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Oral administration of live *Shigella* vaccine candidates in rhesus monkeys show no evidence of competition for colonization and immunogenicity between different serotypes

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ABSTRACT

Live oral monovalent *Shigella flexneri* 2a vaccine candidates as well as bivalent formulations with *Shigella sonnei* were evaluated in a rhesus monkey model for colonization and immunogenicity. Freshly harvested suspensions of *S. flexneri* 2a vaccine candidates WRSf2G12 and WRSf2G15 as well as *S. sonnei* vaccine candidate WRSs3 were nasogastrically administered to groups of rhesus monkeys, *Macaca mulatta*, either in a monovalent form or when combined with each other. The animals were monitored daily for physical well-being, stools were subjected to quantitative colony immunoblot assays for bacterial excretion and blood and stools were evaluated for humoral and mucosal immune responses. No clinical symptoms were noted in any group of animals and the vaccine candidates were excreted robustly for 48–72 h without significant changes in either the magnitude or duration of excretion when given as a monovalent or as bivalent mixtures. Similarly, immunological interferences were not apparent in the magnitude of humoral and mucosal immune responses observed toward *Shigella*-specific antigens when monkeys were fed monovalent or bivalent formulations. These results predict that a multivalent live oral vaccine of more than one serotype can have a favorable outcome for protection against shigellosis.

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1. Introduction

There are more than 50 serotypes of *Shigella*, a gram negative enteroinvasive bacterial pathogen that causes diarrhea and bacillary dysentery [12,13]. These serotypes are derived from four species *Shigella dysenteriae, Shigella flexneri, Shigella.sonnei*, and *Shigella boydii*. Protection against shigellosis is serotype specific, that is based upon the O-antigen structure of the bacterial lipopolysaccharide (LPS), although convalescent sera also demonstrate immune responses to several plasmid-encoded protein antigens [7,8,14,15]. It is generally accepted that an effective vaccine must be a multivalent product composed of, at a minimum, vaccine candidates against *S. sonnei* that is prevalent in developed countries and *S. flexneri* 2a and *S. flexneri* 3a that are mainly present in Asia and Africa [13]. Such a mixture could provide >75% protection against the disease worldwide. However, in the future, with shifting trends and new serotype identifications, other serotypes may have to be included for a more comprehensive coverage [18,23,24].

Recently, several novel live, oral *S. sonnei* and *S. flexneri* 2a vaccine candidates have been described whose principal attenuating feature is the loss of the VirG(or IcsA) function that prevents intercellular bacterial spread within the host tissue [3,19,20]. These new vaccine candidates have additional gene deletions such as the chromosome-based *setAB*, encoding an enterotoxin of *S. flexneri* 2a and invasion plasmid-based enterotoxin genes *senA* and *senB.* In addition, some of the derivatives also lack the *msbB2* gene whose product is normally required for maximal acylation of lipid A and whose loss could potentially reduce or eliminate symptoms due to LPS endotoxicity [6,21]. While the newer *S. sonnei* vaccine candidates, WRSs2 and WRSs3 are in clinical trials, however, the long term goal is to be able to combine a *S. sonnei* vaccine with the corresponding *S. flexneri* 2a vaccine candidates WRSf2G12 or WRSf2G15. The safety and immunogenicity of the *virG*(*icsA*)-based live attenuated *Shigella* vaccines is related to the ability to invade epithelial cells of the gastrointestinal tract (GIT) and undergo limited replication within the host. When different strains are combined to make a bivalent (or a trivalent) formulation there is the potential for

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competition between the serotypes in the intestinal tract. To evaluate the potential for such interactions in a gastrointestinal model of infection, monovalent and bivalent mixtures of WRSs3, WRSf2G12 and WRSf2G15 were nasogastrically administered to rhesus monkeys. Like humans, primates are naturally susceptible to infections with *Shigella*, and show similar symptoms and pathology. Rhesus monkeys in many cases succumb fatally to *Shigella* infections. The data from this current study indicates that bivalent formulations of live *Shigella* vaccine candidates are safe and there is no interference between the *Shigella* serotypes for colonization and immunogenicity in the primate gut. This observation has favorable consequences for human vaccination trials.

2. Material and methods

2.1. Animal immunizations

Five groups of six rhesus monkeys each received either (a) WRSs3 (b) WRSf2G12 (c) WRSf2G15 (d) WRSs3 and WRSf2G12 and (e) WRSs3 and WRSf2G15**.** The monkeys, all male, ranged in weight from 7 to 13 kg and were chosen from a pool of animals housed at WRAIR that had previous exposure to dengue, leishmania and anthrax. Prior to assignment in this study, the monkeys were shown to be negative for *S. sonnei* and *S. flexneri* 2a LPS-specific IgG antibodies by serology (titers of <1:100) and negative for *Shigella* in fecal cultures on Hektoen Enteric Agar (HEA) plates. The vaccine doses were administered nasogastrically in 20 ml of water to sedated animals as previously described [2,4]. The monkeys were observed daily for general activity, feeding habits, and blood and stools were collected for analysis according to a predetermined schedule. Complete blood counts and blood chemistry were also performed pre and post inoculation to denote any signs of clinical illness after inoculation. These studies were carried out under a WRAIR IACUC-approved rhesus monkey protocol and all efforts were made to minimize pain and discomfort as previously described [2,4].

2.2. Preparation of the bacterial inoculums

Research seeds of individual vaccine strains were used to seed bacterial lawns on TSA plates that were incubated at 37 C for 24 h. The confluent bacterial cultures were harvested in sterile water as previously described [2,4]. The OD was adjusted to obtain a final concentration of $\sim 2.5 \times 10^{10}$ CFU in 10 ml of water. Each monkey in group a, b and c was given 10 ml of the vaccine strain combined with 10 ml of water (total volume 20 ml) preceded by 20 ml of a 5% sodium bicarbonate solution to neutralize gastric acidity as previously described [2,4]. The monkeys in groups d and e received a total of 5×10^{10} CFU composed of 10 ml each of two bacterial cultures from the two serotypes.

2.3. Stool processing for qualitative and quantitative analysis of bacterial excretion

Stool samples were collected prior to vaccination and twice daily for 12 days and then only once in the afternoon on days 15, 17, 21 and 28. Stools were weighed in scintillation vials, suspended in 20% glycerol in saline solution, briefly centrifuged to remove debris and the supernatant stored at −80 °C for quantitative colony blot assay. An aliquot of the fresh stool supernatant was streaked out on HEA plates for observation of excretion which is used as a marker for gastrointestinal colonization. Colonies obtained from plating of fresh stool samples were lifted onto nitrocellulose filters and verified by colony immunoblots with either mAb to invasion plasmid antigens IpaB or IpaC to confirm the presence of *Shigella* [3]. Quantitative stool colony immunoblot assay was used for quantitative analysis (CFU/g of stool) of stool samples. Serial dilutions of stool samples were plated on HEA plates and incubated at 37 C. The colonies were lifted onto nitrocellulose filters that were placed, colony side up, on a blotting paper soaked in chloroform for 10 min (this step performed in a chemical hood) followed by several washings in Tris buffered saline (TBS). The filters were dried in a 60 °C oven for an hour and stored at 4 °C for batch processing. The filters were washed for 5 min in TBS prior to incubation in casein and then in the primary antibody for an hour at room temperature with shaking. The primary antibody was used at a 1:250 dilution in casein and was either BactoShigella antiserum Polygroup B (*S. flexneri* typing serum from Difco) or Polygroup D (*S. sonnei* typing serum from Difco) depending upon the animal group to be evaluated. The stool samples from the bivalent treatment groups (groups d and e) were plated out in duplicate and each filter of a pair was treated with one of the two types of primary sera. Protein A linked to alkaline phosphatase was used as the secondary antibody at a 1:500 dilution in casein. The antigen-antibody reactions were developed using Fast Red and naphthol phosphate as substrates as described previously [2]. The number of red spots was counted and the CFU/g of stool was calculated from the dilutions plated.

2.4. Serum immune responses

Serum IgG and IgA endpoint titers specific for *S. sonnei* and *S. flexneri* 2a lipopolysaccharide (LPS), the invasion plasmid antigen (Ipa) proteins, IpaB, IpaC and IpaD as well as *S. sonnei* and *S. flexneri* 2a Invaplex 50 (composed of LPS in a complex with IpaB and IpaC) were determined by ELISA as previously described [2]. 96-well plates were coated with specific antigen and plasma samples collected on day 0, 14, and 28 were diluted in 2% casein and titrated across the plates using 2-fold serial dilutions [2]. The plates were incubated further with goat-anti-human IgG-AP or goat-anti-human IgA-AP. Bound antigen-specific antibody was detected by a phosphatase substrate [2,21]. The endpoint titer was defined as the reciprocal of the last dilution of sample that produced an OD value of 0.2. A responder was defined as having a ≥4-fold increase in post-vaccination titer as compared with pre-vaccination titer. Statistical analysis was accomplished using Prism 4 for Macintosh (Graphpad Software, Inc). Log-transformed endpoint titers were analyzed using a two-way analysis of variance with a Bonferroni post-test.

2.5. Mucosal immune responses

Mucosal immune responses were determined by measuring *S. sonnei* and *S. flexneri* 2a LPS and Invaplex-50 specific antibody secreting cells (ASCs) in peripheral blood mononuclear cells (PBMCs) by ELISPOT and fecal IgA by ELISA. PBMCs were isolated by Ficoll density gradient and cryopreserved in DMSO freezing medium [1,2]. ASCs were determined using goat anti-human antibodies [9]. For fecal IgA measurements stool was collected and frozen immediately until extracted. It was thawed and suspended in PBS containing EDTA, trypsin soybean inhibitor and PMSF [9,10]. The suspension was vortexed and incubated for 20–25 min at 4 °C with intermittent vortexing and then filtered through gauze and centrifuged at 20,000 × *g* for 30 min at 4 °C. The supernatant was frozen and later used for ELISA assays using human-specific reagents. The antigen-specific endpoint titers were determined and the final titer for each animal was adjusted to 10 μg/ml of total IgA [9,10].

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