IMMUNITY IN CHOLERA AND VACCINE DEVELOPMENT: PROBLEMS AND PROSPECTS

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The acute diarrheal illness cholera is considered to be one of the ancient diseases in the recorded history of human civilization. Despite advances made in the health care delivery and research, the disease continues to affect a large population worldwide, particularly those living under low socio-economic conditions. A major effort to control cholera has been to develop a safe and effective vaccine. Unfortunately, the initial efforts, which started as early as in 1884, were unsuccessful due to various reasons. With the advancement of our knowledge on cholera, its causative bacterium *Vibrio cholerae* and the toxin produced by multiplying vibrios in the intestine, a resurgence of activities in the area of cholera vaccine research was noted. This was also prompted by data obtained from epidemiological and human volunteer studies which demonstrated that clinical cholera does provide some form of immunity to the disease. These and other studies also emphasized on (a) the protective role of local antibodies that are secreted in the intestine and, (b) the relative importance of antibacterial over antitoxic immunity in cholera. An understanding of mucosal immunology made it clear that oral, rather than parenteral, vaccines are better suited to stimulate intestinal immunity. These considerations initiated concerted efforts to develop oral vaccines that can be delivered as live (attenuated) or killed (inactivated) organisms. Availability of recombinant DNA technology made it possible to develop genetically engineered live oral vaccine strains with deletion(s) of the gene(s) encoding one or both the subunits of cholera toxin. Although many of these strains induced satisfactory level of protection in human volunteer and/or field studies, adverse side effects and other safety concerns were the important constraints for their further development. The killed vaccines, that are usually free from any adverse reactions and cost-effective, have also generated promising data in some of the recently conducted human trials. One such bivalent cholera vaccine is currently undergoing controlled field trials under a joint public-private partnership venture.

Introduction

Cholera is an acute form of diarrheal illness that had affected millions of people around the world over the centuries. Historically speaking, there are very few infectious diseases which can match classical cholera, caused by the bacterium *Vibrio cholerae* belonging to O1 serogroup, in terms of the rapid onset and severity of the disease symptoms as well as its capacity to cause explosive outbreaks leading to epidemics and pandemics. Further, cholera has been associated with high mortality and morbidity rates in the affected population. The oral-fecal route of transmission of the infective organism is believed to be facilitated due to the lack of safe drinking water and sanitation facilities for people living under low socio-economic conditions. Although considerable progress has been made in recent years to reduce the mortality rate in cholera through rehydration therapy (administered either orally or by the intravenous route), this treatment regimen is primarily capable of reducing the mortality rate associated with severe cases of cholera. Further, such facility may not be

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easily available in the remote parts of the world even now, particularly in the time of an outbreak or epidemic. In view of the above considerations, it is not surprising that cholera continues to haunt us even today with an increasing number of countries reporting cases of cholera in the recent past. The problem is compounded by the recent emergence of \textit{V. cholerae} strains belonging to the serogroup O139 as the additional causative agent of epidemic cholera.

A major emphasis in the area of cholera research has been toward the development of a vaccine against the disease. As a matter of fact, very few diseases have attracted so much attention of biomedical scientists and public health workers as cholera in this regard. The primary objective of all these efforts was to develop a vaccine that would fulfill the following criteria: (a) one or a few doses of the vaccine should be able to induce significant level of protection against the disease, at least, for a number of years, (b) the vaccine should be effective in all age groups and in different population, (c) it should be effective to stop the spread of cholera during the time of an outbreak or epidemic, (d) it should be easily administrable without any side-effects, (e) it should be cheap and easily available and (f) it should be stable when stored in facilities available under field conditions.

\section*{Early Studies on Cholera Vaccines}

Attempts to develop a vaccine against cholera started more than a century ago around the time when Robert Koch identified \textit{Vibrio cholerae} as the causative organism of the disease. Pioneering work in this regard was done by Louis Pasteur as early as in the year 1880. While working on chicken cholera, Pasteur made a serendipitous observation that led to the development of the concept and methodologies of “attenuation” of virulent organisms and their possible use as “live” vaccines to prevent infectious diseases like cholera, anthrax, rabies etc. Pasteur’s work prompted initial researchers to use attenuated strains of \textit{V. cholerae} for vaccination against cholera in humans. Thus, immediately after the discovery of cholera vibrios by Koch (1884), a broth culture of “attenuated vibrios” was used by Jaime Ferran to inoculate a large number of people by the parenteral route during the 1984 cholera epidemic in Spain. Severe adverse effect and lack of protective efficacy were the major drawbacks of the vaccine. Next major attempt in the area was made by Haffkine who also used attenuated strains for vaccination studies in India during 1893 to 1896. Adverse side effects, lack of control and standardization were the limitations towards the determination of its efficacy. Live attenuated vaccines were later on replaced by heat inactivated vaccine administered either orally or parenterally. Field trials were carried out in India (1920) and Indochina (1930) with killed whole cell \textit{V. cholerae} vaccine administered with bile which induced significant level of protection. However, adverse reactions again limited the use of such vaccines. Subsequent decades (1940-1960) saw a drop in the activity toward the vaccine development although cholera cases (epidemics) continued unabated in various parts of the world. During this period, killed whole cell vaccines were widely used to vaccinate military personnel and civilians exposed to the risk of cholera.

\section*{Infection Derived Immunity in Cholera}

The importance of acquired immunity in cholera had earlier been a matter of debate since reinfection was known to occur in people living in cholera endemic areas. Interestingly, Koch (1884) himself was of the opinion that acquired immunity against cholera did exist among cholera patients although it might not persist for a long time. This view was supported by epidemiological studies carried out during the 1960s to 1980s which demonstrated the significance of acquired immunity against the disease. One such study showed that an initial episode of cholera in Bangladesh conferred about 90% protection against a subsequent attack of clinical cholera and such protection lasted, at least, for a few years. This conclusion was validated by subsequent epidemiological analyses of data. These findings, and the fact that the reduction of disease incidence may also be achieved through vaccination, demonstrated the significance of acquired immunity in cholera. Further, human volunteer studies provided compelling evidence in favor of the existence of infection derived immunity in cholera as well as on the quality and duration of such immunity. Thus, clinical cholera was shown to give protection against challenge with live vibrios of homologous serotype (Inaba or Ogawa) and such protection did last, at least, for three years (the longest interval tested in the study). The level of immunity was stronger (100% protection) after an initial infection with the classical biotype strain than that (90%) observable following an initial infection with the El Tor biotype strain of \textit{V. cholerae}. Interestingly, considerable protection was also evident against challenge with organisms belonging to heterologous serotype.

\section*{Protective Immune Mechanisms in Cholera}

\textbf{Role of antibacterial antibodies:} Immunity in cholera is primarily mediated by antibodies that are stimulated in
convalescent cholera patients. Seroepidemiological data available from studies in Bangladesh suggest a relationship between serum vibriocidal antibody titers and protection against cholera. Antibacterial response in cholera has been shown to be directed primarily against the lipopolysaccharides (LPS) of V. cholerae and the serum anti-LPS antibodies are associated with mainly IgM and, to a lesser extent, with IgG and IgA class of antibodies. A crucial role of LPS in the induction of protective immunity was suggested by the results of a field trial carried out with parenterally administered purified LPS vaccine. Further, a recombinant vaccine based on an attenuated strain of Salmonella typhi expressing V. cholerae LPS on its surface induced low, but demonstrable, level of protection in human volunteers. The importance of anti-LPS antibodies in the induction of protection was also suggested through studies in experimental animals.

Besides LPS molecules, the contribution of other V. cholerae antigens towards the induction of protective immunity was also suggested by studies in experimental animals. These include flagellar sheath proteins, hemagglutinins, certain outer membrane proteins, the major subunit protein (TcpA) of toxin coregulated pilus (TCP) etc. Antibody responses against a variety of cell surface proteins have been documented in human convalescent sera and secretions. However, convalescent phase sera from volunteers and cholera patients contained only marginal level, if any, of antibodies to TcpA. Incidentally, the TCP was shown to play an important role in the intestinal colonization of vibrios. Therefore, it is reasonable to believe that the long-term protection against cholera can be achieved in the absence of significant level of anti-TCP immune response. Recently, proteomic analysis of V. cholerae isolated from stool samples of clinical cholera cases provided information on in vivo expressed immunogenic proteins that might be of relevance in the design of an improved cholera vaccine.

Role of antitoxin antibodies: The discovery of cholera toxin (an enterotoxin) and its role in the causation of diarrhea opened up a new approach in the area of cholera vaccine research. Numerous attempts were made to induce antitoxin immunity in man and experimental animal models. These studies were primarily aimed at effective neutralization of toxin activity through the stimulation of antitoxin antibodies. The relative inefficiency of the whole cell cholera vaccines developed earlier prompted several groups of workers to study the efficacy of toxoid vaccines in humans. However, parenteral or oral immunization of volunteers with large and multiple doses of cholera toxoid conferred no or little protection against live vibrio challenge. On the other hand, oral immunization of volunteers with the non-toxigenic mutants of a V. cholerae O1 strain provided significant level of protection against challenge with the toxigenic parent strain. All these considerations emphasized upon the relative importance of antibacterial over antitoxin immunity in cholera. Although antitoxin immunity alone may not be sufficient to protect humans against cholera, evidences are available to show that both antibacterial and antitoxic immunity may act synergistically mediating more efficient and lasting protection against the disease.

Role of mucosal (intestinal) antibodies: The precise mechanism of antibody mediated protection in cholera is yet to be understood completely. The superficial nature of infection process in cholera, which does not require any tissue invasion by the organism, suggests that the protective mechanisms should be operative locally i.e., either at the mucosal surface or in the lumen of the gut. Therefore, the scope of serum (circulating) antibodies to mediate protection in the gut appears to be somewhat limited. It is now believed that vibriocidal antibodies present in the serum may not be the actual mediator of the protective immunity but may serve as the surrogate marker for the presence of local (intestinal) antibodies directed against the same or other critical antigens of V. cholerae. The local immune response in cholera is largely mediated by secretory IgA (sIgA) antibodies that are likely to play an important role in protection against cholera. These antibodies, which usually do not exhibit complement dependent vibriocidal activities, probably induce protection by preventing intestinal attachment and colonization of vibrios. There are several possible mechanisms by which sIgA may interfere with the bacterial adherence process. For example, such antibodies may react with bacterial cell surface antigens thereby leading to their immobilization and/or agglutination. Alternatively, these may bind to bacterial adherence/colonization factors thereby interfering with the intestinal attachment and subsequent colonization process. In addition to their anti-adherence activities, sIgA antibodies have also been shown to possess toxin neutralization properties.

Oral Cholera Vaccines

Since induction of protective immunity by oral ingestion of live V. cholerae organisms closely mimics infection derived immunity in cholera, much attention has recently been focused to develop cholera vaccines that can be administered orally. Two major approaches were primarily followed to this objective. The first was to
generate live (attenuated) strains of *V. cholerae* through genetic manipulations of wild type pathogenic strains. The alternative approach was to use killed (inactivated) organisms as whole cell vaccines with or without the purified B-subunit of CT.

**Live (attenuated) cholera vaccines**: Initial vaccination experiments carried out during 1960 to 1980 utilized avirulent *V. cholerae* O1 strains either obtained from the environment or generated through manipulation of pathogenic O1 strains using mutagenic agents. However, preliminary results were not quite encouraging as many of these failed to colonize the intestine and stimulate adequate levels of antibodies or produced adverse reactions in the immunized hosts. With the advent of recombinant DNA technology 1980 onwards, it was possible to generate attenuated *V. cholerae* strains with precisely defined location of the mutations or deletions of genes. During the past few decades, different groups of researchers developed several vaccine strains of *V. cholerae*, primarily through deletion of genes encoding the B or both A and B subunits of CT. Although many of these strains colonized well in the intestine of human volunteers and stimulated immunologic response, post vaccination adverse reactions in the form of mild to moderate diarrhea and other symptoms restricted the scope of further studies with these strains. Furthermore, concern regarding the safety of recombinant vaccines was raised following the observation that the genes encoding CT are carried by a phage that can be acquired and integrated in the genome of nontoxicogenic *V. cholerae* strains expressing toxin coregulated pilus or TCP. These and other considerations demanded further modifications of the vaccine strains to eliminate residual diarrhea as well as the possibility of reversal to the virulent phenotype. Therefore, it is not surprising that only a few live recombinant strains have so far been developed that meets the criteria of safety, immunogenicity and effectiveness in human trials. One such live vaccine is the strain CVD 103-HgR derived from a *V. cholerae* O1 (Inaba) strain that was genetically modified to produce only the B-subunit of CT. The vaccine (OrocholTM produced in Switzerland) was given orally along with buffer to neutralize stomach acidity. Placebo-controlled, multicentric trials established the safety and immunogenicity of a single dose of the vaccine. Protection efficacy of a single dose of this vaccine was evaluated in adult volunteers in the USA, where it was found to give high level (about 95%) of protection against classical strains and about 65% protection against El Tor strains of *V. cholerae* following a challenge given three months after vaccination. However, the vaccine failed to demonstrate desirable level of protection in a subsequent large-field trial performed in cholera-endemic areas of Indonesia. This apparent failure was partly attributed to the limited number of cholera cases recorded during the trial period. Despite the fact that the vaccine showed protective efficacy in controlling an ongoing outbreak of cholera in Pohnpei Island (Micronesia), production of the vaccine has been discontinued since 2004.

Another live oral vaccine which received considerable attention recently is the attenuated strain (Peru-15) developed from a *V. cholerae* O1 El Tor strain isolated in Peru. The vaccine (manufactured in the USA under the name CholeraGardeTM) was found to be safe and immunogenic in North American adult volunteers and elicited significant level of protection against *V. cholerae* challenge. The vaccine was also found to be well tolerated and immunogenic when tested in Bangladeshi children in Phase I and II trials. Further studies are needed to evaluate its vaccination potential through Phase III and IV trials. Several other live oral candidate vaccine strains are currently undergoing different stages of testing which include strains developed in Cuba (*V. cholerae* 638 derived from a O1 strain) and Kolkata (VA1.3 from a non-toxigenic *V. cholerae* O1 strain).

The emergence of *V. cholerae* O139 organisms as the causative agent of epidemic cholera has also prompted researchers to develop a vaccine that would protect against O139 cholera. Some progress has already been made in this direction with the development of candidate vaccine (O139) strains like Bengal and CVD112 that have been shown to be safe and immunogenic in initial human volunteers trials. A live metabolic auxotroph of *V. cholerae* O139 has also been generated by gene mutation and the resulting strain (VCUSM2) has shown encouraging results in experimental animals.

**Killed (inactivated) cholera vaccines**: It is evident from above paragraphs that the experience gathered from several decades of effort to develop a live oral cholera vaccine has been less rewarding than expected. Clearly there are several issues concerning the safety, reactogenicity, efficacy, cost-effectiveness etc related to the use of a live vaccine (that would probably be the most useful in the developing countries) need to be addressed. All these considerations led to the resurgence of the concept of dead (inactivated) vaccines that may be administrated orally without any apparent reactogenicity or risk of reversion to virulence. One such vaccine was developed by a Swedish group that contained killed (inactivated) whole cells of *V. cholerae* along with purified recombinant B-subunit of CT (WC/rBS). Field trials
carried out in Bangladesh demonstrated that two doses of the vaccine, administered 1-2 weeks apart, conferred about 80-90% protection in all age groups during the first six months after vaccination\textsuperscript{56}. The level of protection declined sharply in young children after six months, although it persisted to a level of about 60% in older children and adults when followed up to a period of five years.

The killed WC/rBS vaccine (commercially produced in Europe as Dukoral\textsuperscript{TM}) is currently a mixture of four preparations of killed (either by heat or formalin) whole cells of \textit{V. cholerae} O1 belonging to two different serotypes (Inaba and Ogawa) and biotypes (classical and ElTor) and containing purified recombinant B-subunit of CT. In 2001, World Health Organization (WHO) recommended it for vaccination against cholera in high risk population facing outbreaks or epidemics\textsuperscript{57}. Since then, it has been used successfully in mass vaccination of high risk population in different parts of Asia and Africa to control cholera outbreaks in times of emergency. More importantly, the vaccine was found to be effective against cholera in Beira (Mozambique) in a population with high prevalence rate of HIV infection\textsuperscript{48}. The vaccine is currently administered in three doses to young children (2-6 years) with booster doses every six months apart. However, a two dose regimen is used for older children and adults with a booster dose every two years apart.

A major concern regarding the large scale use of Dukoral in the developing countries (where it is most needed) is its relatively high cost of production, which is primarily due to the inclusion of the B- subunit component in the formulation. Therefore, attempts were made to develop a cheap and modified version of this inactivated vaccine without the B subunit. Such a vaccine was produced and tested in Vietnam with considerable success. In a field trial conducted in Vietnam, two oral doses of the modified vaccine showed a protective efficacy of about 66% against El Tor cholera in all age groups when followed up for 8 months\textsuperscript{59}. The vaccine (ORC-Vax\textsuperscript{TM}) is currently used in Vietnam to protect against cholera.

In view of the importance of \textit{V. cholerae} O139 in the causation cholera epidemics, a modified version of the ORC-Vax\textsuperscript{TM} vaccine was formulated which contained killed whole cells of \textit{V. cholerae} belonging to both O1 and O139 serogroups. The bivalent vaccine was shown to be safe and immunogenic as it stimulated significant level of vibriocidal antibody response to both O1 and O139 organisms following a two- oral dose regimen\textsuperscript{60}. Large scale production of the modified bivalent vaccine has been licensed to a commercial manufacturer (Shantha Biotech, India) for preclinical as well as clinical evaluation. The licensed vaccine has so far been produced in large doses and used in humans without any major adverse reactions. Preliminary results obtained from a double-blind, placebo-controlled trial carried out in Kolkata has shown that the modified vaccine is safe and efficacious providing nearly 70% protection against clinical cholera for at least 2 years and the protection is evident in children as well as in older individuals\textsuperscript{61}. Further analysis is currently underway to evaluate its long term protective ability.

Other cholera vaccines in experimental stage of development: Several other cholera vaccines are currently in various stages of development in experimental animals\textsuperscript{62}. These include: (a) different preparations of \textit{V. cholerae} O-antigen based polysaccharides conjugated to proteins for parenteral administration\textsuperscript{63-65}, (b) a DNA vaccine expressing B-subunit of CT to be delivered parenterally\textsuperscript{66}, (c) potato\textsuperscript{67} or rice\textsuperscript{68}-based oral vaccines expressing B-subunit of CT, (d) \textit{V. cholerae} outer membrane vesicle preparation- based vaccine for mucosal immunization to elicit intestinal antibodies\textsuperscript{69}, (e) a proteoliposome–based formulation administered by the nasal route eliciting vibriocidal antibodies\textsuperscript{70} etc. However, none of these has reached the stage for advanced trials in humans.

Conclusion

The search for an effective cholera vaccine, which started more than a century ago, has been a long and frustrating experience. Despite tremendous progress made in our understanding of the disease cholera and its causative organism, we are yet to achieve our goal to develop a vaccine that would fulfill all the desirable criteria as outlined earlier. The reasons are manifold. Part of the problem arises as a result of intestinal localization of cholera organisms in the infected host which, unlike many systemic diseases, does not allow a robust and long-lasting immunity to be generated against reinfection. Further, stimulation of high level of antibodies in serum by parenteral vaccination may not necessarily reflect the status of gut-associated immunity that can be more effectively stimulated by oral immunization. Another problem is that cholera, being a disease primarily affecting people living under low socio-economic conditions, has so far failed to receive a high priority either from the health authorities of industrialized nations or from pharmaceutical companies. It is needless to mention that, like any other candidate vaccine against infectious diseases, a prospective cholera vaccine is also required to go through
rigorous screening procedures involving various phases of preclinical and clinical trials that are both labor- and cost-intensive. It is heartening to see that several non-governmental and philanthropic organizations have recently come forward with a mission to alleviate the sufferings of the poor people living in the developing world through the delivery of improved health care facilities. This development, as well as the evolution of the concept of public-private partnership based consortium involving participation of pharmaceutical corporate houses, has considerably changed the scenario of vaccine research.

As a result, there has been a recent resurgence of activities toward the development of a cholera vaccine. As the proverb goes that “the taste of pudding lies in eating”, the applicability of a cholera vaccine can only be decided eventually through a controlled field trial in the cholera endemic areas on a prospective basis. Evidently, the feasibility and success of such a trial depend on many factors which include the choice of an appropriate study area and incidence of cholera during the study period. Despite these constraints, it is gratifying to note that considerable progress has been made in the area of cholera vaccine research during the past few decades. Finally, one may conclude with a note of optimism that the performance of one or more of the candidate vaccines currently under trial will be found to be quite satisfactory to be recommended for large scale use in the immunoprophylaxis of cholera.

“Dr. Marco Aurelio Urbino, the father of Juvenal, was a civic hero during the dreadful time (of cholera) as well as its most distinguished victim. ………..his diligence and his self sacrifice and above all his personal courage deserved the many honors rendered him when the city recovered from the disaster (epidemic). ……… He did not live to see his own glory. When he recognized in himself the irreversible symptoms that he had seen and pitted in others, he did not even attempt a useless struggle but withdrew from the world so as not to infect anyone else. Locked in a utility room at Misericordia Hospital, deaf to the calls of his colleagues and the pleas of his family, removed from the horror of the plague (cholera) victims dying on the floor in the packed corridors, he wrote a letter of feverish love to his wife and children, a letter of gratitude for his existence in which he revealed how much and with how much fervor he had loved life. It was a farewell of twenty heartrending pages in which the progress of the disease could be observed in the deteriorating script, and it was not necessary to know the writer to realize that he had signed his name with his last breath. In accordance with his instructions, his ashen body was mingled with others in the communal cemetery and was not seen by anyone who loved him”

From “Love in the time of cholera” by Gabriel García Márquez
(Nobel Prize winner in literature, 1982)

References
