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Safety and immunogenicity of a *Shigella flexneri* 2a Invaplex 50 intranasal vaccine in adult volunteers☆

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ABSTRACT

Shigellosis is a leading cause of diarrhea worldwide prompting vaccine development. The *Shigella flexneri* Invaplex 50 is a macromolecular complex containing IpaB, IpaC, and LPS, formulated from an aqueous extract of virulent *Shigella* delivered via nasal administration. Preclinical vaccine testing demonstrated safety, immunogenicity and efficacy. An open-label dose-escalating phase 1 study evaluated a 3-dose (2-week intervals) regimen via nasal pipette delivery. Thirty-two subjects were enrolled into one of four vaccine dose groups (10, 50, 240, or 480 μg). The vaccine was well tolerated with minor short-lived nasal symptoms without evidence of dose effect. Antibody-secreting cell (ASC) responses were elicited at doses ≥50 μg with the highest IgG ASC, Invaplex 50 (100%) and *S. flexneri* 2a LPS (71%), as well as, serologic responses (43%) occurring with the 240 μg dose. Fecal IgA responses, Invaplex 50 (38.5%) and LPS (30.8%), were observed at doses ≥240 μg. The Invaplex 50 nasal vaccine was safe with encouraging mucosal immune responses. Follow-on studies will optimize dose, delivery mechanism and assess efficacy in a *S. flexneri* 2a challenge study.

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1. Introduction

Shigellosis is a leading cause of diarrheal disease worldwide particularly in developing countries where it is estimated that over 163 million cases with 1 million fatal cases occur annually [1]. In addition shigellosis is a continuing problem for civilian and military travelers visiting endemic regions [2–5]. Vaccine development remains a high priority given the disease burden and increasing antibiotic resistance [6]. *Shigella flexneri* account for 30–60% of shigellosis cases in developing regions necessitating coverage of predominant *S. flexneri* serotypes in a multivalent *Shigella* vaccine [1].

Shigella pathogenesis is attributed to the organism's ability to invade, replicate intracellularly, and spread intercellularly within the colonic epithelium [7–9]. Essential components in the invasion process and subsequent immunity include several highly conserved, virulence-plasmid-encoded proteins (IpaA, IpaB, IpaC, and IpaD) [10,11]. *Shigella* LPS, the chemical basis of *Shigella* serotypes, has been demonstrated to be a protective antigen in field efficacy studies and is a key factor involved in the functionality of surface proteins such as VirG and the type III secretion system (TTSS) [12,13]. The *S. flexneri* invasin complex (Invaplex) vaccine is an ion exchange-purified, high molecular weight complex isolated from virulent *Shigella* that consists of LPS and many proteins, including the invasins IpaB, IpaC and IpaD, that exhibits native biological activities and antigenicity [14,15]. Intranasal delivery of Invaplex stimulates protective immunity in small animal models for shigellosis [14]. Nasal delivery of the Invaplex vaccine has the potential to require low antigen doses possibly due to the product's native affinity for epithelial cells and M-like cells found in the nasal cavity, reduce antigen degradation and dilution as compared to an oral route, and allow administration without needles [16]. This first-in-human, dose-escalating study provides an initial assessment of the

☆ This study was approved by the ethical review committee of the Naval Medical Research Center, Silver Spring, MD (DOD # 32522, HSRRB Log # A-12528) in compliance with all Federal regulations governing the protection of human subjects and registered on ClinicalTrials.gov (NCT00082069). Animal research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals (adhered to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 ed.).

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S. flexneri 2a Invaplex 50 vaccine safety and immunogenicity as a 3-dose, biweekly intranasal regimen.

2. Methods

2.1. Investigational vaccine

2.1.1. Preparation of Invaplex vaccine

The cGMP Invaplex vaccine was prepared from a Production Cell Bank of virulent *S. flexneri* 2a, strain 2457T that had been previously used in human challenge studies and is stored at the WRAIR Pilot Bioproduction Facility. An isolated, smooth, Congo red positive *S. flexneri* 2a colony was used to inoculate 3 L (1 L per flask) of Bacto Antibiotic Medium 3 (Becton Dickinson, Sparks, MD). After 6 h of growth at 37 °C the early log phase (mean OD_{600} = 0.125) cultures were combined and transferred aseptically to a 400 L fermentor (New Brunswick) containing 300 L of Bacto Antibiotic Medium 3 and 0.003% antifoam. The culture was incubated at 37 °C with an agitation speed of 400 rpm and airflow of 300 L/min. After 18 h of growth the *Shigella* cells were harvested by centrifugation in a Sharples AS-26 continuous feed centrifuge. At the time of harvest an aliquot of the final culture was used for quantitation of cfu/mL, gram stain, purity, colony uniformity, per cent Congo red positive colonies, culture identity and serotype (Denka Seiken Co., Ltd.).

The bacterial cells were suspended in 15 L of sterile water using a mechanical mixer and then incubated for 2 h with stirring (250 rpm) at 37 °C. After centrifugation (13,700 × *g* for 30 min, 4 °C), the resulting supernatant was filtered (0.22 μm membrane, Millipak 200) and then stored at −80 °C. The bulk water extract was analyzed for total protein (bicinchoninic acid assay, Pierce Chemical Co) and for IpaB, IpaC and LPS content by immuno spot blot using monoclonal antibodies specific for IpaB (mAb 2F1), IpaC (mAb 2G2), and *S. flexneri* 2a LPS (mAb 2E8). For final purification the water extract was thawed, filtered twice (0.1 μm membrane, Millipak-200), adjusted to a final concentration of 20 mM Tris and pH 9.0 and applied to an anion exchange column (Q Sepharose High Performance, Pharmacia) equilibrated in 20 mM Tris buffer, pH 9.0. Next, using buffer steps, the Invaplex 24 peak was collected in 240 mM NaCl in 20 mM Tris, pH 9.0 followed by collection of the Invaplex 50 peak in a step consisting of 500 mM NaCl in 20 mM Tris, pH 9.0. The Invaplex fractions were placed immediately at 4 °C. This procedure follows the elution strategy (altered for scale) described by Turbyfill et al. [14]. The final Invaplex 50 product was adjusted to 250 mM NaCl and a final protein concentration of 1.2 mg protein per ml, sterilized by filtration (0.22 μm Millipak-20 filter unit), dispensed to sterile glass vials (1.0 mL per vial) and stored at −80 °C without preservative. Although collected the Invaplex 24 product was not used in this study.

2.1.2. Analysis of Invaplex 50 vaccine

SDS-PAGE Coomassie blue stained gels were used to assess the total protein profile of Invaplex [14]. Western blots were probed with purified anti-IpaB mAb 2F1 (1 μg/mL) or purified anti-IpaC mAb 2G2 (2 μg/mL) [17] and developed as previously described [14]. For LPS analysis, SDS-PAGE gels and western blots were loaded with proteinase K-treated samples and stained with silver [14,18] or probed with mAb 2E8 (anti-*S. flexneri* 2a LPS) and developed as described above, respectively. Standard LPS preparations included purified LPS from *S. flexneri* 2a and a reference Invaplex preparation (lot 0808).

The quantity of IpaB and IpaC in Invaplex was determined using a modified ELISA procedure using purified recombinant IpaB or IpaC proteins as standards [18]. The *Limulus* amebocyte lysate (LAL) assay for LPS detection was performed by the gel clot method (Pyrotell, Associates of Cape Cod Inc.). Control standard LPS and LAL reagent water used in this assay were purchased from Associates of Cape Cod Inc. *S. flexneri* 2a LPS content in each Invaplex preparation was also measured by determining the 2-keto-3-deoxyoctonate (KDO) concentration [19].

2.2. Preclinical animal studies

2.2.1. Toxicology study in mice

To determine acute toxicity of the Invaplex product, groups of male and female Balb/c mice were immunized on days 0, 14 and 28 with *S. flexneri* Invaplex 50 at doses of 0.5 and 5.0 μg (Groups 2 and 3, respectively) or with USP saline (group 1), all delivered intranasally in a total volume of 5 μL. Prior to dosing, all animals were anesthetized by intraperitoneal injection using a ketamine/acepromazine/xylazine cocktail. The animals were observed twice daily for moribundity and mortality, and once daily for clinical signs of toxicity to include nasal irritation and weight loss. Prior to necropsy on days 30 (2 days post-final immunization) and 42 (2 weeks post-final immunization), animals (up to 10 mice of each sex per group) were fasted overnight, bled from the retro-orbital sinus on the next day and then sacrificed by CO_2 overdose. An extensive necropsy was performed, and selected tissues from the control and high dose groups were examined microscopically. Nasal tissues were processed and sectioned as described by Gizurarson et al. [20] and the Registry of Industrial Toxicology Animal data [21] to permit evaluation of the nasal mucosa, underlying submucosa, and underlying immune tissue. Microscopic evaluation of target organs was also performed for the low dose group of both sexes.

2.2.2. Immunogenicity and protective efficacy of *S. flexneri* 2a Invaplex in mice and guinea pigs

Mice (female, Balb/cByJ) or guinea pigs (male, Hartley) were immunized intranasally with 5 μg (mice) or 25 μg (guinea pigs) protein/dose of *S. flexneri* 2a Invaplex 50 or with saline on days 0, 14, and 28 as previously described [14,15]. Three weeks (day 49) after the final immunization, all mice (15 per group) were challenged intranasally with a lethal dose of *S. flexneri* 2a (1.0×10^7 cfu/30 μL) as described for the mouse lung model [22]. Mice were weighed and observed daily for overt signs of illness including fur ruffling, hunched posture, lethargy and difficulty breathing for 14 days after challenge. Death was the endpoint for the mouse challenge model. Guinea pigs were challenged intraocularly 3 weeks after the final immunization with *S. flexneri* 2a (2457T) (6.0×10^8 cfu/mL) and observed daily for 5 days for the occurrence of keratoconjunctivitis. The degree of inflammation and keratoconjunctivitis was scored using a scale of 0–3, as described by Hartman et al. [23]. Blood was taken from mice (tail bleed, 5 mice per group) and guinea pigs (ear bleed, 5–6 animals per group) on days 0, 28, 42, and 63. Prior to intranasal immunization or challenge, animals were anesthetized with a mixture of ketamine hydrochloride (Ketaset®, Fort Dodge Laboratories, Inc., Fort Dodge, Iowa) and xylazine (12 mg/kg) (Rompun®, Bayer Corp., Shawnee Mission, Kansas). Efficacy was calculated by the formula: [{% death or disease (controls) − % death or disease (vaccines)}/% death or disease (controls)] × 100.

2.3. Clinical trial design

The study was conducted as an open-label dose-escalating trial with 8 subjects receiving one of four Invaplex 50 vaccine intranasal doses (Group A – 10 μg, Group B – 50 μg, Group C – 240 μg, and Group D – 480 μg). An interval of no less than 60 days following the first dose separated volunteer groups receiving subsequent increasing doses with prospectively defined stopping criteria and independent medical review prior to dose escalation.