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Correlates of protection for enteric vaccines

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Abstract

An immunological Correlate of Protection (CoP) is an immune response that is statistically interrelated with protection. Identification of CoPs for enteric vaccines would help design studies to improve vaccine performance of licensed vaccines in low income settings, and would facilitate the testing of future vaccines in development that might be more affordable. CoPs are lacking today for most existing and investigational enteric vaccines. In order to share the latest information on CoPs for enteric vaccines and to discuss novel approaches to correlate mucosal immune responses in humans with protection, the Foundation Mérieux organized an international conference of experts where potential CoPs for vaccines were examined using case-studies for both bacterial and viral enteric pathogens.

Experts on the panel concluded that to date, all established enteric vaccine CoPs, such as those for hepatitis A, Vi typhoid and poliovirus vaccines, are based on serological immune responses even though these may poorly reflect the relevant gut immune responses or predict protective efficacy. Known CoPs for cholera, norovirus and rotavirus could be considered as acceptable for comparisons of similarly composed vaccines while more work is still needed to establish CoPs for the remaining enteric pathogens and their candidate vaccines.

Novel approaches to correlate human mucosal immune responses with protection include the investigation of gut-originating antibody-secreting cells (ASCs), B memory cells and follicular helper T cells from samples of peripheral blood during their recirculation.

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Conflict of interest

ED and SPN are employees of Sanofi Pasteur. Other authors declare that they have no conflicts of interest to report.

Disclaimers

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

Keywords

Enteric vaccines; Enteric pathogens; Correlates of protection; Conference

1. Introduction

An immunological Correlate of Protection (CoP) is an immune response that is statistically interrelated with protection and may be either a mechanistic CoP (mCoP) or a non-mechanistic CoP (nCoP) [1]. There may be more than one CoP for a disease, which are usually referred to as «co-correlates» [2]. Most currently known CoPs relate to neutralizing serum or mucosal antibody, but other functions of antibody may be more important in particular cases. In addition, cellular immune responses often synergize with antibody to protect.

Vaccines are licensed against some enteric pathogens and several candidate vaccines against other pathogens are in development or testing. For most both existing and not yet licensed enteric vaccines, established CoPs are lacking today. To examine correlates of enteric vaccine-induced protection, the Fondation Mérieux organized a conference from March 21–23 2016 (“Les Pensières” Conference Centre, Annecy-France). The purposes of this workshop that gathered immunologists, epidemiologists, statisticians, infectious disease and regulatory experts were to provide state of the art information on CoPs for enteric vaccines and to discuss novel approaches to correlate mucosal immune response with protection in humans.

Key note introductory lectures by *Stanly Plotkin* (University of Pennsylvania, USA) and *Jan Holmgren* (University of Gothenburg, Sweden) set the scene for the conference by summarizing current knowledge on “CoPs induced by vaccines with special reference to enteric vaccines” and “The links between mucosal and systemic immunity: what is known and what is not known”. Enteric pathogens differ in the way they cause infection and disease, especially whether they are invasive or not and to which extent they cause mucosal inflammation. This influences what type of immune responses they elicit, how vaccination by different routes may protect, and what immune CoPs there may be. Second generation enteric vaccines that could be cheaper, more protective and possibly need only a single dose are under development. Evaluation of these new generations of vaccines which might be preferred to the existing vaccines may encounter ethical objections to future placebo-controlled efficacy trials in endemic populations. Identification of a CoP could facilitate a non-inferiority study to support licensure. It would also help design lower sample size studies to better understand the sometimes large variation in vaccine efficacy in different settings and the effect of interventions to improve vaccine performance in low income settings. The finding of a CoP would also facilitate the testing and licensure of future vaccines that might then be faster, more affordable and could help increase the global vaccine supply especially in developing countries. Some of these aspects were also addressed by *Nicholas Grassly* (Imperial College of London, the UK) who discussed how experiences from use of CoPs in polio vaccination might apply to other enteric vaccines.

The prioritization of enteric vaccine candidates requires a better understanding of the incidence, etiology, and adverse clinical consequences of the most life-threatening and disabling episodes of diarrhea among young children. Karen Kotloff (University of Maryland, USA) reviewed the main findings of the Global Enteric Multicenter study (GEMS), a prospective, age-stratified, matched case/control study of moderate-to-severe diarrhea (MSD) in children aged 0–59 months in Africa and Asia [3]. This study found that most attributable cases of MSD were due to five pathogens: rotavirus, *Cryptosporidium, Shigella*, heat-stable enterotoxin (ST)-producing Enterotoxigenic *Escherichia coli* (ETEC), and to a lesser extent Adenovirus 40/41. *Campylobacter jejuni*, Aeromonas and *Vibrio cholerae* O1 had regional importance (and it should be noted that especially *V. cholerae* continues to be an important pathogen responsible for many deaths in children above age 5 years and adults). Reanalysis of original samples by quantitative molecular diagnostic approach based on real-time PCR, led to revised estimates of the most important causes of MSD which were now in descending order, *Shigella* spp, rotavirus, adenovirus 40/41, ST-producing ETEC, *Cryptosporidium* spp, and *Campylobacter* spp [4]. These results suggest that targeted interventions for a limited number of pathogens, e.g. in the form of vaccines, might have a substantial impact.

2. Case studies of correlates of protection

Established and/or possible new CoPs for a number of existing or in-pipeline vaccines against important enteric infections/-pathogens were specifically addressed.

2.1. Bacterial pathogens

2.1.1. Cholera—The causative agent of cholera, *V. cholerae*, is a non-invasive pathogen causing severe and often life-threatening diarrhea through the action on the small intestinal epithelium of the cholera enterotoxin released by the bacteria during their extensive multiplication in the intestine [5,6]. Of >200 *V. cholerae* serogroups, serogroup O1 (with two major serotypes, Inaba and Ogawa) currently causes >99% of all cholera cases globally.

Knowledge gained by challenged volunteer model studies regarding immune protection in cholera and immune response to oral cholera vaccines (OCVs) were reviewed by *Myron Levine* (University of Maryland, USA). Such studies were successful in predicting the substantial protection afforded by killed whole cell OCVs in phase 3 clinical trials [8,9] suggesting that this challenge model could serve as surrogate for field evaluation. However, the protective efficacy induced by a single-dose live, attenuated OCV (CVD-103HgR) observed in human challenge studies [10–12] was not reproduced in a placebo-controlled large field trial [13]. This discrepancy may reflect differences in microbiota and preexisting immune exposure between cholera endemic and non-endemic populations, both factors being likely to have a greater impact on the immunogenicity of a live as compared to a killed OCV. Further work, ideally also evaluating the model in a cholera endemic setting, is needed before the challenged human volunteer model can be used as a reliable surrogate for field evaluation of OCVs.

Serological studies have shown an inverse relationship between naturally acquired serum vibriocidal antibody titer and susceptibility to cholera infection [14,15]. In human challenge

studies, almost 100% of challenged volunteers who developed clinical illness mounted strong serum vibriocidal antibody responses which were largely IgM. The titers peaked very early and fell towards baseline between one and 6 months post-challenge but remained above pre-challenge levels [16]. The usefulness of serum vibriocidal antibody seroconversion as a CoP has been recently investigated in a human cholera challenge model that showed strong correlation between serum vibriocidal antibody seroconversion and protection against severe and mild cholera in vaccinees challenged at 10 days or 3 months post-vaccination [10]. However, as mentioned the protective effect of a single dose of live oral cholera vaccine (CVD-103 HgR) observed in human challenge studies [10–12] was not confirmed in a placebo-controlled field efficacy trial [13]. Hence, the utility of serum vibriocidal antibody as a proxy in assessing the protective efficacy of cholera vaccines is not demonstrated at a trial aggregate level and may need separate evaluation in cholera endemic settings.

John Clemens (International Centre for Diarrheal Disease Research, Bangladesh) discussed CoPs based on knowledge of the immune response induced by cholera vaccines and he also suggested novel types of studies for licensure of new OCVs. Parenteral cholera whole cell vaccines were developed soon after the isolation of the pathogen but were withdrawn in the 1970s due to their reactogenicity and limited and transient protection. Oral ingestion of antigens has been found to be the most effective method of eliciting mucosal immunity and immune protection. The latter is mediated by mucosal secretory IgA (SIgA) antibodies produced locally in the intestine that are primarily directed against the cell wall lipopolysaccharide (LPS) and/or the binding (B subunit) part of cholera toxin (for a recent review see [6]). In accordance with this, the incidence of cholera in breast-fed infants and children in Bangladesh was inversely correlated to the levels of SIgA anti-LPS and anti-cholera toxin B subunit antibodies (both independently and when they were combined synergistically) in their mothers' breast-milk [7]. Since intestinal SIgA levels induced by OCVs vane within the first year after cholera infection or OCV immunization but significant protection lasts for several years, the development of immunologic memory that can be activated into renewed protective SIgA production upon exposure to cholera pathogen is pivotal; consistent with this Swedish volunteers who received initial immunization with two doses of OCV displayed a strong anamnestic SIgA response when exposed to a single low-dose booster immunization as late as >10 years after the initial immunizations [17]. Currently, three WHO-prequalified OCVs are available, all of which are based on killed *V. cholerae* O1 Inaba and Ogawa cholera bacteria and one of which in addition contains cholera toxin B subunit [7].

For licensure of second-generation killed OCVs a potential attractive alternative to large and expensive (and, given the proven efficacy of similar licensed OCVs, arguably unethical) placebo-controlled field efficacy trials might include randomized non-inferiority trials to compare serum vibriocidal antibody response and safety of new candidates against the existing WHO prequalified vaccines followed by large-scale, non-placebo controlled demonstration projects in targeted settings assessing protective effectiveness. These demonstration projects will also provide opportunities for identification and evaluation of improved immune CoP(s) for use in evaluating future cholera vaccines.

2.1.2. Typhoid—*Salmonella enterica* serovar Typhi (*S. Typhi)* causes significant morbidity and mortality, particularly in developing countries. Currently available vaccines, the oral live-attenuated Ty21a and the Typhoid Vi Polysaccharide vaccines, have moderate efficacy. A new conjugate typhoid vaccine (based on tetanus toxoid (Typbar TCV) is licensed and widely used in India. Other conjugate vaccines are under development.

Information on the relative importance of mucosal and humoral immunity to *S. Typhi* based on the results of challenge studies in humans was provided by Marcelo Sztein (University of Maryland, USA). Immunity to *S. Typhi* is associated with a complex immune response comprising in addition to serum and intestinal-mucosal antibodies, mucosal and systemic memory B cells and cell-mediated immunity components. However, their relative contribution in inducing protection is unknown. Antibodies to *S. Typhi* antigens are likely to play an important role in defense against extracellular typhoid bacilli. However, as *S. Typhi* also persists intracellularly in macrophages and other cells, the cell mediated immune response is expected to be essential for eliminating *S. Typhi* from the infected cells. Analysis of lymphocytes from volunteers immunized orally with Ty21a vaccine or attenuated typhoid vaccine candidates demonstrated the induction of *S. Typhi*-specific CD4+ and CD8+ effector T cells, particularly T effector/memory (TEM) cells, which persist for extended periods of time and have the potential to home both to the gut and peripheral lymphoid tissues [18]. The importance of these immune responses was investigated in several human oral challenge studies with *S. Typhi* wild-type by comparing volunteers who developed typhoid fever and those who did not. These studies found a direct association between the baseline levels of multifunctional *S. Typhi*-specific CD8+ T cells and protection against typhoid disease and delayed disease onset [19]. Moreover, following challenge, development of typhoid fever was accompanied by decreases in circulating *S. Typhi*-specific CD8+ TEM cells with gut and peripheral lymphoid tissue homing potential and upregulation of *S. Typhi*-specific regulatory T (Treg) cells [20]. These results suggest that the tissue distribution of activated Treg cells, their characteristics and activation status may play a pivotal role in typhoid fever, possibly through suppression of *S. Typhi*-specific effector T cell responses. Challenge studies have helped to elucidate the immune events, but CoP is likely multi-factorial. In line with what was mentioned for cholera, in the absence of a clear CoP, non-inferiority trials could be a basis for the evaluation of second generation typhoid vaccines.

2.1.3. Shigella—There is no vaccine for Shigellosis, which is an important public health problem in developing countries particularly in over-crowded urban or peri-urban areas [3,21,22].

The immune response and the usefulness as CoPs of different serological or other immune parameters following exposure to natural infection and candidate vaccines were evaluated by Daniel Cohen (Tel Aviv University, Israel). Serum IgG, IgA, and IgM antibodies, intestinal SIgA antibodies, urinary SIgA antibodies, antibody secreting cells (ASCs), B memory cells and T cell responses are the major components of the immune response to *Shigella* LPS antigens following natural infection and could be considered as potential CoPs for *Shigella*. Sero-epidemiological and vaccine efficacy studies of *Shigella* LPS/polysaccharide-carrier protein conjugate vaccines have shown that high levels of serum IgG antibodies to *Shigella* LPS correlated with protection against shigellosis caused by the homologous serogroup

[23–26]. The magnitude of IgG response following vaccination was significantly higher than what is observed after natural infection. The association between serum IgG antibody to *S. sonnei* LPS and protection was also confirmed in double-blinded vaccine-controlled randomized efficacy trials in young adults and children 3–4 years old [27,28]. Furthermore, significant correlations between the IgA B memory cell response and IgA as well as IgG serum responses to homologous LPS were found in children with *S. sonnei* and *S. flexneri 2a* shigellosis [29]. These findings suggest that in addition to serum IgG antibodies to *Shigella* LPS that emerged as a CoP with mechanistic capabilities, the B memory cell response may be another CoP candidate.

2.1.4. Enterotoxigenic Escherichia coli—Enterotoxigenic *Escherichia coli* (ETEC) is among the top 3 pathogens causing diarrhea-associated deaths in children <5 years old and is also the most common cause of travelers' diarrhea. ETEC immune responses induced following infection as well as findings from vaccine studies were reviewed by Ann-Mari Svennerholm (University of Gothenburg, Sweden) and Firdusi Quadri (International Centre for Diarrheal Disease Research, Bangladesh). The main virulence factors of human ETEC are the heat-labile enterotoxin (LT), the heat-stable enterotoxin ST (STh and also STp) and different colonization factors (CFs). Oral immunization with the Dukoral cholera vaccine containing cholera toxin B subunit, which immunologically cross-reacts with LT, has provided significant but short duration of protection against ETEC diarrhea caused by LT producing strains, both in endemic population and in travelers [30,31]. There is also evidence of CF-specific immune protection both in animal models and in humans after natural infection. Thus, in a birth cohort study of 321 children in Bangladesh followed during their first 2 years of life, those with symptomatic or asymptomatic infections by CFA/I, CS1 plus CS3, CS2 plus CS3, or CS5 plus CS6 strains, were found be protected against a repeat episode of ETEC diarrhea or infection by the homologous CF type [32]. In Bangladeshi adults, clinical ETEC infection induced LT and CF specific memory B cell responses with a peak at day 7 (compared to days 2 and 30) after onset of the illness [33,34]. These patients also developed IgA and IgG antibodies to LTB and CFs at day 7 that remained significantly elevated at day 30 and were of higher avidity than that of corresponding antibodies at the acute stage of infection, i.e. day 2 [33,34]. There was a significant positive correlation between LTB and CF specific memory B cell responses and the increase in avidity of the corresponding antibodies. Increased levels of gut homing total and antigen-specific IgA and IgG antibody-secreting cells (ASCs) were observed at day 7 after onset of ETEC diarrhea. Thus, natural infection with ETEC induces ASCs, memory B cells and high avidity antibodies to LTB and CF antigens. On re-exposure to ETEC with a shared antigen, the infection-induced memory can elicit a rapid anamnestic response. Assessment of functional immunologic memory or specific memory cells may therefore alongside with (appropriately time-collected) intestine-derived IgA ASCs be important as potential CoPs in the design and evaluation of candidate ETEC vaccines.

Efforts to develop an effective ETEC vaccine have been focused on candidate vaccines containing CF and LT antigens and administered orally to induce good intestinal immune responses. The most promising human ETEC candidate vaccines are oral inactivated or live *E. coli* strains expressing the most prevalent ETEC CF antigens, i.e. CFA/I, CS3, CS5 and

CS6, together with LTB antigen. Such vaccines may have the potential to protect against up to 80% of all clinical ETEC infections [35]. Possible CoPs might be identified through studies of the relations between vaccine-induced antibody levels in serum and/or mucosal specimens against LT/LTB and/or CF antigens, antigen-specific ASCs in peripheral blood samples, or B and T memory cells and protection [36]; such correlation studies may be done both in vaccine trials in endemic populations and in travelers, and in the challenged volunteer model. Induction of anti-CFA/I IgG titers at or above a certain level was associated with significantly reduced incidence of ETEC diarrhea in Egyptian children [37], suggesting that in endemic settings serological anti-CF antibody responses should also be evaluated as a possible CoP for homologous CF ETEC diarrhea.

2.1.5. Campylobacter—*Campylobacter* species, mainly *C. jejuni*, are among the most common causes of bacterial food and water-borne gastroenteritis in children and adults especially in the developing world [38,39]. In addition to the acute disease, infection by *Campylobacter* may lead to long-term post-infection nutritional consequences and other sequelae such as reactive arthritis and Guillain-Barre's syndrome. Patients with AIDS have almost 40-fold increased rate of *Campylobacter* infection [40].

Epidemiologic studies of children, human challenge models and early phase 1 vaccine trials that allowed a better understanding of *Campylobacter* CoPs were revised by Beth D. Kirkpatrick (University of Vermont, USA). Evidence for acquired immunity has been obtained from early epidemiological observational studies in developing countries that reported seroconversion in young children due to endemic exposure and documented a correlation between the level of *Campylobacter*-specific antibodies and protection [41]. The presence of more severe and protracted disease in individuals with hypogammaglobulinemia suggests the role of IgG in protective immunity. Human challenge studies using C. *jejuni* CG8421 strain showed that immune responses after primary infection included serum IgA, IgG, ASC, and IFN-γ production [42–44]. In these studies, IgA and IFN-γ levels were associated with resistance to clinical disease, suggesting that they might be used as CoPs against *Campylobacter* infection. Re-challenged subjects showed 100% homologous protection at 4–6 weeks which decreased to 43% in 12 weeks in one study [43] while a complete absence of protection was reported by another study [42], indicating that immune-mediated protection from disease may be short-lived and strain-specific.

Currently, vaccine development is hampered by an incomplete understanding of pathogenesis and development of immunity. In the absence of an appropriate animal model, CoPs could be assessed by developing an effective vaccine and determining correlates of vaccine-induced efficacy or by investigation of mechanisms of immune protection from natural disease. The first approach cannot work currently because different vaccines in the pipeline have not been usefully immunogenic. Human challenge models and early phase 1 vaccine trials have clarified the gaps in knowledge (the "known unknowns") needed for vaccine development and evaluation of protective immunity. The pending questions are how to design a vaccine to extend the duration of clinical protection and if an empiric vaccine (and a CoP) can be developed in the absence of a better understanding of the development of protective immunity.

2.2. Viral pathogens

2.2.1. Rotavirus—Two orally administrated live-attenuated rotavirus vaccines licensed in 2006 showed good efficacy against severe rotavirus disease in large clinical trials [45,46]. Umesh D. Parashar (Centre for Disease Control, USA), Gagandeep Kang (Christian Medical College, India) and Manuel Antonio Franco (Pontificia Universidad Javeriana, Colombia) discussed the immune response and protection against rotavirus, and lessons learned from rotavirus vaccine studies and birth cohort studies, respectively. Following rotavirus vaccine implementation, large decreases in childhood diarrhea-related hospitalization and death were reported in different countries [47,48]. Vaccine efficacy ranges from 84% to 98% in high income countries [49,50] to 50% or below in low income settings [51–53] but even at modest efficacy the benefits of vaccination for reducing childhood diarrhea-related hospitalization rates remain high [54].

Serum antibodies play an important role in natural protection from rotavirus infection and disease and could be a potential CoP candidate. Total serum rotavirus IgA (RV-IgA) reflects the duodenal RV-IgA levels four months after natural infection and correlates with protection. In a birth cohort of Mexican children, those with an IgA titer higher than 1:800 had significantly lower risk of both rotavirus infection and disease [55]. An IgG titer higher than 1:6400 was associated with 49% reduction against any rotavirus infection [55]. Similarly, higher levels of IgG and IgA were independently associated with reduced risk of rotavirus infection after adjustment for age as well as the number of rotavirus infections in a birth cohort study in Vellore, India [56]. Total serum RV-IgA has also been correlated with protection [57] and vaccine efficacy in different vaccine settings [58]. However, IgA seems to be a non-mechanistic CoP as vaccinees without RV-IgA also have significantly less rotavirus gastroenteritis than placebo recipients [59], suggesting that factors other than serum RV-IgA play a role in protection. The presence of RV-IgA is “reasonably likely to predict clinical benefit” classifying it as a level 3 endpoint surrogate of protection [59]. Validating RV-IgA as a level 2 endpoint surrogate marker (i.e. validated surrogate for a specific disease setting and class of interventions) for clinical evaluation will be of great help in regulatory approval of the next generation of RV vaccines. Serum RV-Secretory Ig (SIgA and SIgM retro-transcytosed from intestine to serum) may be complementary to RV-IgA as a CoP in vaccine trials [59].

2.2.2. Hepatitis A—Hepatitis A virus (HAV) is the cause of the most common form of self-limited, acute viral hepatitis infection. One of the major target populations for HAV is children who are usually asymptomatic. Pre- or post-exposure immunization by several licensed vaccines available throughout the world appears to be very effective in prevention of disease and spread of the infection [60].

Mechanisms of protective immunity to HAV were reviewed by Stephen Feinstone (George Washington University School of Medicine, USA). HAV cellular receptor 1 (HAVCR1), also known as T-cell immunoglobulin and mucin domain 1 (TIM-1), is expressed in liver, kidney, lung, T helper 2, and NKT cells and is important in immune regulation.

Structural phylogenetic analysis revealed that HAV diverged from picornavirus [61]. However, during acute infection, this non-enveloped virus circulates in the blood in a

membrane enveloped form (eHAV) by high-jacking cellular membrane, thereby evading neutralizing antibodies and facilitating its spread within infected hosts [62]. At the same time, virus shed from the host into feces lacks the envelope, increasing opportunities for inter-host transmission [63]. Bile salts presumably strip the envelope from the stool excreted HAV [63]. The detection of CD8+ T cells in patients with acute hepatitis A provided an early conceptual framework for both acute liver injury and immune control of HAV, but more recent work suggests that cytotoxic CD8+ T cell activity is variable in hepatitis A and that CD4+ effector T cells and possibly also CD4+ Treg cells and their released cytokines may be more important determinants of both pathogenesis and infection control [64].

A very robust IgM response is seen early after infection, suggesting that antibody response can be considered as a CoP. Follow-up studies in vaccinees have provided evidence of antibody persistence up to 20 years after the initial vaccination in 97% of subjects [65].

2.2.3. Norovirus—Noroviruses (NoV) belonging to *Caliciviridae* family are divided into 6 genogroups GI-GVI [66]. Viruses in genogroup II and in particular GII4 NoV strains are a leading cause of epidemic and sporadic outbreaks of gastroenteritis worldwide [67], and the most important cause of foodborne illness [68]. NoV have become the leading cause of acute gastroenteritis in US young children after the introduction of rotavirus vaccination [69]. Expression of the major capsid protein i.e. VP1 lead to spontaneous formation of virus-like particles (VLP) that serves as the basis of vaccines being developed.

Robert Atmar (Baylor College of Medicine, USA) and Lisa Lindesmith (University of North Carolina, USA) provided a summary of data on immune responses to NoV in human experiment human infection challenge studies and clinical trials using VLPs as vaccines. Human challenge studies back to 1977 found that immunity to NoV was not due to previous exposure, suggesting that genetic factors may determine susceptibility or resistance to NoV [70]. Higher pre-infection serum ELISA antibody titers were associated with lower rate of infection in Panamanian children [71] while other studies failed to demonstrate a correlation between serum antibody levels and protection from NoV disease [72–74]. However, recent studies reported a correlation between titers of serum antibody that blocks binding of NoV VLPs to histo-blood group antigens (HBGA) and protection against clinical NoV induced gastroenteritis, suggesting that such antibodies could be considered as a CoP [72,73]. GII4 strain has a broad HGBA binding profile compared to non GII4 strains [75]. Pre-challenge levels of NoV-specific salivary IgA antibody and circulating NoV-specific IgG memory B cells were also recently identified as potential new CoPs against NoV gastroenteritis [76]. Notably, 2 of the potential CoPs, i.e. HBGA blocking antibody and NoV-specific salivary IgA have been shown to be correlated [76]. The identification of several CoPs raise the question on the relative importance of each of them, and whether they work together or are all of them surrogates of protection. Understanding the relative importance of the different CoPs is essential to inform decisions on vaccine strategies, including the route of immunization and the use of specific adjuvants.

Absence of validated small animal models, unclear effect of host genetics and pre-exposure history, and strain diversity (>40 genotypes infect humans) are major challenges to successful vaccine design. But the primary obstacle to the development of an efficacious

NoV vaccine may be to design a vaccine that elicits an immune response broad enough to accommodate GII4 antigenic drift. This can be overcome by the use of a multivalent VLP vaccine engineered to contain neutralizing antibody-inducing epitopes from multiple GII4 strains. Volunteers vaccinated simultaneously with GI1 and GII4 VLPs generated broad cross-genotype blockade antibody responses, a surrogate measurement for protective immunity [75]. Importantly, breadth of “blockade antibody” response extended to novel GII4 VLPs that had not circulated prior to sample collection, indicating that vaccination may provide protection from emergent strains and suggesting that immunization primarily activated a memory antibody response to multiple GII4 strains [75]. Finally, few studies have been conducted in children [77–79] and it remains to be determined whether the immune markers found in adults also correlate with protection in this age group.

3. Novel approaches to correlate mucosal immune responses with protection in humans

Mucosal antibody and cellular immune responses such as antigen-specific ASCs and T cells that are currently under investigation for their use as potential CoPs and novel approaches to study these responses were discussed during the meeting and are summarized in the following section.

3.1. Mucosally derived antibody-secreting B cells

The mucosal immune system exhibits a fair degree of anatomical compartmentalization [80]. As such, immunity induced or expressed in a given tissue may not be reflected in distant mucosal tissues and secretions. This compartmentalization calls for tissue-targeted vaccine formulations and for reliable techniques to measure corresponding immune responses.

Following active immunization, naïve B as well as T lymphocytes are activated at local priming sites, whereupon the cells circulate to local lymph nodes and further, via lymphatic and blood, back to the mucosa [80]. After oral immunization, with particular attention to optimal timing, such circulating B cells/plasmablasts recently activated in the intestine can be captured from the peripheral blood and identified as ASCs. ASCs are found in the circulation following either systemic or mucosal infection and after vaccination regardless of the route of administration. The characteristics of a mucosa-derived ASC response and challenges in exploring ASCs in vaccination studies were evaluated by Cecil Czerkinsky (University of Nice-Sophia Antipolis, France) and Anu Kantele (University of Helsinki, Finland). Capturing mucosa-originating ASCs during their transit in blood - the most accessible lymphoid compartment in humans – may offer an opportunity to evaluate mucosal antibody responses to vaccines. The challenges in using blood ASCs or other mucosal immune cells as surrogate of tissue-specific protection in relation to enteric infections include: (i) their validation in human challenge studies or field-based vaccine trials (rotavirus, cholera, ETEC, *Shigella*, typhoid vaccines); (ii) pheno-typic definition and homing properties of mucosal ”memory” B cells; (iii) the same for various mucosal effector T cells such as Th1, Th17, CTL, NKT, and Treg cells; and (iv) identification of mucosal innate immune markers. Furthermore, due to the transient nature of the response, the kinetics of the different immune parameters needs investigation.

The value of ASCs as a potential non-mechanistic CoP in blood was reported following oral and inactivated poliovirus (OPV and IPV) recall vaccination in previously OPV-immunized individuals. Results showed stimulation of gut memory B cell response by boost either with IPV or with OPV that correlated with protection from subsequent challenge (with OPV) [81].

Applying the oral S. Typhi Ty21a typhoid vaccine as a model, ASCs appeared in the blood a few days after antigen encounter, peaked around day 7, and then declined within the following week, the magnitude depending on the dose, type of antigen and vaccine formulation [82,83]. A stronger response was achieved after booster but only if the booster dose was not administrated too early [84]. Persisting antigen exposure prolonged the response, IgA- or IgM-ASCs representing the dominating Ig isotypes, followed by IgG-ASCs [85]. One should note that the ASC response after intestinal antigen encounter is not equal to or even correlating with serum antibodies. ASCs showed a distinct homing profile determined by the site of antigen encounter and oral boosting was more efficient than parenteral boosting in up-regulating the gut-homing receptors [86]. The site of *S. Typhi* Ty21a typhoid vaccine elicited cross-reactivity against *S. paratyphi* A and B [87] and some non-typhoid salmonella strains (i.e. *S. enteritidis* and *S. typhimurium*) [85]. The Vi capsular polysaccharide vaccine has also been shown to elicit cross-reactivity to *S. paratyphi* C and non-typhoid salmonella strains [87,88], probably due to the persistence of minor amounts of contaminating LPS (O-Antigen) in the Vi vaccine.

3.2. Th1, Th17 and T follicular helper cell responses to oral vaccination

T helper (Th) cells can play important roles in the generation of mucosal IgA responses. Thus, T follicular helper (Tfh) cells provide essential support for antibody affinity maturation and generation of memory B cells in germinal centers and Th17 cells may promote production and secretion of mucosal SIgA. To advance the knowledge about how mucosal antibody responses to oral vaccines are generated, and to identify biomarkers related to mucosal immunological memory, peripheral blood Th cell responses to an oral multivalent ETEC vaccine, ETVAX comprising inactivated CF-expressing *E. coli* and a hybrid LT/CT B subunit, have been investigated in adult volunteers. The results, as presented by Anna Lundgren (University of Gothenburg, Sweden), showed that the multivalent ETEC vaccine was highly immunogenic and induced ASC and fecal antibody responses to all 4 CF antigens as well as the B subunit in the vaccine [36,89]. The vaccine also induced a potent T cell response (with production of INF-ϒ and IL-17A) to LT B subunit [89], and when a double-mutant heat-labile toxin (dmLT) mucosal adjuvant was co-administered this further enhanced T cell INF-ϒ and IL-17A responses [90].

Notably, vaccination also resulted in increased proportions of activated Tfh-like (CD4 + CXCR5+) cells expressing the gut homing marker β7-integrin in peripheral blood. Tfh cells, but not Tfh-depleted peripheral blood cells, promoted total and vaccine specific IgA production from co-cultured B cells *in vitro*. Importantly, magnitudes of Tfh responses after primary vaccination correlated significantly with vaccine specific IgA ASC memory responses to a single oral booster dose given 1–2 years after the primary two-dose immunization. The gut homing phenotype of activated blood Tfh cells and the correlation

with vaccine specific antibody-in-lym phocyte-supernatant (ALS) responses suggest that blood Tfh cells may reflect ongoing mucosal germinal center responses and that Tfh responses in blood may be used as a surrogate for mucosal memory B cell development and possibly as a CoP (Unpublished data, Anna Lundgren, University of Gothenburg, Sweden).

4. Discussion and conclusions

The expert group stressed that identification and validation of accurate CoPs are needed in order to (i) enable correct choice of vaccine antigens; (ii) ascertain consistency of potency; (iii) monitor consistency of vaccine production; (iv) study the susceptibilities of individuals and populations after vaccination; (v) validate vaccines for which placebo-controlled efficacy trials are no more ethical, as when a prior generation vaccine is already licensed (e.g. rotavirus vaccines); (vi) bridge from first-generation vaccine to second generation; and (vii) enable the licensure of combination vaccines.

They emphasized the fact that to date, all established CoPs are based on humoral immune response parameters that measure functional or total IgG antibody in serum. Examples of well-established CoPs for vaccines discussed during the meeting include those for hepatitis A, Vi typhoid and poliovirus. Known CoPs for cholera, norovirus and rotavirus vaccines could be considered as acceptable for comparing similar types of vaccine while more work is still needed to establish CoPs for the remaining candidate enteric vaccines.

Rotavirus vaccination arguably provides the most complex and controversial puzzle with respect to definition of CoP in current vaccinology. Neutralizing antibodies, non-neutralizing antibodies, secretory antibodies, and cellular immune responses have all been proposed as CoPs, and indeed, it may be that all of these play a role, depending on the situation [91].

For cholera, serum vibriocidal antibodies are traditionally used as a marker of appropriate immune stimulation by cholera vaccines although it should be kept in mind that these antibodies neither represent a surrogate nor a CoP [92]. Standardization, harmonization and validation of vibriocidal measurement methods and development of reference and standardized reagents would increase the usefulness of this potential CoP and would enable to compare candidates.

The case of ETEC is very complex because similar to what was discussed for cholera, serum antibody responses may at best confirm vaccine take and reflect the protective intestinal-mucosal SIgA antibody responses. The ALS assay measuring antibodies in super-natants from cultured peripheral blood lymphocytes is better reflective and more sensitive than serum titers to CF antigens but is laborious and difficult to do in large scale vaccine trials and in infants. Measuring SIgA antibodies in fecal extracts has shown promise also in young children and infants in ongoing ETEC vaccine studies in Bangladesh.

Several types of *Shigella* vaccines, i.e. oral live attenuated, oral killed and injectable conjugate vaccines, are under development [64]. As the induced immune mechanism will depend on the vaccine type, identification of CoPs will depend on correlation with efficacy for each type of vaccine.

Campylobacter vaccines are even more complicated and have shown conflicting findings both with regard to immune response and efficacy [42,43]. Furthermore, the association of *Campylobacter* infection with Guillain-Barre's syndrome limits the ability to carry out challenge studies with a variety of strains.

As illustrated by different case studies in this paper, only a few vaccine-induced CoPs are currently available. The reasons for the paucity of established CoPs are multiple. First, immune protection is usually complex, often not well understood, and influenced by many factors such as age and previous exposure, nature of the vaccine (e.g. live or killed; subunit or whole organism), time to exposure (dependence on acute response or memory), and nutritional status. Second, there is a general lack of good animal models and the challenged volunteer method is underused and only available in a few places. Finally, immunological methods are rarely functional. Mucosal immunity is even more complex and less understood than systemic immunity because it is more tightly regulated in both magnitude and kinetics and more difficult to measure.

Since the mid-80s, much effort has focused on capturing gut-derived antigen-specific immune cells from peripheral blood, and then enumerating ASCs (mainly IgA) or cytokine-secreting T cells by ELISPOT or measuring antibodies or cytokines in ALS fluid by ELISA. Recent method miniaturizations now allow automatized analyses from "pediatric" blood volumes. The ASC or ALS antibody response is a reflection of the immune response to an infection or vaccination that has occurred recently and is useful in optimizing responses and searching for cross-reactivity. Thus, investigation of gut-originating ASCs from samples of peripheral blood during their recirculation could serve as a valuable and a less invasive (compared to tissue biopsy) measure for studying mucosal immune response in humans. In addition, even though the acute ASC responses after enteric infection or oral vaccination are transient and absent after a few weeks, mucosal immunologic memory seems to be very long-lasting; e.g. a strong anamnestic ASC response to an oral cholera vaccine recall immunization was seen in Swedish volunteers as late as 9–14 years after primary vaccination [17]. At the cohort level IgA ASCs have largely correlated with intestinal immune responses to different oral vaccines and regimens, but correlation of individual ASC numbers with individual protection remains to be demonstrated. ASC responses may also be used to develop a new generation of diagnostic tools, for example by testing ALS samples to multiple antigens in microspot assays to detect recent infections.

The measurement of shedding of live vaccines could also be considered as a CoP and should be more investigated. In addition to its potential use as a CoP at individual level, shedding level is also probably an important mechanism by which herd immunity occurs.

In conclusion, CoPs are a subject of continued interest for both theoretical and practical reasons. The availability and quality of CoPs are key parameters for vaccine development, licensure and effectiveness measurement. However, different aspects of this topic (e.g. laboratory methods, statistical tools, study design, etc.) require further clarification. The WHO has released a document on this issue in order to facilitate communication and to encourage the development of a broad research agenda on this topic [93]. Understanding the

relative importance of different CoPs is important to form decisions on vaccine strategies for disease prevention, including the route of immunization and use of specific adjuvants.

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