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Single-strain improved oral cholera vaccine

Scientific report- Jan Holmgren

FORSKNING

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Summary

There is an urgent need for effective and affordable cholera vaccines both for use in cholera outbreaks and in mass vaccination campaigns. The main goal of the project has been to develop a simplified killed oral cholera vaccine based on a single *Vibrio cholerae* O1 vaccine strain and a single inactivation method, which could replace three virulent O1 strains inactivated by two different methods used in current licensed vaccines thereby reducing production costs. The vaccine should ideally also contain recombinantly produced low-cost cholera toxin B subunit (rCTB) and be in a formulation that would avoid both the need for a cold chain and for co-administration of a bicarbonate buffer.

By substituting the inactive *wbeT* methyl transferase gene in a *Vibrio cholerae* O1 Inaba strain with an engineered *wbeT* gene encoding a partially functional gene product we have created non-toxigenic “Hikojima” strains that stably express both the Ogawa and Inaba serotype antigens. When mice were orally immunized with formalin-inactivated Hikojima whole-cell vaccines (El Tor or Classical biotype) we could detect O1 lipopolysaccharide (LPS) cross-reactive as well as Ogawa- and Inaba-specific serum antibodies using both LPS-specific ELISAs and vibriocidal assays. Serum antibody titers and, most important, intestinal mucosal IgA anti-LPS antibody responses were fully comparable with those obtained by immunization with a licensed oral cholera vaccine (Dukoral™).

Our new primary vaccine candidate, which is now being further developed together with the Wellcome Trust Hilleman Laboratories in India, consists of a single, genetically engineered El Tor Hikojima strain (MS1568) replacing the three virulent O1 strains inactivated by two different methods used in current licensed vaccines; in addition, our vaccine includes low-cost recombinantly produced cholera toxin B-subunit (rCTB) which further improves short term protection against cholera and offers cross-protection against ETEC diarrhea. The vaccine is provided orally in a 2ml formulation that eliminates the requirement of clean drinking water following intake and, together with pH- and thermo-stabilization technologies that enhance stability and avoid need for co-administration with a buffer, this should make the vaccine attractive for worldwide use including stockpiling purposes. Clinical studies with the new vaccine are now planned to take place in collaboration with icddr,b in Bangladesh. In a later stage we also hope to evaluate whether the addition of a promising new oral-mucosal adjuvant mmCT also developed in the project will further add to the immunogenicity and efficacy of the vaccine to allow the current two-

dose administration to be replaced by a single-doseregimen without any loss in efficacy even in young children.

Scientific report

"Single-strain improved oral cholera vaccine"

1.1 Background

Cholera is endemic in over 50 countries with an estimated mortality of 100,000-120,000 and an attributed morbidity of 3.8-4.4 million cases annually. Since 2010 the WHO recommends the use of oral cholera vaccines (OCVs) in association with other control measures both for routine preventive use in high-endemic settings and for intervention during cholera epidemics. Two licensed OCVs are available, both of them comprising a mixture of heat- and formalin-killed *V. cholerae* O1 strains of Inaba and Ogawa serotypes and El Tor and classical biotypes: one (Dukoral™) additionally contains cholera toxin B-subunit (CTB) and the other (Shanchol™) while lacking CTB also contains killed *V. cholerae* O139 bacteria [1]. The composition of these vaccines is based on knowledge that immune protection against cholera is mediated by locally produced secretory IgA antibacterial and antitoxic antibodies in the gut, directed against the O1 lipopolysaccharide (LPS) antigen and CTB, respectively, which two antibodies have a synergistic protective effect [1]. Both vaccines have been shown in several field trials to be well tolerated and to confer several-year long protection against cholera; however, they are relatively complex to manufacture due to their multiple strain and dual inactivation formulation. It would be a significant advantage if an efficacious OCV could be based on a single inactivated *V. cholerae* O1 strain co-expressing the Inaba and Ogawa O1 antigens and inactivated by a single method. Preferably it should also contain a low-cost CTB component, which both improves short term protection against cholera from ca 65 to 85% and offers ca 50% cross-protection against ETEC diarrhea [1]; ideally the CTB should be pH-protected to avoid the need (as for Dukoral™) to give it together with a buffer.

Both Ogawa and Inaba bacteria have a common O1 LPS antigen, referred to as the A-antigen. The difference between the Ogawa and Inaba serotypes lies in the terminal perosamine of the O1 LPS, which is methylated in the Ogawa LPS forming the Ogawa-specific B-antigen and not methylated in the Inaba LPS resulting in Inaba-specific C-antigen. The methylation is catalyzed by an enzyme encoded by the *wbeT* gene and mutations that inactivate this enzyme such as insertions, deletions or truncation of this gene result in the Inaba serotype. A third serotype called Hikojima has been described that expresses all three antigens (A,B and C), but this serotype is rare and evidence indicates that it is an unstable transitional serotype observed when a strain

undergoes serotype switching from Ogawa to Inaba and does not occur in nature. We have worked to develop a new generation of killed OCVs based on a single, genetically engineered stable Hikojima type strain that co-expresses the Ogawa (AB) and Inaba (AC) LPS antigens. A first such strain (MS1342) was achieved by the insertion into the chromosome of a parent Inaba strain, a wild-type *wbeT* gene that was stably expressed at low levels [2]. A disadvantage of this approach was the presence of two copies of the *wbeT* gene in the constructed strain with a low risk for instability due to recombination. We have now developed two additional Hikojima strains, one El Tor (MS1568) and one classical (MS1580), in which the activity of the endogenous *wbeT* gene is instead reduced by site-directed mutagenesis [3]. We demonstrate the genetic and phenotypic stability of these novel strains, as well as their ability after formalin inactivation to elicit strong intestinal as well as serum antibody responses reacting with both Ogawa and Inaba LPS in mice following oral route immunizations. A vaccine has been developed comprising, in a 2-ml thermostable formulation with no need for co-administration with a buffer, 10^{11} formalin-killed MS1568 Hikojima bacteria and 1-mg low-cost enteroprotected rCTB, making the vaccine attractive for worldwide stockpiling purposes [4].

1.2 Results

1.2.1 The generation of stable Hikojima vaccine strains

We determined that a naturally occurring point mutation in *wbeT* gene product (S158P) completely destroyed its LPS methylation activity thus resulting in the Inaba serotype. We hypothesized that this site is important for the activity of the *wbeT*-encoded protein and therefore could be used as a target site for directed mutagenesis in order to generate mutants with reduced activity of the *wbeT* gene product. A library of mutants was constructed in the classical Inaba strain JS1569 and colonies tested using an antibody-based colony blotting assay. This procedure demonstrated that the *wbeT* S158F mutation resulted in activity giving balanced Inaba/Ogawa serotype expression. Based on this, *wbeT* with an S158F encoding mutation together with its native promoter was inserted into the suicide vector pMT-Suicide1-sacB and conjugated into the classical strain JS1569 and the El Tor strain Phil6973. Colonies that acquired chloramphenicol and kanamycin resistance were screened for the Hikojima phenotype using agglutination with serotype-specific antisera. Finally, the antibiotic resistance genes were removed using recombination. Two different Hikojima strains, MS1568 El Tor from Phil6973 and MS1580 Classical from JS1569, were generated. Both

strains proved to have a stable phenotype over more than 100 generations of cultivation [3].

1.2.2 Determination of Ogawa and Inaba antigens in the generated Hikojima strains

Both live and formalin-inactivated MS1568 and MS1580 bacteria agglutinated equally well with both Ogawa and Inaba antisera. The relative amounts of Ogawa and Inaba antigens were determined by three immunological assays (inhibition ELISAs and dot blot testing of formalin-killed bacteria, and ELISAs on isolated LPS preparations) and by mass spectrometric measurements of the amounts of specifically methylated and non-methylated perosamine in the LPS preparations. The strains MS1568 and MS1580 were consistently estimated to have 50-56% and 32-42% of their total LPS being of the Ogawa type, respectively.

1.2.3 Immune responses to formalin-inactivated whole-cell vaccines of the novel Hikojima strains

Both inbred Balb/c and outbred CD1 mice were immunized orally/intragastrically with equivalent amounts of formalin-killed MS1568 or MS1580 whole-cell vaccines or with Dukoral™. Following immunizations, fecal pellet extracts and small intestinal tissue extracts (Perfext method) were tested by ELISA for specific IgA anti-LPS antibody levels, and serum samples were analyzed for vibriocidal antibodies against *V. cholerae* O1 Ogawa and Inaba target bacteria and by ELISA for IgG + IgM antibodies against Ogawa and Inaba LPS; similar analyses were also performed with serum samples that had been absorbed with formalin-inactivated Inaba or Ogawa bacteria to determine serotype-specific antibodies. In both mouse strains all the vaccines induced strong IgA antibody responses in fecal and small-intestinal extracts. In no cases were the Hikojima- vaccinated groups inferior to the Dukoral-vaccinated group (Figure 1A). The immunizations also gave rise to strong vibriocidal antibody responses (Figure 1B) as well as serum IgG+IgM ELISA anti-LPS antibody responses (not shown), which again did not differ significantly between the different vaccine groups.

We also examined the ability of immune sera collected from mice orally immunized with the different vaccine preparations to protect against oral challenge with live, virulent *V. cholerae* O1 bacteria of Inaba and Ogawa serotypes, as tested in the well established infant mouse model. Results showed that immune serum from mice orally immunized with formalin-inactivated MS1568 or MS1580 whole-cell vaccine strongly

protected baby mice against experimental cholera caused by either Inaba or Ogawa bacterial to at least the same extent as did immune serum from mice orally immunized with the Dukoral vaccine (not shown).

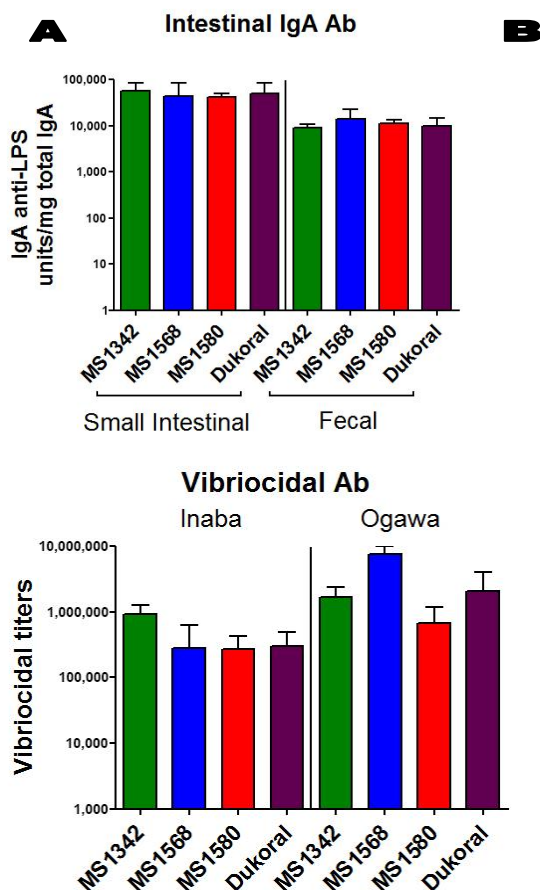


Figure 1. Immune responses to formalin-inactivated whole-cell vaccines of the novel Hikojima strains. Antibody responses elicited by two rounds of oral immunizations in Balb/c mice two weeks apart with formalin-killed MS1342, MS1568, and MS1580 Hikojima whole cell vaccines as compared to immunizations with Dukoral vaccine. (A) Intestinal-mucosal IgA anti-LPS antibody (Left panel) levels in small intestinal tissue extracts (expressed as units per mg of total IgA measured by ELISA); and (Right panel) the same in fecal extracts. (B) Vibriocidal antibody titers against Inaba (left panel) and Ogawa (right panel) test organisms. Bars represent geometric mean values + SEM. Analyses of data by ANOVA showed that post-immunization antibody levels did not differ significantly between any of the immunization groups.

The results show that both the El Tor (MS1568) and the classical (MS1580) Hikojima formalin-killed whole cell vaccines were strongly immunogenic when given orally and induce strong intestinal IgA and serum vibriocidal and IgG + IgM anti-LPS responses that were fully comparable to those induced by the Dukoral™ vaccine.

1.2.4 Low-cost production and enterocoating of rCTB

For maximal early protection against cholera, which would be of special importance for use of OCVs in cholera outbreak situations, OCVs should in addition to a whole-cell component also contain CTB, which both improves short term protection against cholera from ca 65 to 85% and offers ca 50% cross-protection against ETEC diarrhea [1]. Preferably, the CTB should be pH-protected to avoid the need (as for Dukoral™) to give it together with a buffer.

We have developed a low cost production method for industrial manufacturing of CTB for use in OCVs. We have engineered a suitable production strain MS1012, which is a classical *V. cholerae* O1 Inaba strain derived from the wild type isolate 569B. It carries a high-copy number recombinant plasmid allowing high level expression of rCTB and a deletion in the chromosomal *ctxA* gene ensures that no bioactive CT is produced. The plasmid pMT-ctxB/thyA complements the *thyA*⁻ phenotype by supplying a *thyA* gene. Thus MS1012 can grow on minimal and syncase media without the need for supplementation with thymine. The plasmid also carries a constructed *ctxB* gene encoding the classical variant of mature CTB.

Large-scale fermentation of MS1012 is followed by a high-yield two-step purification of rCTB from the medium. This provides for low-cost production of >1000 1-mg doses of purified rCTB per liter, which then using enterocoating has been made resistant to acid pHs for combination with the formalin-killed Hikojima (MS1568) whole-cell vaccine. The combined Hikojima whole-cell/ enterocoated rCTB OCV can be stored for long times at 30-37 C without loss in immunogenicity and can be administered orally without any neutralizing buffer.

1.2.5 Development of a promising cholera toxin-derived adjuvant (mmCT) for potential additional inclusion in OCVs

The development of highly effective oral vaccines, especially those based on isolated or recombinantly produced antigens (subunit vaccines), has been much hampered by the lack of safe, yet effective mucosal adjuvants that could enhance the immunogenicity. Experimentally cholera toxin (CT) and the heat labile enterotoxin from *Escherichia coli* (LT) are two of the most powerful mucosal adjuvants known but much too toxic for human use. They are closely related so-called AB₅ toxins that when administered orally give rise to watery diarrhoea due to the enzymatic action of the A subunit which ADP-ribosylates the G_s protein of adenylyl cyclase and leads to active export of electrolytes and water into the intestinal lumen. For LT it has been found

that toxicity can be virtually eliminated with retention of significant adjuvant activity by mutations that inhibit trypsin-sensitive cleavage of A1 from the remaining A2-B5 toxin complex, such as in e.g. the recently described dmLT adjuvant.

For potential use in OCVs we have now developed a multiple-mutated cholera toxin (mmCT) in which mutations not only inhibit trypsin-sensitive intracellular A1-A2 cleavage, but importantly for stable detoxification also the cleavage mediated by bacterial proteases of *Vibrio cholerae* in which the mutated toxin is produced [5]. The mmCT molecule is easily purified from the growth medium of *V. cholerae*, stable and non-toxic. We have demonstrated that it exhibits strong adjuvant activity when mucosally administered in experimental animal models enhancing the activation of both CD4 and CD8 lymphocytes and mucosal IgA production. Given together with OCVs, both the MS1568 Hikojima whole-cell vaccine and Dukoral, mmCT strongly further potentiates the intestinal IgA anti-LPS as well as antitoxigen antibody responses. We have also found that mmCT is strongly adjuvant-active also on human immune cells and defined its mode of action. Despite the 1000-fold reduced cyclic AMP-inducing activity compared to CT, the adjuvant activity mainly on antigen-presenting cells resulting in strong Th17 cell activation is still dependent on the residual low such activity associated with inflammasome activation and IL-1 production [6].

We believe that mmCT is a promising candidate adjuvant that could be used to both further potentiate our novel Hikojima-rCTB OCV and other OCVs and could have wide application also in the development of other future mucosal vaccines.

1.3 CONCLUSIONS

In developing a new generation of killed whole cell OCVs, a central aim is to make it affordable for the populations in greatest need. This can be achieved by simplifying manufacture whilst maintaining the levels of protection afforded by currently available OCVs. In currently licensed OCVs, the use of several strains and two inactivation methods makes their manufacture complex and relatively expensive. Using a single vaccine strain and one inactivation method as described here will considerably simplify and reduce the cost of manufacture.

We describe here that it is possible to generate stable Hikojima strains by substituting the inactive endogenous *wbeT* genes in Inaba strains of either El Tor or classical biotype *V. cholerae*. A mutated *wbeT* gene with a single amino acid substitution reduces the activity of the gene product resulting in only partial methylation of the surface LPS. This leads to a balanced co-expression of Inaba and Ogawa LPS on the

bacterial surface. The generated strains, MS1568 and MS1580 respectively, both stably expressed approximately 50% each of Ogawa and Inaba LPS as tested over more than 100 generations. Our results suggest that either of these Hikojima strains could replace the mixture of strains in currently licensed OCVs. However, MS1568 may be preferable since it is of the El Tor biotype and is derived from Phil6973, which is used as a formalin killed strain in both the Dukoral™ and Shancol™ OCVs and hence has been extensively tested in humans. The described low-cost, thermo- and pH-stable vaccine formulation, comprising formalin-killed MS1568 Hikojima bacteria and enteroprotected rCTB, should be attractive for worldwide use including stockpiling purposes.

The first generation of our novel Hikojima-rCTB OCV will not include the promising novel oral-mucosal mmCT adjuvant developed in order to speed up the introduction of the Hikojima-based OCV to the market. Given the safety and promising immune response enhancing properties of mmCT we would hope however that future clinical studies could include also OCV with added mmCT to determine if this would allow replacement of a two-dose vaccination regimen with single-dose administration without loss in efficacy.

1.4 Acknowledgements

The project was led by Prof. Jan Holmgren and Dr. Michael Lebens. Dr. Stefan Karlsson, who did his PhD thesis work in the project, contributed importantly to all aspects of the work, and other scientists and technical staff also contributing significantly in the different studies are named in the reference list below. In addition to the financial support from MSB-Sida, the project was supported by grants from the Bill and Melinda Gates Foundation, the Swedish Research Council and The Swedish Science Foundation (Infection Biology program)

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