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Development of a bivalent conjugate vaccine candidate against malaria transmission and typhoid fever

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ABSTRACT

Immune responses to poorly immunogenic antigens, such as polysaccharides, can be enhanced by conjugation to carriers. Our previous studies indicate that conjugation to Vi polysaccharide of *Salmonella* Typhi may also enhance immunogenicity of some protein carriers. We therefore explored the possibility of generating a bivalent vaccine against *Plasmodium falciparum* malaria and typhoid fever, which are co-endemic in many parts of the world, by conjugating Vi polysaccharide, an approved antigen in typhoid vaccine, to Pfs25, a malaria transmission blocking vaccine antigen in clinical trials. Vi-Pfs25 conjugates induced strong immune responses against both Vi and Pfs25 in mice, whereas the unconjugated antigens are poorly immunogenic. Functional assays of immune sera revealed potent transmission blocking activity mediated by anti-Pfs25 antibody and serum bactericidal activity due to anti-Vi antibody. Pfs25 conjugation to Vi modified the IgG isotype distribution of antisera, inducing a Th2 polarized immune response against Vi antigen. This conjugate may be further developed as a bivalent vaccine to concurrently target malaria and typhoid fever.

1. Introduction

Malaria and typhoid fever are co-endemic in large parts of the world, particularly in tropical areas [1–3]. Despite significant control efforts, approximately 21 million typhoid cases and 222 000 typhoid-related deaths were reported in 2014 [3], while 214 million malaria cases and 438 000 malaria-related deaths were reported in 2015 [4]. Typhoid fever results from *S.* Typhi infection transmitted through contaminated water and food [5]. Malaria is a parasitic infection transmitted by female *Anopheles* mosquito [6]. Though the source and route of these two infections are different, their prevalence has significant regional overlap in Africa and other tropical countries, and disproportionately affect children under 5 years of age [7,8]. In co-endemic regions, malaria infection may enhance susceptibility to typhoid fever, and co-infection may lead to misdiagnosis due to similar symptoms [9–11].

Currently, two typhoid vaccines, Vi capsular polysaccharide vaccine (Typhim Vi®) and oral live attenuated vaccine (*S.* Typhi Ty21a), are licensed and marketed. However, both vaccines provide poor protection for infants and children under 2 years of age, and are not recommended in this age group.

Bacterial polysaccharide conjugation to carrier proteins has become a common approach to overcome the poor immunogenicity of polysaccharides, starting with *Haemophilus influenzae* type b (Hib) vaccine, the first conjugate vaccine licensed in 1987 [12–14]. Conjugation technology has enabled the development of more immunogenic typhoid conjugate vaccines. Two Vi-TT (Tetanus Toxoid) conjugate vaccines, Typbar TCV® (Bharat Biotech) and Peda Typh™ (Bio-Med), have been licensed and marketed in India [15,16], while Vi-rEPA (recombinant ExoProtein A), [17–19], Vi-DT (diphtheria toxoid) [20,21], and Vi-CRM_{197} (nontoxic mutant of diphtheria toxin) are being evaluated for use in infants and children [22].

In studies exploring the immunogenicity of Vi conjugated to carrier proteins such as PspA (pneumococcal surface protein A), HBsAg (Hepatitis B virus surface antigen) and DT (Diphtheria Toxoid), all proteins were found to enhance the antibody response to Vi. Interestingly, conjugation also enhanced immune responses

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to PspA and HBsAg but not DT [23,24]. These findings suggested the potential of Vi conjugation to enhance responses to some protein antigens.

Based on this observation, we explored development of a bivalent conjugate vaccine against both typhoid fever and *P. falciparum* malaria. Malaria vaccine development has been hindered by the complexity of the parasite and its life cycle, as well as poor immunogenicity of many malaria antigens. The most advanced malaria vaccine candidate is a pre-erythrocytic vaccine called RTS,S, which is a virus-like particle formulated in AS01 adjuvant. RTS,S has demonstrated partial efficacy against clinical malaria in infants and young children in Phase 3 trials that wanes with time [25].

Other major vaccine efforts against malaria include pre-erythrocytic whole organism vaccines, blood stage vaccines, and transmission blocking vaccines (TBV) [26–29]. TBV have received increased attention owing to renewed interest in malaria elimination and eradication. TBV antigens are expressed in the mosquito stages of the parasite life cycle and induce antibodies that, when taken up by mosquitoes during blood meals, can prevent mosquito infection and subsequent transmission [30]. TBV might be developed as stand-alone products, or can be combined with components that prevent human infection as vaccines to interrupt malaria transmission (VIMT) [31].

Among the antigens identified as targets for TBV, Pfs25 has been the most extensively studied vaccine candidate and has received most attention for clinical development [32,33]. Pfs25 is poorly immunogenic and strategies to enhance immunogenicity have included conjugation to carrier proteins [34,35] or Outer Membrane Vesicles [36], and incorporation in virus like particles [37] or nanoparticles [38]. We have shown that conjugation of Pfs25 to different carriers increases antibody titers in animals [34–36,39,40] and humans [32].

Here we describe the functional immunogenicity of a bivalent vaccine candidate generated by conjugation of Pfs25 to Vi polysaccharide. We observed significant enhancement of antibody responses against both antigens, suggesting that this concept can be pursued as a bivalent vaccine to block malaria transmission and prevent typhoid fever.

2. Materials and methods

2.1. Vi capsular polysaccharide

Vi polysaccharide used in this study was purified from *S.* Typhi isolate number C6524 strain, originally obtained from a patient by the National Institute of Cholera and Enteric Diseases (NICED) in India [41]. Larger scale manufacturing including fermentation of *S.* Typhi, Vi purification, and Vi characterization was performed by SK Chemical, Gyunggido, South Korea. Vi contains 2.3 mmol *O*-acetyl per gram, and 79% of Vi was eluted from Sepharose CL-4B gel column with a distribution constant (K_D) of 0.25. Protein and nucleic acid contamination in purified Vi were less than 0.05% and 0.5% respectively, and endotoxin level was 1.5 EU per microgram. Vi quality met WHO requirements for Vi polysaccharide vaccine.

2.2. Malaria transmission blocking vaccine (TBV) antigen

Recombinant Pfs25 (MW 18,735 Daltons) used for the synthesis of Vi-Pfs25 and EPA-Pfs25 conjugates was produced in *Pichia pastoris* according to the method previously reported by Tsai et al. [42].

2.3. Preparation of Vi-Pfs25 conjugates

Vi-Pfs25 conjugates were synthesized by two synthetic methods (Fig. S1). Details of conjugate syntheses are given in the supplementary data.

2.4. Characterization of conjugates

Pfs25 content was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS PAGE)/Western blot analysis of conjugate (2.5 μg Pfs25 equivalent), using 4–20% Tris-Glycine gel (ThermoFisher) and 30 mA constant current, and transfer to nitrocellulose membrane using iBlot device (Invitrogen). Blots were incubated with primary antibody (anti-Pfs25 mAb, 4B7) followed by secondary antibody labeled with alkaline phosphatase (goat anti-mouse IgG, KPL), and developed with BCIP/NBT phosphatase substrate (KPL).

2.5. Immunogenicity and IgG subclass composition

All animal studies were conducted per the guidelines and approval of Animal Care and Use committee at the National Institutes of Health. CD1 female mice received intramuscular injections of various conjugates in PBS or adsorbed to Alhydrogel® adjuvant (2% Alhydrogel® Brenntag, Biosector) on day 0 and 28 with 2.5 μg dose equivalent of Pfs25 in 50 μl injection volume. Alhydrogel® formulations contained 450 μg/ml of aluminum (22.5 μg/dose) and were prepared in PBS, pH 7.4. The conjugate/Alhydrogel® formulations were incubated at room temperature with gentle rotation for 1 h and stored at 2-8° C for up to 7 days until use. Control mice received 2.5 μg Vi alone in PBS or formulated in Alhydrogel®.

Mice were bled on day 42, then variously on day 83 or 91 for different groups. Mice immunized with PBS formulations were bled on day 83, and those with Alhydrogel® formulations were bled on day 91. Antibody titers against Pfs25 and Vi were measured by ELISA as described in Supplementary Data section, using un-conjugated Pfs25 and Vi respectively as coating antigens [43,44]. Vi-titer of sera diluted 1:500 is expressed in terms of optical density (OD) at 405 nm. IgG subclasses were determined in pooled sera from the second bleed using Mouse Monoclonal Antibody Isotyping Kit (ISO2, Sigma Aldrich). Each IgG subclass (IgG1, IgG2a, IgG2b and IgG3) titer was calculated as the reciprocal of serum dilution giving an OD of 0.5 for Vi and Pfs25, and expressed as a% contribution to the sum of subclasses.

2.6. Transmission blocking activity and bactericidal activity assays

2.6.1. Standard membrane feeding assay (SMFA)

Transmission blocking activity of immune sera was evaluated by SMFA, which measures the reduction of oocyst formation in mosquitos fed on *P. falciparum* gametocytes mixed with test sera versus control sera [33]. Briefly, *Anopheles stephensi* mosquitoes fed on cultured *P. falciparum* gametocytes mixed with sera through a Parafilm membrane stretched across a heated membrane feeding apparatus. Mouse test and control sera were tested at 1:5 dilution. In 8 days, mosquitoes were dissected, and the number of oocysts counted. A reduction in the number of midgut oocysts in mosquitoes fed on immune sera versus control sera indicates transmission blocking activity.

2.6.2. Serum bactericidal assay (SBA)

Bactericidal activity against *S.* Typhi was evaluated in the SBA, as described previously [45]. Serial dilutions of heat-inactivated (56 °C) sera (50 μl) were mixed with *S.* Typhi bacteria in triplicate, incubated for 1hr at 37 °C with 10% baby rabbit complement, then plated on square LB agar plate and overlaid with top agar. Bacterial

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