Vaccination against shigellosis: is it the path that is difficult or is it the difficult that is the path?

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Abstract

Following several decades of research, there is not yet a convincing vaccine against shigellosis. It is still difficult, in spite of the breadth of strategies (i.e. live attenuated oral, killed oral, subunit parenteral) to select an optimal option. Two approaches are clearly emerging: (i) live attenuated deletion mutants based on rational selection of genes that are key in the pathogenic process, and (ii) conjugated detoxified polysaccharide parenteral vaccines, or more recently conjugated synthetic carbohydrates. Some of these approaches have already undergone phase I and II clinical trials with promising results, but important issues have also emerged, particularly the discrepancy between colonization and immunogenic potential of live attenuated vaccine candidates depending upon the population concerned (i.e. non endemic vs. endemic areas).

Efforts are needed to definitely establish the proof of concept of these approaches, and thus the need for clinical trials which should also soon explore the possibility to associate different serotypes, in response to serotype specific protection against shigellosis. More basic research is also required to improve what we can still consider as first-generation vaccines, and to explore possible new paradigms including the search for cross-protective antigens.

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Shigellosis (bacillary dysentery) is endemic throughout the planet, although essentially a major health concern in its most impoverished areas, particularly in the pediatric population between 1 and 5 years old. It can be caused by any serotype belonging to four groups: group A (Shigella dysenteriae), group B (Shigella flexneri), group C (Shigella boydii), and group D (Shigella sonnei). The serotype 1 of S. dysenteriae (SD1) emerges as one of particular concern, due to its expression of the Shiga toxin, a potent cytotoxin that not only aggravates the intestinal symptomatology, but also causes major systemic complications such as the Hemolytic Uremic Syndrome (HUS). S. sonnei incidence tends to increase over other groups as living standards improve, thus dominating as an endemic strain in western countries. The disease is characterized in its classical forms, by a short period of watery diarrhea with increasing intestinal cramps and general malaise, followed by the appearance of a dysenteric syndrome that comprises intestinal cramps and tenesmus, leading to permanent emission of bloody, often mucopurulent stools. Acute complications may occur in absence of quick antibiotic treatment, such as toxic megacolon, peritonitis, and septicemia that is mostly observed in severely malnourished children. Conversely, repeated shigellosis episodes may lead to severe malnutrition, thus a vicious circle. When poor conditions are concentrated in a single epidemiological crisis, like in refugee camps, the attack and mortality rates may be quite high, as observed in Goma, Zaire, in 1994 in the course of an SD1
epidemiological. Projections based on methodologically convincing epidemiological studies from the three previous decades allowed, back in 1999, to evaluate the number of cases of shigellosis to 169 million per year, with a death rate ranging between 500,000 and 1.1 million, 69% being children below 5 years in the developing world [1]. These impressive figures have undoubtedly led the community to realize that shigellosis is a high-impact disease, particularly in the poorest populations. The WHO has set it at the top of its priority list, along with ETEC, for the development of a vaccine, and this has recently emerged as a “Shigella-ETEC vaccine initiative” by the Bill & Melinda Gates Foundation. Current figures may not be that high however, although the epidemiological situation is evolving and figures are lacking in key areas, particularly in Africa. Recent surveys indicate that in general, the incidence of diarrheal diseases remains stable worldwide, although mortality shows sustained decrease, being currently evaluated at a level of 4.9/1000 per year [2]. A recent epidemiological survey conducted in six Asian countries [3] has established that shigellosis was likely to be following a similar trend with a stable incidence of cases (4.6% of cases of diarrhea), and decreased severity and mortality. The rationale for this switch in disease profile is still unknown. Several modes of explanation may be envisioned, such as better nutrition and hygiene paralleling economic development of the Asian continent, absence of current epidemics of SD1, better education of mothers, improvement of primary health care, and extended use of ORS and antibiotics. The issue of antibiotics is likely to be an important one. Beyond the possibly positive impact of free, uncontrolled use of antibiotics on the disease profile at this stage, one may just as soon have to face a new crisis associated with multidrug resistance. In some areas, the prevalence of strains resistant to all first-line antibiotics, including fluoroquinolones, reaches 5% and is clearly on the rise. It is also unlikely that the epidemiological situation in Asia can be generalized, thus there is a need for an exhaustive evaluation of the incidence of shigellosis, particularly in the sub-Saharan part of the African continent. Current economic stagnation and frequent social instability are creating conditions for shigellosis to remain a leading cause of morbidity and mortality. In order to facilitate such studies, there is a need for efficient and durable surveillance networks benefiting from good microbiological expertise and novel quick, reliable, and robust diagnostic tools such as immunochromatographic dipsticks that could be used directly on fecal samples [4].

All elements being considered, including the permanent risk of massive re-emerging epidemics, the need for a Shigella vaccine clearly remains. Its major target would be the pediatric population of the developing world, essentially infants around the age of 1 year, and possibly also the elderly population that represents the other peak of disease susceptibility. Such a vaccine could also benefit travelers to high-risk areas, particularly those working or intervening in these areas, e.g., members of NGOs, army personnel, etc.

Shigella infection prevents disease during subsequent exposures. Lipopolysaccharide (LPS), the major bacterial surface antigen, is the main target of the host adaptive immunity. Anti-LPS antibodies (Abs) are elicited upon infection, both locally as secretory IgA (SIgA), and systemically as serum IgG. Ab-mediated protection has been shown to be mostly serotype specific [5], pointing to the O-specific polysaccharide moiety of LPS, also termed O-antigen (O-Ag), as the target of the protective immune response. Indeed, Shigella serotypes are defined by the structure of their O-Ag repeating unit (RU) [6].

This has been a strong incentive to considering that protection was an achievable goal with an oral vaccine reproducing key steps of the natural infectious process. Still, natural protection is not absolute and rather short lasting, and again, essentially serotype specific [7]—not necessarily good news for Shigella vaccine developers. Nevertheless, phase III trials carried out in Yugoslavia in the 1960s [8], using Streptomycin-dependent (SmD) mutants of S. flexneri and S. sonnei, had shown that oral vaccination was an achievable goal. This has remained over the years the gold standard, even though reversion of the mutation, in some cases, had illustrated the need for an association of attenuated mutations consisting in gene deletion, whose selection would be rationally based on the increasing knowledge in the pathogenic mechanisms of Shigella [9]. Following these encouraging initial results, attempts were indeed made at rationally attenuating virulence of candidate strains representing the most frequently isolated serotypes, such as S. flexneri 2a and S. sonnei, as well as S. dysenteriae serotype 1, due to severity of cases. Two major strategies have been considered: (i) altering key metabolic pathways affecting bacterial growth in tissues, or (ii) knocking out virulence genes selected upon their expected capacity to affect one or several key steps of the infectious process. Some recent vaccine candidates have combined both approaches. An initial candidate belonging to the first category, an aro mutant of S. flexneri (SFL124) expressing the S. flexneri group antigen, was constructed in an attempt to obtain cross-protection. This mutant appeared too attenuated, thus very well tolerated by volunteers in clinical trials, but weakly immunogenic [10,11]. This vaccine candidate raised an important issue regarding the bases of its attenuation. It is likely that the wild-type strain that had been selected was already weakly pathogenic, therefore its further attenuation by aro mutation likely accounted for insufficient colonization potential and immunogenicity. A recent review has stressed the need to confirm full pathogenicity of the strains that serve as a basis for vaccine construction [7]. This is ethically complicated, but the mere isolation from a patient may not guarantee that the isolate shows “optimal” pathogenicity. Other metabolic mutations have been considered, particularly guaAB that introduces a severe auxotrophy impairing synthesis of nucleic acids [12], as well as mutations impairing the strain’s capacity to scavenge ferric iron (Fe³⁺), a property required to compete for vital Fe³⁺, via the production of siderophores (i.e. aerobactin or enterochelin), against iron-chelating molecules of mucosal surfaces (i.e. lactoferrin), or of tissues (i.e. aerobactin) [13]. The most recent Shigella vaccine candidates have undergone a combination of metabolic and virulence mutations. This combination can lead to various
degrees of attenuation. Current vaccine candidates, on these bases, can fall into the category of weakly attenuated or strongly attenuated strains.

In the category of weakly attenuated candidates belong icsA-based mutants, IcsA is an outer membrane protein of Shigella that nucleates cellular actin, thereby allowing intracellular motility and cell to cell spread of the microorganism. Mutation in this gene impairs the capacity of Shigella to spread extensively in the epithelium, away from its initial site of entry [14]. It has been shown that such mutants were directly targeted to colonic solitary nodules, the actual inductive sites of the mucosal immune response [15]. Combined with a deletion of the aerobactin system (iuc iut), in S. flexneri 2a, icsA has provided a vaccine candidate (SC602, Institut Pasteur) that has undergone phase I and II clinical trials (Walter Reed Army Institute of Research and US Army Institute for Research in Infectious Diseases) whose results were considered encouraging in Western volunteers [16]. In brief, the strain was strongly immunogenic, eliciting a high percentage of circulating plasmocytes producing anti-LPS IgA by the ELISPOT assay, although showing residual reactivity with limited fever and diarrhea in about 15% of the recipient volunteers. Moreover, when vaccinees who had received a dose of 10^8 CFU as vaccine inoculum were challenged with a wild-type, pathogenic S. flexneri strain of similar serotype, they appeared fully protected against dysentery, and subsequent studies carried out in the USA and Israel demonstrated the absence of accidental transmission [17]. In short, this was a quite encouraging series of studies that certainly confirmed the concept that a live attenuated Shigella vaccine is an achievable goal. A ΔicsA S. sonnei vaccine candidate constructed by scientists at WRAIR (WRSS1) showed similar results with regard to tolerance and immunogenicity [18]. More recently, a S. dysenteriae 1 vaccine candidate (SC599, Institut Pasteur) has been tested in phase I and II trials (Saint George Vaccine Institute, London, UK, and Centre de Vaccinologie Cochin-Pasteur, Paris, France). In this strain, three deletions have been introduced: ΔicsA, Δent fep fes (genes encoding the enterochelin system), and ΔstxA, the gene encoding the catalytic subunit of Shiga toxin. Unlike its S. flexneri and S. sonnei ΔicsA counterparts, this strain has shown good tolerance, limited systemic immunogenicity (as judged by seric IgM, IgG and IgA titers), and average to good mucosal immunogenicity as judged by percentage of anti-LPS IgA measured by ELISPOT, in comparison to SC602 and WRSS1 (in press, and in preparation). In the absence of clear correlates of protection, it is currently difficult to anticipate the potential of this family of vaccines for the future. This is a particularly important issue, as the serotype-dependent nature of protection would necessitate further construction of strains, particularly S. flexneri 3a, 1b and 6, in order to cover a broader spectrum of serotypes [19], and testing of a combination of these strains to address issues such as interference. Last but not least, only a phase III efficacy trial conducted in an endemic area may provide the final piece of information required to validate the approach. To add to the difficulty, SC602 was tested in a phase II trial in a highly endemic area of Bangladesh (collaboration between ICDDR,B, AFRIMS, and WHO). The strain showed excellent tolerance in all age categories, including 1-year old infants with inocula up to 10^7 CFU (unpublished data). On the other hand, colonization appeared limited and immunogenicity very weak. Beyond possible technical issues, it remains that one dose of such ΔicsA-based vaccine appears immunogenic and possibly protective in (Shigella-naïve) Western populations, but clearly shows less immunogenicity and weak capacity to colonize in endemic areas. Several possibly combined hypotheses may account for this issue: the protective role of breast feeding against the vaccine strain in infants, the high level of innate stimulation of the intestinal mucosa by recurrent enteric infections in a highly endemic zone, thereby severely affecting the capacity of the vaccine strain to colonize the mucosa (in this context, the nature of the selected mutation may need to be discussed), the high exposure of children, at an early age, to multiple enteric pathogens, including the most prevalent serotypes of Shigella, thus a quickly acquired status of adaptive immunity. In any event, these observations are important to consider because they are very unlikely to apply only to this particular category of vaccine candidate. Considering at least two oral doses as a possible solution to discuss, it would require a second phase II study in similar epidemiological conditions.

To the category of strongly attenuated strains belongs a series of strains constructed at the Center for Vaccine Development (University of Maryland). In the most recent generation of vaccine constructs, a S. flexneri 2a strain has undergone a guaAB mutation that has been combined with a sen and a set mutation, thereby knocking out the genes encoding two putative enterotoxins of this serotype. A strain named CVD 1208 has been tested in a phase I trial [20,21]. The tolerance appeared excellent, including the lack of residual diarrhea, validating the elimination of Sen and Set expression, thereby allowing administration of vaccine doses up to 10^9 CFU without side effects. At such doses, systemic and mucosal responses reached good levels, similar to those observed with SC602 with a 10^4 CFU inoculum, if one tries a comparison [16]. This is clearly an alternative option that also needs to be validated in further trials, including in the field.

In spite of the encouraging results observed in the course of these different studies, issues clearly remain such as:

- Will it be possible to immunize with a single oral dose?
- What is the acceptable limit of serotype numbers to be introduced in a multivalent oral vaccine, considering the increasing serotype diversity observed depending on the region considered?
- Should the current strategies of vaccine design take into account the most recent evidence that Shigella is able to strongly interfere with the innate and adaptive immune response of the host, thereby creating an immunosuppressive environment that is not questionable in a vaccine concept? ([9,22]; Gamelas Magalhaes et al., submitted)

Subunit vaccines based on the use of the major protective antigen, i.e. LPS, and administered parenterally are an
An alternative option for the development of a *Shigella* vaccine. However, LPS cannot be used as a parenterally administered immunogen, due to its highly toxic lipid A. Strategies involving constructs whereby LPS toxicity is masked, or LPS analogues devoided of toxicity, were investigated instead. Similarly to the successful conversion of bacterial capsular polysaccharides from T-cell independent antigens (Ag) to T-cell dependent ones [23–25], the non-toxic acid-detoxified *S*. *flexneri* 2a LPS (pmLPS) was turned into a potent T-cell dependent immunogen through its covalent coupling to a protein carrier. Since the early 1990s, several *S*. *flexneri* 2a pmLPS–protein conjugates have been shown to be safe and immunogenic in adults [26,27] as well as in young children [28]. The most encouraging results were obtained with a *S*. *sonnei* pmLPS–rePA conjugate vaccine administered parenterally to young adults, showing protection in about 75% of the vaccinees during a *S*. *sonnei* outbreak [29]. Nevertheless, *Shigella* pmLPS–protein conjugate vaccines remain complex constructs obtained from randomly activated pmLPS. Potential loss of antigenicity may occur during detoxification and/or coupling to the carrier. Thus, accurate controls of these two crucial steps are required to ascertain both the complete removal of LPS toxicity and maintenance of LPS antigenicity. In addition, appropriate consideration should be given to the increasing requirements from regulatory agencies for always better-defined molecules to be used in humans.

An alternative to conventional pmLPS–protein conjugates is the use of synthetic mimics of the bacterial O-Ag. Indeed, following early reports in the 1940s [30], increasing evidence supports the concept that carbohydrate epitopes, which are made of short oligosaccharide (OS) sequences, are immunogenic in animal models once conjugated to appropriate carriers [31–33]. Most importantly, recent and anticipated future improvements in glycochemistry are expected to give access to better-defined and standardized complex carbohydrates. Thus, besides the search for peptide mimics [34–37], the use of OS mimicking the carbohydrate determinants recognized by anti-O-Ag protective monoclonal antibodies (mAbs) has been developed [38,39]. Such mimics are expected to induce a protective anti-LPS Ab response when appropriately presented to the immune system. Along this line, a new vaccine candidate targeting *S*. *flexneri* 2a infection has recently emerged [40]. This synthetic OS-based conjugate was designed in order to obey to the following rules: (i) the use of carbohydrate hapten suitable for single-point attachment onto a carrier to overcome the limitations due to LPS random chemical modifications and/or detoxification; (ii) the control of various parameters such as the length and nature of the carbohydrate hapten, its loading onto the carrier, as well as the choice of the carrier, to allow the design of glycoconjugates with optimal immunogenicity. It was issued from a four-step process encompassing (i) identification of the protective *S*. *flexneri* 2a epitopes, (ii) conception of the candidate glycoconjugates, (iii) study of the immunogenicity of the glycoconjugates in mice, and (iv) when appropriate, analysis of the protective efficacy of the anti-*S*. *flexneri* 2a LPS Abs induced by the glycoconjugates.

A rational strategy was undertaken for the identification of protective *S*. *flexneri* 2a epitopes. The methyl glycosides of di- to pentasaccharides representing frame-shifted fragments of the basic *S*. *flexneri* 2a O-Ag RU (a branched pentasaccharide AB(E)CD as shown in Fig. 1), together with an octa- and a decasaccharide, were synthesized by multi-step chemical synthesis [41–46]. Analysis of the contribution of each monosaccharide residue to the recognition of *S*. *flexneri* 2a LPS by several serotype 2a-specific protective mlgGs showed that most mAbs bind internal epitopes repeatedly exposed on the LPS. In addition to outlining the key role played by ECD in Ab recognition, B(E)CD was shown to represent an immunodominant protective determinant. However, since chain elongation improved mlgG binding, a deca- and a pentadecasaccharide representing 2 and 3 biological RU, respectively, were synthesized [47] in addition to selected short haptens [48].

Upon coupling of several OSs including ECD, B(E)CD, AB(E)CD, [AB(E)CD]2, and [AB(E)CD]3, to the classical carrier protein, tetanus toxoid (TT), by controlling both the coupling chemistry and the OS loading, immunogenicity of the resulting glycoconjugates was investigated in mice. [AB(E)CD]2 clearly appeared as the best sequence among those tested for the induction of anti-LPS IgGs specific for *S*. *flexneri* 2a. Indeed, AB(E)CD and [AB(E)CD]2 were eliciting a significant, albeit lower, anti-LPS IgG response [40]. More importantly, IgGs induced by the glycoconjugate incorporating [AB(E)CD]2, were shown to be protective, and protection was clearly shown to be dependent on the anti-LPS Ab titer [49] (Phalipon et al., submitted).

A phase I clinical trial is envisioned and preclinical investigation is under way, the major goal being the production of GMP batches of the selected conjugate by companies expert in the synthesis of complex carbohydrates and/or polysaccharide–protein conjugates. The good news is that the multi-step chemical synthesis leading to the obtention of [AB(E)CD]3 has been recognized as feasible at large scale and reasonable cost, suggesting that the semi-synthetic glycoconjugate vaccine approach remains an attractive option that fulfills most of the WHO requirements concerning the availability of vaccines for developing countries.

Further development will integrate the need for multivalency, and a lot of effort is currently invested in the identification of a cross-protective antigen. Besides the testing of new routes of systemic delivery, immunization is being investigated.

Recent evidence indicates that some structures involved in the pathogenesis of *Shigella*, the protein components of which are highly conserved throughout groups and serotypes, could be considered as antigens that may elicit neutralizing Abs expected to disarm *Shigella* in the course of its pathogenic process. Such antigens that may provide reasonable—if not

\[
\alpha-D-GlcP \quad E \\
(1 \rightarrow 4)
\]

2) \(\alpha-L-Rhap-(1 \rightarrow 2)\) \(\alpha-L-Rhap-(1 \rightarrow 3)\) \(\alpha-L-Rhap-(1 \rightarrow 3)\) \(\beta-D-GlcP\)NAc-(1 \(\rightarrow \))

Fig. 1. Structure of the basic repeating unit of the O-antigen moiety of *S*. *flexneri* 2a LPS [6].
complete—cross-protection could also be used as carriers for the major polysaccharide-based serotypes, and thus a creative combination able to generate a new paradigm of protection against the diverse *Shigella* serotypes.

As a matter of fact, one of the dominant issues soon to appear will be the capacity to obtain the strongest possible immunogenicity combined with the highest level of cross-protection, in the simplest possible vaccine preparation.

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References


W.F. Goebel, Studies on antibacterial immunity induced by artificial antigens. II. Immunity of experimental pneumococcal infection with antigens containing saccharides of synthetic origin, J. Exp. Med. 72 (1940) 33–48.


