Insights from Natural Infection-Derived Immunity to Cholera Instruct Vaccine Efforts

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Diarrhea caused by Vibrio cholerae O1 (and occasionally O139) remains a notable disease burden in much of the developing world, affecting several million individuals and leaving an estimated toll of >100,000 deaths annually (52). Outbreaks with unacceptable high case fatality rates continue to occur in areas of extreme poverty, overcrowding, and poor sanitary conditions, often accompanying natural disasters, civil disruption, or social unrest (11, 15, 17, 52).

It is known from epidemiological studies and experimental challenges in healthy adult volunteers that a prior episode of clinical cholera induces serogroup-specific protection against clinical disease upon subsequent exposure (25, 27). The precise immunological effectors that mediate protection against V. cholerae are not fully understood. Vibriocidal antibodies (mainly, but not entirely directed against lipopolysaccharide [LPS] antigen) are a measure of immunity and have been inversely correlated with susceptibility to infection (13, 29, 31). Studies performed in the 1960s with household contacts of cholera cases showed that vibriocidal titers of ≥160 were associated with an ~86% lower risk of developing cholera infection. Of 59 household contacts with vibriocidal titers of ≥160, only 1 (1.7%) developed cholera diarrhea and only 1 (1.7%) other developed inapparent infection, compared with 28 (14.7%) of 190 nonimmune individuals (titer, <20) who developed cholera and another 24 (12.6%) who developed inapparent infection (29). Among these household contacts of cholera patients, every 2-fold rise in vibriocidal titer was associated with a 50% decrease in risk of cholera infection. These data mimic what Mosley et al. also found in large population-based surveillance studies accompanied by serosurveys in which every 2-fold rise in geometric mean vibriocidal titer was accompanied by a halving of the annual incidence of clinical cholera cases (30).

Two types of oral cholera vaccines consisting of whole killed organisms are commercially available: Dukoral (Crucell, Sweden), which contains V. cholerae O1 serotype Inaba and Ogawa strains of both El Tor and classical biotypes admixed with recombinant cholera toxin B subunit (CTB), and Shanchol (Shantha Biotechnics-Sanofi Pasteur, India) or mORCVAX (VaBiotech; Vietnam), which contain a mix of several V. cholerae O1 strains and an O139 strain, without CTB. In large-scale, randomized controlled field trials, these vaccines (or their prototypes) were found to be safe and immunogenic and conferred ~60 to 80% efficacy in preventing cholera in adults and older children (9, 47). The efficacy of the oral inactivated-cholera vaccines is substantially less in children. The protection afforded is also short-lived (~2 to 3 years) (52). In contrast, clinical cholera due to wild-type infection generally leads to a more robust and durable protection upon reexposure, which is evident in both young and old individuals and lasts up to a decade (2, 10, 25).

A thorough understanding of the bacterium-host interactions that lead to disease, the immune responses induced, and the mechanisms that mediate protection against subsequent V. cholerae illness can help guide efforts to develop even more effective vaccines (e.g., requiring only a single dose and more protective in young children). Because humans are the only natural hosts for V. cholerae O1 and O139, an obstacle to this endeavor is having access to infected patients and individuals exposed to V. cholerae. Our current understanding of cholera pathogenicity and immunity comes from studies conducted in areas where the disease is prevalent or through experimental infection of healthy adult volunteers (human challenge models). When human challenge studies were performed in the 1970s, 1980s, and 1990s, some of the most critical techniques to assess immune responses of modern immunology were not available and therefore many relevant questions could not be answered. Although instructive, it could be argued that some results from challenge studies in subjects in industrialized countries may not be generalizable to endemic cholera, since they are performed in a different (nonendemic) population and in a nonnatural environment (2). However, challenge studies in Thai volunteers that used the same virulent cholera strain given to North American volunteers achieved 90% attack rate with a dose that was only 1 log higher (46). Investigation of disease in its natural context is ideal but very difficult. Assembling cohorts, procuring clinical specimens, and performing sophisticated immunological assays during field studies in resource-poor areas where cholera is endemic can be logistically complicated, requiring infrastructure, equipment and expertise, and substantial financial resources. Cultural differences and social and political conflicts can complicate the work further.

Undeterred by these difficulties, a group of very talented and committed scientists from the International Centre for Diarrheal Disease Research in Bangladesh (ICDDR,B), and the Massachusetts General Hospital and Harvard Medical School joined efforts to establish a highly successful and productive collaborative research program focused on dissecting host-pathogen interactions and human immune responses to V. cholerae. This work has generated a wealth of new knowledge that has informed the cholera field, while also advancing our understanding of the immunology of enteric diseases in general. In a series of papers published recently in Clinical and Vaccine Immunology (4, 22, 33, 48), including the accompanying article by Johnson et al. (19), as well as in...
other relevant publications (1, 14, 16–18, 20, 21, 23, 41, 49), this collaborative research group has extensively described the immune responses to *V. cholerae* in adults and children living in rural and urban Bangladesh, comparing clinical infection versus vaccination. They meticulously investigated systemic and mucosal immunity by measuring serum and mucosal antibodies, antibody-secreting cells (ASCs), antibodies in lymphocyte supernatants (ALS), and B- and T-cell memory in a variety of clinical specimens (including some that are difficult to obtain, such as mucosal biopsy specimens and intestinal fluids) using state-of-the-art technology. Several aspects make this work novel. Not only did these investigators explore in detail immunological markers of infection and vaccination, but they also examined how these markers were associated with (and possibly contribute to) protection in a population in an area of endemicity. They also studied younger and older children, which is particularly relevant considering the different susceptibilities and immune competencies of children compared to adults.

In analyzing serum antibodies, these investigators found that both clinically infected and vaccinated individuals had comparable levels of antibodies to LPS and CTB. However, infected individuals developed stronger virobicidal responses than those who had been vaccinated. While these antibodies have been traditionally the marker of cholera protective immunity, these investigators found that serum IgA against CTB and LPS could also predict protection independent of virobicidal antibodies (16). However, a subsequent study failed to confirm this association. Nevertheless, the authors point out that infected patients who became ill had lower IgA LPS baseline titers than the household contacts, suggesting that lack of antibodies might indicate a risk for more severe disease (33). Interestingly, serum antibodies against cholera antigens typically return to baseline levels within a year, while protection lasts much longer (14, 16). Mucosal secretory IgA (sIgA) antibodies were detected in duodenal fluids, but these also returned to baseline within 6 months (49). No correlations were found between IgG antibodies and protection.

The ICDDR,B-Massachusetts General-Harvard research consortium also found that individuals from areas of endemicity infected with *V. cholerae* developed IgA ASCs specific for LPS and CTB, which peaked 7 days after the onset of disease (36, 37) and expressed the gut-homing receptors α4β7 (35). Circulating IgA ASCs are typical markers of mucosal priming after enteric infection or oral vaccination, and they can be detected transiently in circulation while migrating back to mucosal effector sites. Mucosal LPS-specific IgA ASCs were demonstrated in duodenal biopsy specimens from cholera-infected patients; the cells remained detectable for at least 6 months, even in the absence of detectable anti-LPS IgA in secretions. These cells may resume antibody production when appropriately stimulated (49).

Mucosally primed antigen-specific B cells may also become memory B cells (B<sub>M</sub> cells), which will differentiate into ASCs upon antigen reexposure. IgG and IgA B<sub>M</sub> cells specific for LPS, CTB, and TcpA were found in cholera-infected patients for up to 1 year following disease (14, 18). In contrast, individuals given Dukoral oral cholera vaccine failed to develop LPS B<sub>M</sub> responses; IgA and IgG B<sub>M</sub> responses to CTB were detected, but only for 1 to 6 months (1). The ICDDR,B-Massachusetts General-Harvard group was the first to report the presence of B<sub>M</sub> cells against *V. cholerae* antigens. In fact, they were the first to report B<sub>M</sub> responses against a noninvasive intestinal mucosal pathogen in humans (18). The observation of IgA and IgG B<sub>M</sub> cells specific for LPS was surprising based on the traditional notion that T-independent antigens lack B<sub>M</sub> cell production. Subsequently, investigators studying other enteric pathogens (e.g., *Shigella* and *Salmonella*) showed induction of LPS-specific B<sub>M</sub> cells, some of them with gut-homing capacity (α4β7<sup>+</sup>), in healthy adults orally immunized with live attenuated organisms (43, 44, 50, 51). It has been shown recently that T-cell-independent class-switched B<sub>M</sub> cells (CD27<sup>+</sup> IgA<sup>+</sup>) can be generated locally in the human gastrointestinal tissue, independent of germinal center reaction (5). A high point of the Ryan, Qadri, and Calderwood’s work has been the association between IgG B<sub>M</sub> responses against *V. cholerae* O1 LPS and protection against infection in household contacts of cholera patients. These findings were published in *Clinical and Vaccine Immunology* in June 2012 (33). The authors did not find associations between protection and CTB-specific IgG and IgA B<sub>M</sub> cells or IgA B<sub>M</sub> cells specific for LPS, but they cautioned that due to the high heterogeneity of the individual responses (33), the number of samples might not have been sufficiently numerous to provide statistical power to detect such associations.

An article published in *Clinical and Vaccine Immunology* in March 2012 focused on immune responses in younger (2 to 5 years of age) and older (6 to 17 years of age) children after either vaccination or infection (22). Although the responses to the oral killed-cholera vaccine Dukoral were comparable between the two groups, they were much lower than those detected in infected children. Infected children had higher virobicidal antibodies, systemic IgA and IgG anti-LPS, and IgG and IgA LPS and CTB-specific B<sub>M</sub> responses, whereas those vaccinated with Dukoral had lower responses to CTB and lacked B<sub>M</sub> cells against LPS. The point was made that the lack of responses against LPS may explain, in part, the shorter duration and lower level of protection in vaccinated younger children compared to the responses in older individuals.

Looking at these results together, the ICDDR,B-Massachusetts General-Harvard investigators argue that serum antibodies represent a “surrogate” (i.e., nonmechanistic correlate) rather than a “true correlate” of protection (i.e., a mechanistic correlate), since serum antibody titers drop quickly, while protection persists for years, and no consensus “protective” threshold has been identified (34). Likewise, mucosal antibodies decline even faster. The investigators propose instead that a mucosal anamnestic response mediated by B<sub>M</sub> cells (directed primarily to the LPS) appears to be necessary for long-term protection against cholera (7). It would make sense for the host to mount a quick, refined, and local response when reexposed to the pathogen instead of producing antibodies continuously. This hypothesis is in agreement with results obtained in North American volunteers who were rechallenged 3 years after experimental infection with *V. cholerae* O1. These individuals exhibited impressive anamnestic responses (indicative of immune memory) and remained protected in the absence of the repetitive exposure to the organism and immune reinforcement of populations in areas of endemicity (25). In areas where cholera is endemic, moderate baseline responses can control low-level repeated exposure, but a strong anamnestic response would still be required to fend off an overloading, infecting dose. The idea of B<sub>M</sub> cells being key contributors to protection is very plausible and probably true for other enteric bacteria as well. B<sub>M</sub> cells (with mucosal-homing capacity) are being increasingly used as predictors of long-term protection against enteric disease.
To facilitate the study of B<sub>M</sub> responses in vaccine studies, the ICDDR,B-Massachusetts General-Harvard group offers a technical improvement. Based on the strong correlation observed between the frequency of IgG B<sub>M</sub> cells measured by enzyme-linked immunosorbent spot (ELISPOT) assay and the level of antibodies produced by these cells, they propose the analysis of antibodies in a culture supernatant of expanded B<sub>M</sub> cells as a proxy for B<sub>M</sub> responses (18). Measurement of antibodies is easier, multiple antigens can be tested, and supernatants can be frozen and assayed at a later time point (18). This method has been successfully applied in the evaluation of other vaccine candidates (38, 50, 51).

The Ryan, Calderwood, and Qadri group also performed a thorough analysis of T-cell responses in cholera-infected or vaccinated individuals, including children (4). They showed that cholera induces an early proinflammatory response (clinically silent) and priming of antigen-specific CD4<sup>+</sup> Th1- and Th17-type cells that were not observed in Dukoral recipients. The failure of this oral vaccine to activate adequate CD4<sup>+</sup> T-cell responses may account for the limited efficacy of this vaccine (20). In an article published in *Clinical and Vaccine Immunology* in August 2012, they showed for the first time that natural infection resulted in significant CTB-specific effector memory T cells (T<sub>EM</sub>) expressing follicular helper (CXCR5) and gut-homing (β7) receptors along with production of Th1 (gamma interferon [IFN-γ]), Th17, and Th2 (IL-13) cytokines (4). Vaccination, on the other hand, induced modest T<sub>EM</sub> responses (without IL-13), but only in older children (6 to 14 years old). Younger children (2 to ~6 years old) did not develop T<sub>EM</sub> but did apparently develop regulatory T (Treg) cells that produced interleukin-10 (IL-10). T-cell help is critical for the development of B memory. In fact, the early T<sub>EM</sub> responses in older vaccine recipients correlated with their IgG B<sub>M</sub> responses. The authors associated the lack of T<sub>EM</sub> and reduced B<sub>M</sub> responses in vaccinated younger children with the lower efficacy and shorter duration of the protection afforded by the vaccine in this age group. The link that is still missing is that LPS, not CTB, BM responses. The authors associated the lack of T<sub>EM</sub> and reduced B<sub>M</sub> responses in older vaccine recipients correlated with their IgG B<sub>M</sub> responses. The authors associated the lack of T<sub>EM</sub> and reduced B<sub>M</sub> responses in vaccinated younger children with the lower efficacy and shorter duration of the protection afforded by the vaccine in this age group. The link that is still missing is that LPS, not CTB, BM responses, was associated with protection. The article does not address, nor do the authors discuss, a possible (CTB-mediated) bystander T-helper support for the induction of LPS IgG B<sub>M</sub> cells. Since CTB is immunologically cross-reactive with the *Escherichia coli* heat-labile toxin (LT), it is difficult to ascertain the origin of these responses and their role in protection is also unclear (27). The two other oral cholera vaccines on the market (Shanchol and mORCVAX) do not contain CTB but still provide similar levels of protection. Live vaccines also lack CTB and were found to be protective, although they contain other immunogenic proteins. Even though CT may not be directly involved in protection, it may possibly modulate responses to other antigens. An early study in Bangladesh showed that an initial episode of cholera caused by El Tor resulted in relatively poor and short-lived protection against subsequent El Tor exposure and virtually no protection against the classical biotype vibrios. In contrast, an initial episode of classical biotype cholera conferred strong and enduring protection against exposure to either biotype (10). One possible explanation is that CT expressed by the classical biotype modulates the responses to critical protective antigens differently than CT expressed by El Tor. Currently circulating hybrid strains of El Tor that express a toxin similar to that produced by classical biotype may help explain why recent episodes of El Tor (due to hybrids expressing classical CT) may be better immunizing events than those described 2 decades ago. Other T-dependent antigens might also contribute to host defenses against cholera and account for some of the differences between naturally acquired and vaccine-induced protection.

Aggregate results from this group and others provide extensive evidence underscoring the relevance of LPS-specific responses in protection against cholera. In fact, LPS and antigenic derivatives are being studied as potential vaccine candidates. In the article published in this issue of *Clinical and Vaccine Immunology*, Johnson and colleagues investigated potential immunogenic targets of the *V. cholerae* LPS focusing on the O-polysaccharide antigen (OPS) (19). The OPS of *V. cholerae* defines serogroup specificity, and as mentioned above, protection derived from natural disease is serogroup specific. The authors examined circulating levels of IgA, ASC, and ALS responses to LPS and the O-antigen in infected patients and found them to be similar and strongly correlated. IgM antibodies to Inaba OSP were significantly correlated with the vibrioidal responses. They concluded that the O-antigen region seems to capture most of the humoral response to whole LPS and contributes substantially to the observed vibrioidal responses. These results support efforts for O-antigen conjugate vaccines, an approach that is being pursued to prevent disease caused by *Salmonella*, *Shigella*, and other enteric pathogens (32, 45).

Overall, the reduced and short-lived efficacy of the oral killed-cholera vaccine appears to result from its reduced immunogenic capacity and inability to induce key immunological effector mechanisms needed to engender a robust and long-lived protection. Conceivably, live vaccines that can mimic cholera infection could harness protective immunity similar to that achieved through natural infection, ideally after a single immunization. Among the most promising live attenuated cholera vaccines are CVD 103-HgR (PaxVax, United States) and Peru 15 (Haikou VTI Biological Institute, China), both genetically engineered toxin-deficient strains. CVD 103-HgR was shown to be safe and highly effective in North American volunteers (26). This vaccine strain was also tested in a randomized large-scale controlled field trial in an area of high endemicity in north Jakarta (39). Following administration of vaccine or placebo in this densely crowded urban slum, where the majority of eligible subjects participated in the trial, the incidence of cholera fell by >80% compared with pre-field-trial rates. However, evaluating the cases that did occur, the calculated vaccine efficacy was low (39). It is now widely believed that indirect protection provides an explanation for the Jakarta results. In an innovative reanalysis of data from the mid-1980s field trial of a killed-whole-cell–B subunit combination vaccine (precursor of Dukoral), Ali et al. showed that when approximately one-half of subjects in the community receive cholera vaccine, the incidence of cholera plummets both in the vaccines and in the controls, through indirect protection (3). Indeed, as the incidence drops drastically, the levels of vaccine efficacy become insignificant as the disease largely disappears. CVD 103-HgR served as a licensed single-dose cholera vaccine before manufacture was discontinued in 1994 (6, 24, 42). Production of this live vaccine by a new manufacturer (PaxVax) is under way, with the intention to make it available once again as a single-dose cholera vaccine (NCT01585181; [http://ClinicalTrials.gov](http://ClinicalTrials.gov)).

Peru 15 was safe and immunogenic in adults and children in Bangladesh but has not yet been tested in phase 3 studies. Other live oral vaccine strains that have been found to be well tolerated and immunogenic in humans are *V. cholerae* 638 (12), and CTB-expressing strain VA1.3 (28). Both are attenuated EL Tor deriva-
tives; strain 638 protected adult volunteers in a challenge study (12). Other vaccine candidates, including protein subunits and lipopolysaccharide conjugates, are under development (8, 42).

An interesting new approach pursued by the ICDRR,B-Massachusetts General-Harvard investigators and published in *Clinical and Vaccine Immunology* in February 2012 is the use of a neoglycoconjugate vaccine made from a synthetic terminal hexasaccharide of the Ogawa O-antigen conjugated to BSA (48). Oral priming with a live attenuated *V. cholerae*, followed by a transcutaneous boost with the neoglycoconjugate cholera vaccine and cholera toxin or *E. coli* LT as adjuvants resulted in fecal IgA as well as serum IgG and IgA anti-LPS and vibriocidal antibodies. Immune serum transferred to neonatal mice resulted in 79% protection against wild-type *V. cholerae* challenge (48). Interestingly, the vaccine was not immunogenic when used alone (40), but only when it was preceded by oral priming; higher responses were induced by transcutaneous as opposed to subcutaneous boosting. The authors suggest that a boosting vaccine may prolong natural and vaccine-induced protection (48). It remains to be seen whether this approach elicits gut-homing ASC and Bm cells.

More effective and affordable vaccines that confer greater and longer-term protection following administration of just a single dose, along with strategies to improve sanitation and access to clean water, could reduce the risk of cholera in a cost-effective manner. International health organizations have recommended large-scale vaccination to reduce disease and improve public health in areas where cholera is endemic and to alleviate the risk of outbreaks (52). Insights into the elements of protective immunity derived from natural infection can instruct vaccine development efforts and advance the goal of disease control. The ICDRR,B-Massachusetts General-Harvard team has unraveled many of the intricacies of the host immune responses against *V. cholerae* and identified key effector responses that should be required of any new cholera vaccine to increase the likelihood of being effective in high-risk groups.

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We dedicate this article to the memory of Ana Fraccaroli de Pasetti (1917–2012).

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