gene was removed and ctxB and mercury resistance gene were inserted in the hemolysin gene. A mixed vaccine of CVD 103-HgR and CVD 111 increased the serum vibriocidal antibody titer, but an adverse effect (diarrhea) of CVD 111 was a large obstacle. Further attenuation is necessary for CVD 111 (34).

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 2.5×10^{10} each of heat-killed classical Inaba strain (Cairo 48), heat-killed classical Ogawa strain (Cairo 50), formalin-killed El Tor Inaba strain (Phil 6973), 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 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/rBS 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).

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

Vaccine	Study period (study place)	Study content (administration)	Results	Reference
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: 4.9×10^2 CFU/g in the vaccine group vs. 1.1×10^7 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 CVD103-HgR and CVD 111	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

Table 2. Results of filed, volunteer, and animal studies of killed cholera vaccine

Vaccine	Study period (study place)	Study content (administration)	Results	Reference
Killed bacteria[a] -B subunit (WC/BS=first generation)	1985-1988 (Bangladesh)	Filed study for El Tor cholera (three doses)	• Protection in 85% (for 4-6 months) • Protective efficacy decreased to 50% after 3 years (26% at 2-5 years old, 63% over 5 years old)	37, 38
Killed bacteria[a] (WC)	1985-1988 (Bangladesh)	Filed study for El Tor cholera (three doses)	• Protection in 58% (for 4-6 months) • Protective efficacy decreased to 52% after 3 years (23% at 2-5 years old, 68% over 5 years old)	37, 38
Killed bacteria[a] -rB subunit (WC/rBS=second generation)	1992 (Sweden)	Immune response test in volunteers (two, three doses)	• Defense index of immunity (vibriocidal antibodies) increased: WC/BS = WC/rBS two doses = three doses	39
Killed bacteria[a] -rB subunit (WC/rBS)	1992 (Columbia)	Field immune response test (two doses)	• Defense index of immunity (vibriocidal antibodies) increased: 44% in the vaccine group vs. 9.2% in the placebo group. Weak response under 4 years old Weak response in blood type O	40
Killed bacteria[a] -rB subunit (WC/rBS)	1994 (Peru)	Filed study for El Tor cholera (two doses)	• Protection in 86% (for 18 weeks)	41
Killed bacteria[a] -rB subunit (WC/rBS)	1993-1994 (Peru)	Filed study for El Tor cholera (two doses) (three doses)	• Protection in 4% (for 1 year) • Protection in 61% (for 1 year)	42
Killed bacteria[a] (Vietnamese WC)	1992-1993 (Vietnam)	Filed study (two doses)	• Protection in 68% even in children aged 1-5 years (for 8-10 months) • Protection in 66% over 5 years old (for 8-10 months)	43
Killed bacteria[b] (Irradiated WC)	2000 (Japan)	Rabbits (three doses)	• Increased protection, compared with the previous WC. • High levels of intestinal IgA and IgG.	Unpublished data

[a] Killed by heat/ formalin treatment.
[b] Killed by ^{60}Co-irradiation.

(2) WC vaccine

In an achievement of technology transfer, an inexpensive vaccine (cost: 0.1 dollars) was prepared in Vietnam, and examined in a filed study in 1992 (43) (Table 2). The vaccine compositions are 2.5×10^{10} of each of the following: heat-killed classical Inaba strain (Cairo 48), heat-killed classical Ogawa strain (Cairo 50), formalin-killed El Tor Inaba strain (Phil 6973), and formalin-killed classical Inaba strain (569B). This Vietnamese WC vaccine differs from the Swedish WC vaccine in the use of the 569B strain, which expresses well the colonization factor, TCP pilli. Two doses of the vaccine were administered at a 2-week interval. A good result was obtained, and the low protective efficacy in younger children, the disadvantage of Swedish vaccine (WC/rBS, WC), was overcome (Table 2).

(3) Irradiated WC vaccine

Heat and formalin treatment denatures proteins on the bacterial cell surface and destroys bacterial cell surface functions such as motility and adhesion. In contrast, ^{60}Co irradiation first destroys macromolecular chromosomal DNA. Therefore, the bacteria could be killed, preserving the cell surface functions such as motility and adhesion by controlling the irradiation conditions.

Irradiation at 1.5×10^3 Gy (1.5×10^5 rad) destroys 90% or more of 200 kbp plasmid DNA. Under this condition, no viable bacteria are detected. However, 5-38% of the bacteria still swim at 4-39 μm/s; the hemagglutinating activity (detected with human erythrocytes) is observed in 6-50%; and adhesion to M cells in human Peyer's patches is observed in 43-65% (unpublished data).

This new irradiated WC vaccine was examined for immunological properties and protection against challenge in rabbits (RITARD model). The vaccine contained 2.5×10^{10} of both classical Inaba strain (CI3) and El Tor Ogawa strain (EO8). As the controls, heat-killed CI3 and EO8 and formalin-killed CI3 and EO8 were used. EO8 was used as the challenge strain. The irradiated WC induced the secretion of not only IgA but also IgG 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.

IgG is a vibriocidal antibody, while IgA 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,
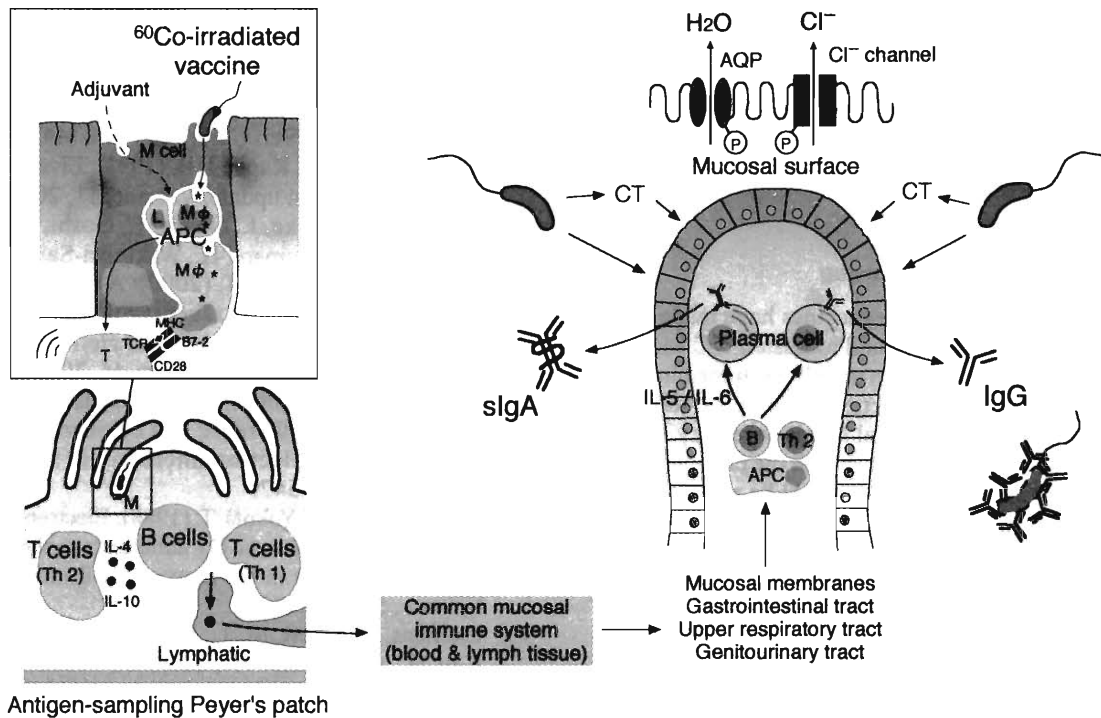
Fig. 3. Irradiated WC vaccine and mucosal immunity mechanism (hypothesis).

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 passing 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 1-6 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); a 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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