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 lpaB, lpaC, 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 (lpaA, lpaB, lpaC, and lpaD) [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 invasions lpaB, lpaC, and lpaD, 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
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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<sub>600</sub> = 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 x 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 immunoblot 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 Turkbeyli 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 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 morbidity 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<sub>2</sub> 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 x 10<sup>7</sup> 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 intracranially 3 weeks after the final immunization with S. flexneri 2a (2457T) (6.0 x 10<sup>6</sup> 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). 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.

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.
2.3.1. Study population and enrollment criteria
Healthy adults (18-55 years) were recruited from the greater Washington, DC area and enrolled after informed consent. Exclusion criteria included: specific health conditions (chronic sinusitis, chronic/respiratory disease, reactive airway disease, chronic lung disease, facial nerve paralysis (Bell's Palsy)), or inflammatory arthritis (including a family history); current smoker; abnormalities on physical examination (specifically including rhinophaty, nasal polyps, ulcers, or a clinically significant deviated nasal septum) or laboratory screening (CBC, serum chemistry, HIV-1 ELISA, hepatitis B surface antigen, hepatitis C virus ELISA, HLA-B27, and serum pregnancy test); abnormal bowel habits; regular use of anti-diarrheal, anti-constipation, or antacid therapy; immunosuppressive drug use; ongoing investigational product research participation; or prior Shigella exposure by history (including infection, vaccinations, potential occupational exposure or travel/residence in high Shigella endemic region in past 3 years).

2.3.2. Immunization procedures in humans
Prior to vaccination, subjects used a facial tissue to gently dean their nasal passages. For vaccination, subjects were placed in a supine position with their head tilted slightly backward using a rolled towel under the neck. Subjects in Groups A, B, and C received 100 μL per nostril (200 μL per vaccine dose) delivered as 5 aliquots of 20 μL. Subjects in Group D received two separate administrations, 10 min apart, of 100 μL per nostril (total of 400 μL per vaccine dose). Vaccine was administered using a single-channel, multi-dispensing electronic pipette (Rainin EDP3-Plus) fitted with an individually wrapped, pre-sterilized 100 μL pipette tip. The pipette tip was placed in each nostril less than 1/4 of an inch. Vaccine was administered slowly over 1-2 min allowing ample time for local absorption and to prevent formation of large drops. Subjects remained supine for 10 min post-vaccination. Three doses of the vaccine were given at days 0, 14, and 28.

2.3.3. Safety monitoring
Clinical assessments, including symptom survey and targeted physical examination, were conducted pre-vaccination (at screening, 7 days prior to first dose, and the day of each dose) and post-vaccination at 30 min, 24 h, 7 days, and after completion of the 3-dose series at day 42 and 56. In addition to the above assessments, adverse event monitoring included symptom diary logs (daily for 10 days post-vaccination). Vaccine was administered slowly over 1-2 min allowing ample time for local absorption and to prevent formation of large drops. Subjects remained supine for 10 min post-vaccination. Three doses of the vaccine were given at days 0, 14, and 28.

2.3.4. Specimen collection and processing
The following specimen types (and relevant study time points) were collected: plasma (days -7/0, 14, 28, 35, 42, and 56), peripheral blood mononuclear cells (PBMC) for antibody-secreting cell (ASC) assays (days 0, 7, 21, and 35), stool (days -7/0, 7, 21, 35, and 56), nasal swabs (IgA measurements on days -7/0, 7, 21, 35, and 56), and nasal cytology on days -7, 1, 15, and 29, nasal lavage (Group D only, days -7/0, 7, 21, 35, and 56), and intestinal lavage stool specimens (Group D only, days -7 and 35). Blood samples were separated into plasma and PBMC fractions using a Ficoll-hypaque gradient technique with plasma stored at -70°C (+10°C) with PBMCs stored in vapor phase liquid nitrogen until testing. Stool specimens were frozen (-70°C) immediately after collection and stored frozen until extracted for IgA determinations. Nasal swab specimens for immunological analyses were collected from each nostril using a water-moistened cotton swab placed approximately 2 cm into the anterior nares and rolled across the nares for 7-10 s. Nasal swabs were stored at -70°C in preservative until tested for total and vaccine-specific IgA response. Caligistics™ were used to collect specimens for nasal cytology then transferred to a glass microscope slide, fixed, and stained using methodology described by Hansel [24]. Semi-quantitative scoring of eosinophilia [25] was as follows: no cells, 0; few scattered cells or small clumps, 1++; moderate number of cells and larger clumps 2++; many cells, easily seen, do not cover entire field, 3++; larger number, covering entire field, 4++

Supplemental specimen collection, limited to the high dose Group D, included nasal lavage and intestinal lavage. Nasal lavage was accomplished by instilling 5 mL of sterile saline into the subject's nostril while the subject closed off the back of his/her nose with his/her soft palate during the procedure. The procedure was repeated in the other nostril and combined for testing. The fluid was processed for storage at -70°C until testing for total and vaccine-specific IgA response. The intestinal lavage procedure uses a commercial preparation of balanced electrolyte solution with polyethylene glycol (NulVTELY®). Brantree Laboratories, Brantree, MA) to induce a transient watery diarrhea as previously described [26-28]. In brief, following an overnight fast, subjects drank approximately 240 mL of the lavage solution every 10-20 min (not exceeding 4 L) until watery stool was clear. The collected specimens were immediately filtered through gauze, centrifuged, treated with protease inhibitor cocktail (Sigma–Aldrich) and stored at -70°C until assayed.

2.4. Immunogenicity assessment

2.4.1. Antigens for immunological assays
The battery of antigens used to measure the primary vaccine-specific immune response included S. flexneri 2a LPS (GLP grade, phenol-extracted, Commonwealth Biotechnology Lab, Richmond, VA), water extract (small animals only), and purified recombinant invasin plasmid antigen (Ipa) proteins, IpaB, IpaC, S. flexneri 2a Invaplex 50 and Invaplex 24. For immunoassays, antigen plates were coated overnight at 4°C with 100 μL of LPS, water extract, IpaB, IpaC, or Invaplex 50 diluted to 10, 10, 1, 2, or 0.5 μg/mL, respectively, in carbonate coating buffer (pH 9.8). All Shigella antigens, other than LPS, were prepared in the Invaplex Research Lab (WRAR).

2.4.2. Serum immune response
Serum (animal studies) or plasma (human studies) IgG and IgA endpoint titers specific for Shigella antigens (see above) were determined by ELISA as previously described [14]. For small animals, blood was collected on Whatman paper filter strips on days 0, 28, 42 and 63. For human studies plasma was collected as described.
above. The endpoint titer was defined as the reciprocal of the last dilution of a given sample that produced an OD 405 value of ≥0.2.

2.4.3. Mucosal immune response

For human studies, nasal and fecal samples were assessed by ELISA for anti-S. flexneri 2a LPS and anti-S. flexneri 2a Invaplex 50 IgA responses as previously described [29] using horseradish peroxidase (HRP)-conjugated goat antihuman IgA and ABTS (KPL, Gaithersburg, MD). For extraction of IgA from stool, frozen stool was thawed and suspended in 2 mL of ice-cold extraction buffer (PBS containing 0.05% Tween 20, 0.2 mg/mL Soybean trypsin inhibitor, 1 mg/mL each EDTA and BSA and 1.7 mg/mL PMSF) per gram of stool. The suspension was incubated on ice for 20-25 min with intermittent vortexing. Following centrifugation (20,000 × g for 30 min at 4°C) the clear supernatants were collected and stored at −70°C until assayed.

For nasal secretion specimens collected on cotton swabs, the swabs (one for each nostril) were placed in 2.0 mL cold extraction buffer (see above). The specimen was next vigorously vortexed, maintained on ice until centrifugation (10,000 × g for 20 min at 4°C) and then stored at −70°C until assayed. Total IgA in stool extracts and nasal extracts was determined by a capture ELISA [30] using goat antihuman F(ab')2 Jackson Laboratories, 1 μg/mL as capture antibody and isotype-specific HRP-conjugated goat-antihuman IgA as the detecting antibody. Known concentrations of human milk stIgA (Sigma) was included as a standard to interpolate IgA concentrations of individual samples.

Shigella antigen-specific (antigen and antigen concentration described above) IgA in stool and nasal extracts were determined by a modified ELISA described elsewhere [31]. After coating ELISA plates with antigen and blocking with 3% BSA-0.05% Tween 20 (1 h, 37°C), dilutions of each fecal or nasal extract samples were added and the plates were incubated for 3 h at 37°C. After vigorous washing (5 times with PBS−1%T), HRP-conjugated goat antihuman IgA (0.25 μg/mL in 1% BSA−0.1%T) was added and incubated for an additional 2 h. The bound antibody was detected using ABTS-H2O2 substrate system (KPL, Gaithersburg, MD). The optical density at 405 nm was measured with a SpectraMax plate reader (Molecular Devices). Endpoint titers were calculated as the reciprocal of the highest dilution giving a net (antigen wells-control well) absorbance value of ≥0.15. Antigen-specific titers were adjusted to 1000 μg/mL of total IgA.

2.4.4. Antibody-secreting cells (ASCs)

IgA and IgG ASCs specific for S. flexneri 2a LPS and S. flexneri 2a Invaplex 50 were enumerated by ELISPOT on days 0, 21, and 35 as previously described [32].

2.5. Study endpoints and definitions

The primary outcome for safety evaluation was local or systemic reactions occurring in the 7-day post-vaccination period. The primary outcomes for vaccine-induced immune responses were Invaplex-specific ASC, serologic and fecal IgA responses, Seroconversion, fecal IgA, and nasal IgA conversion all used ≥4-fold increase over the reciprocal baseline titer against a specific vaccine antigen, Invaplex 50 or LPS, as a responder definition. An ASC response was defined as ≥10 antigen-specific (Invaplex 50 and/or LPS) ASC per 109 peripheral blood mononuclear cells.

2.6. Statistical analysis

Baseline comparability of the groups was assessed. Rates of all adverse events (overall and at least possibly related) observed during the follow-up period after vaccinations were analyzed using Fisher's exact test to compare dose levels. For immunological analyses, qualitative (responder rates) and quantitative (log-transformed values) assessments were made in addition to evaluation of the kinetics of the immune response. Baseline titers were the average of two pre-vaccination time points when available. Median increases (fold rises) of anti-Shigella antibody titers and seroconversion rates were calculated. Geometric mean titers were also determined and presented with the standard deviation. Between groups comparisons were examined with nonparametric tests (Kruskal–Wallis for continuous data and Fisher's exact test for categorical data) unless assumptions were fulfilled for Student's t or Pearson's Chi-square. Paired t-tests were used to compare individual post-vaccination to baseline response within each treatment group and Cochran–Armitage trend tests were used to assess changes across dose ranges. All statistical tests were interpreted in a two-tailed
titers (log-transformed geometric means and standard deviations) are reported as an average of two pre-vaccination time points when available.

Table 2

Baseline characteristics of S. flexneri 2a Invaplex 50 study participants by study group.

<table>
<thead>
<tr>
<th>Group (N=8/group)</th>
<th>Group A 10 µg</th>
<th>Group B 50 µg</th>
<th>Group C 240 µg</th>
<th>Group D 480 µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (SD)</td>
<td>28.8 (5.3)</td>
<td>24.3 (3.7)</td>
<td>26.5 (5.5)</td>
<td>30.0 (7.7)</td>
</tr>
<tr>
<td>Gender (% male)</td>
<td>62.5</td>
<td>75</td>
<td>62.5</td>
<td>87.5</td>
</tr>
<tr>
<td>Ethnicity (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>African-American</td>
<td>50</td>
<td>75</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Caucasian</td>
<td>50</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
</tr>
<tr>
<td>Hispanic</td>
<td>0</td>
<td>12.5</td>
<td>12.5</td>
<td>0</td>
</tr>
<tr>
<td>Other</td>
<td>0</td>
<td>0</td>
<td>25</td>
<td>12.5</td>
</tr>
<tr>
<td>Complete 3-dose series (%)*</td>
<td>6 (80)</td>
<td>7 (87.5)</td>
<td>6 (80)</td>
<td>8 (100)</td>
</tr>
<tr>
<td>Serum antibody – geometric mean titer (SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invaplex 50 IgG</td>
<td>835 (3.6)</td>
<td>1181 (2.6)</td>
<td>3200 (3.4)</td>
<td>542 (3.2)</td>
</tr>
<tr>
<td>Invaplex 50 IgA</td>
<td>55 (1.3)</td>
<td>65 (1.4)</td>
<td>57 (1.4)</td>
<td>50 (1.0)</td>
</tr>
<tr>
<td>S. flexneri 2a LPS IgG</td>
<td>1131 (2.0)</td>
<td>1671 (2.6)</td>
<td>1181 (2.0)</td>
<td>617 (2.7)</td>
</tr>
<tr>
<td>S. flexneri 2a LPS IgA</td>
<td>84 (1.3)</td>
<td>109 (2.4)</td>
<td>55 (1.3)</td>
<td>81 (2.0)</td>
</tr>
<tr>
<td>Fecal IgA – geometric mean titer (SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invaplex 50</td>
<td>5.0 (1.5)</td>
<td>13.1 (4.5)</td>
<td>7.8 (3.0)</td>
<td>5.0 (2.1)</td>
</tr>
<tr>
<td>S. flexneri 2a LPS</td>
<td>4.6 (1.4)</td>
<td>17.9 (4.4)</td>
<td>9.2 (3.1)</td>
<td>13.1 (3.7)</td>
</tr>
</tbody>
</table>

* Subject disenrollment: Group A (N=2) – subject with mild nausea without vomiting (2–3 h after 1st dose) then noncompliant with follow-up; subject with asymptomatic grade 1 AST elevation associated with increased alcohol consumption, day 28 follow-up normal; Group B (N=1) – subject with intermittent respiratory viral illness 1 week after 2nd dose; Group C (N=2) – subject noncompliant with follow-up; subject anxiety (after 2nd dose) related to new onset malaise (moderate severity), intermittent headaches (minimal severity) and persistent nasal congestion (minimal severity) with complete resolution by study day 31; Group D – no disenrollments.

3. Results

3.1. Preclinical evaluation of S. flexneri 2a Invaplex 50 vaccine

Research-grade Shigella Invaplex produced at small scale is an effective vaccine and mucosal adjuvant [14,15,29]. The current study used similar manufacturing procedures to produce cGMP-grade S. flexneri 2a Invaplex at the 301 (lot 0808) and 300 L (lot 0994) scale. Table 1 highlights the results of key preclinical studies for both cGMP lots. Essential components of Invaplex are the presence of LPS, IpaB and IpaC. SDS-PAGE and western blots of lots 0806 and 0994 (Fig. 1) indicate that each lot contains similar amounts of LPS, IpaB and IpaC with minimal degradation. Overall protein composition of the Invaplex product is consistent between different research-grade and cGMP lots as determined by Coomassie stained gels (data not shown).

Each cGMP lot of Invaplex was evaluated for immunogenicity and efficacy in mice and guinea pigs. Each lot stimulated robust immune responses to both LPS and the Invaplex product and was protective in both the mouse lethal lung model and the guinea pig keratoconjunctivitis model (Table 1). Preclinical
Table 3
Frequency of signs and symptoms (%) referable to local (nasal) adverse events.

<table>
<thead>
<tr>
<th>Vaccine group</th>
<th>Dose/follow-up period</th>
<th>Reported symptoms</th>
<th>Physical exam findings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Rhinorrhea</td>
<td>Nasal congestion</td>
</tr>
<tr>
<td>Group A 10 μg</td>
<td>Pre-vaccine</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Dose 1 – 1st 24 h</td>
<td>13</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Dose 1 – days 2–7</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Dose 2 – 1st 24 h</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Dose 2 – days 2–7</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Dose 3 – 1st 24 h</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Dose 3 – days 2–7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Any – post-vaccine</td>
<td>38</td>
<td>50</td>
</tr>
<tr>
<td>Group B 50 μg</td>
<td>Pre-vaccine</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Dose 1 – 1st 24 h</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Dose 1 – days 2–7</td>
<td>38</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Dose 2 – 1st 24 h</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Dose 2 – days 2–7</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Dose 3 – 1st 24 h</td>
<td>29</td>
<td>0</td>
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Safety (CGT), intranasal pyrogenicity, and 3-dose acute toxicity studies) demonstrated no evidence of unusual or unacceptable toxic properties. Complete necropsy and microscopic examination of nasal cavity tissue, including the olfactory bulb, did not reveal any significant histopathology attributable to the Invaplex vaccine.

3.2. Trial enrollment and baseline characteristics

Seventy-eight subjects were screened for this vaccine study of which a total of 32 subjects were enrolled. No significant differences between participants (n = 32) and non-participants (n = 46) were observed for age, gender, or race/ethnicity (data not shown). A total of 27 subjects (84%) completed the 3-dose vaccine series. Table 2 provides baseline characteristics of study participants by vaccine dosing group. Overall, the median age of subjects was 25.5 years (interquartile range [IQR] 22.5, 31.0) with a male predominance (71.9%). Baseline immunology, serology, and fecal IgA, was not different between groups with the exception of elevated Invaplex 50 serum IgG titers in Group C. Higher baseline fecal IgA titers were observed in Group B subjects relative to the other groups; however, all groups demonstrated low baseline fecal IgA levels.

3.3. Safety assessment

There were no severe or serious adverse events reported during the study monitoring period, or at the 180-day follow-up telephone check. Surveyed pre- and post-vaccination signs and symptoms are detailed in Table 3. The most commonly reported symptoms and signs were rhinorrhea (50%), nasal congestion (47%), nasal discharge (63%), and inferior turbinate edema (50%). The majority (99%) of the observed symptoms and signs were graded as minimal or mild severity. In addition, most adverse event findings were commonly observed at comparable severity levels pre-vaccination as summarized in Table 3 (particularly common in the two highest dose groups). Less common solicited symptoms and physical exam findings (pre- vs. cumulative post-vaccination) observed were headaches (31% vs. 19%), transient nasal burning (0% vs. 16%), nasal itching (0% vs. 19%), sore throat (0% vs. 22%), and cough (13% vs. 13%). A few additional symptoms, not in the serial survey, were reported: exess salivation (n = 1), transfusion (n = 1), excess salivation (n = 1), medi­ nal/metallic taste post-vaccination (n = 2), sore left nostril (n = 1) and nausea and vomiting (n = 1). Symptoms or signs referable to local nasal findings were more frequently reported in the 24 h after receipt of vaccine irrespective of dose than at any other time during the surveillance period (Table 3). No statistically significant change in adverse event frequency was observed across dose series or with escalating dose. Interestingly, reported local symptoms, such as nasal congestion, were not commonly associated with local exam findings of nasal discharge, hyperemia, and/or edema.

Exploratory analyses of nasal cytology showed pre-vaccination nasal eosinophilia in 10% of subjects with cumulative post-vaccination frequency of 36%, most commonly at low semi­ quantitative levels [1+ (12.9%), 2+ (12.9%), 3+ (6.5%) 4+ (3.2%)]. Nasal neutrophilia was more commonly observed than eosinophilia and at higher levels both pre-vaccination (32.6%) and post-vaccination [1+ (3.0%), 2+ (16.1%), 3+ (25.8%) 4+ (16.1%)]. There was no relationship between local signs and symptoms and nasal eosinophilia or neutrophilia. An evaluation of the serial hematometry and serum chemistry results showed no significant changes from baseline among any of the study groups.
was low for both antigens and isotypes (Fig. 2). Interestingly, levels of seroconversion were somewhat better with two other antigens (S. flexneri 2a Invaplex 24 and purified recombinant IpaC), although peak fold rises remained low (Table 4).

### 3.4. Immunogenicity

A total of 30 subjects received at least two vaccinations required for immunologic evaluation. Positive ASC responses specific for S. flexneri 2a antigens were detected at vaccine dosage as low as 50 μg (Table 4, Fig. 2). Immunization with 240 μg of Invaplex 50 resulted in the highest percentage of responders, with 100% and 71% of subjects having an IgG ASC response to Invaplex 50 and LPS, respectively, and three (43%) with IgA ASC specific for either LPS or Invaplex 50. Subjects immunized with the highest dose of vaccine (480 μg) also had positive ASC responses; IgG ASC specific for Invaplex 50 (50%) and LPS (38%), and two (26%) subjects with both Invaplex 50 and LPS-specific ASC-IgA-ASC. The two higher dose groups also induced the ASC peak responses with the greatest magnitude.

Fecal IgA response frequency showed a dose–response against Invaplex 50 (Cochran–Armitage trend test p-value: 0.05) and LPS (Cochran–Armitage trend test p-value: 0.02). This was also apparent in the peak fold rises of antigen-specific fecal IgA titers (Figure 2). However, this trend was not as apparent in nasal IgA secretions collected with nasal swabs. Use of a nasal wash in Group D identified an increased number of immune responders to both Invaplex 50 and LPS identifying a potential sample collection limitation with the nasal swab that may have precluded the observation of a dose response. An intestinal lavage performed on a subset of subjects in Group D (n = 4) failed to identify any antigen-specific immune responses.

Serologic response rates were low across all 4 study groups (Fig. 2). One subject in the 50 μg dose group seroconverted (IgA) to S. flexneri 2a LPS and to Invaplex 50. Additionally, 1 subject in Group C had a serologic response to Invaplex 50 (IgA and IgG) and 2 had a response to LPS (IgG only). The peak fold rise in serum titers was low for both antigens and isotypes (Fig. 2). Interestingly, levels of a dose response. An intestinal lavage performed on a subset of subjects in Group D (n = 4) failed to identify any antigen-specific immune responses.

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### 4. Discussion

Development of successful Shigella vaccines has been elusive. Over a span of multiple decades various approaches, including killed whole cell, live attenuated and subunit vaccine strategies have been investigated [33,34]. Although a clear correlate of protective immunity has not been identified, previous nonhuman primate studies [35], epidemiologic cohorts [36], and challenge studies assessing homologous protection [37] provide strong evidence that an immune response directed at LPS should provide protective immunity. This response, when measured by ASC assay, seems to be most promising but is largely unconfirmed. One of the difficulties in achieving adequate mucosal immunity is the identification of an effective vaccine delivery route capable of stimulating intestinal immune responses. Oral vaccines provide an effective advantage in this regard but often are difficult to administer in doses that are immunogenic, safe and well tolerated. The Invaplex vaccine is designed to deliver key antigens by an intranasal mucosal route in a manner that utilizes native biological activities of the Ipa proteins to enhance uptake and immunogenicity [9,18,29]. Preclinical studies of Invaplex nasal immunization have demonstrated the induction of a predominantly Th2-like humoral immune response and cytokine pattern, directed to Invaplex, LPS, IpaB, and IpaC, along with Invaplex-specific cellular immune responses. Antigen-specific IgA responses in fecal extracts and intestinal washes are induced indicating stimulation of the common mucosal immune system leading to a protective immune response in mice and guinea pigs with no evidence of toxicity [14,15,29].
adverse events, such as headache, malaice, fever, and myalgias have been observed in similar healthy adult populations between placebo and the best studied nasal vaccine, the licensed trivalent live attenuated influenza virus (LAIV); whereas, local reactogenicity, manifested by runny nose and sore throat, was observed at higher rates in LAIV vaccine recipients [38]. As the Invaplex study was not placebo-controlled, interpreting the significance of adverse event frequency is limited. A prior dose escalation study tested a proteosome-S. flexneri 2a LPS intranasal vaccine and found a dose-related effect on nasal symptoms although adverse events were generally mild and self-limited [39]. The weight ratio of protein/LPS for the proteosome vaccine was 1.12 with a studied dose range from 0.1 to 1.5 mg total protein. The localized symptom of rhinorrhea was more frequent, severe, and of longer duration (approximately 3 days) in the highest dose (1.5 mg) group that equates to 1.34 mg LPS per dose. For comparison, the highest Invaplex dose (480 μg) contained approximately 140 μg of LPS, which is about 10-fold lower than that used in the proteosome-S. flexneri 2a lipopolysaccharide intranasal vaccine trial.

Overall, specific immune responses were evident in Invaplex doses of ≥50 μg with dose-response most apparent with APC and fecal IgA measures although the response rates in subjects receiving 240 μg exceeded that of subjects receiving 480 μg. The 240 μg dose may be optimal, or it may have been due to the fact that the 480 μg dose was delivered in a greater volume (400 μL compared to the 200 μL used for the other doses), a longer delivery time (for the higher dose requiring two administrations), or potentially related to the higher Invaplex 50 pre-immunization serum IgG titers in the 240 μg group. Given the small numbers of subjects in each group, it is not possible to determine if a delivery-related effect (possibly promoting greater contact time with nasal inducive immune sites), boosting of pre-existing immune responses, or random occurrence is the basis for this unexpected difference. Nasal vaccination induced a range of mucosal responses, most encouragingly fecal IgA, although the response rates were not optimal. This may have been due to the use of a pipette to deliver the vaccine. The pipette delivers the vaccine in droplets, which may not allow for optimal uptake. A nasal spray device will increase the surface area of the mucosa exposed and may improve particulate delivery of the vaccine to inductive sites of the mucosal immune system thus increasing the immune response [40].

Invaplex immunogenicity compares favorably with prior clinical studies of other S. flexneri investigational vaccines, both oral live attenuated [41] and nasal S. flexneri proteosome vaccine [39], as well as, immunological responses following S. flexneri 2a challenge [32,42]. An immune measure used commonly across these various studies is the LPS IgG ASC per 10^6 mononuclear cells reported as geometric mean and percent subject responders. Using this immune measure, the current study (restricted to the highest response group) demonstrated a geometric mean of 23 with 71% responders. In comparison, live attenuated S. flexneri vaccines delivered via the oral route have reported a range of geometric means from 8-310 with a wide range of responder rates (14-100%), as summarized by Venkatesan and Ranallo [41]. S. flexneri 2a post-challenge IgA-ASC geometric mean titers in naïve subjects have been reported in the range of 14-58 [32,42]. The nasal Shigella proteosome vaccine had a maximum of 1.34 mg of LPS content compared to 140 μg of LPS in the highest Invaplex dose studied and reported a geometric mean of 16 ASC with an 80% response rate [39]. Invaplex produced a comparable response with a 10-fold lower LPS content. The enhanced induction of LPS immunogenicity is likely related to the Invaplex vaccine's ability to maintain an active, native virulence structure, with the inclusion of IpaB and IpaC, similar to that found on the surface of invasive Shigella enabling Invaplex to efficiently interact with M-like cells in the nasal mucosa [18,29]. The administration of Invaplex directly to

In this clinical trial, the vaccine was well tolerated with post-vaccination adverse events limited to mild severity without increasing frequency upon Invaplex dose escalation. Local nasal symptoms and signs were commonly observed pre-vaccination with some increase following vaccination. Comparable systemic

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**Fig. 2.** Peak immune responses following intranasal vaccination with S. flexneri 2a Invaplex 50. Blood and mucosal washes (fecal and nasal) collected from individuals immunized with 10, 50, 240, or 480 μg of Invaplex 50 (A-D, on horizontal axis, respectively) were assayed for ASC responses (Panel A), mucosal IgA (Panel B) or serum antibodies (Panel C) directed to Invaplex 50 (closed symbols) or LPS (open symbols). Fold-increases (mucosal antibody and serology) over baseline were determined by dividing the titer determined in a sample collected post-vaccination by the titer prior to vaccination. Panel A: Antibody-secreting cell responses to Invaplex 50 (IgG: ○, IgA: □) and LPS (IgG: ◦, IgA: ◇). Panel B: Mucosal responses to Invaplex 50 (fecal IgA: ○, nasal IgA: □) and LPS (fecal IgG: ◦, nasal IgA: ◇). Panel C: Serologic responses to Invaplex 50 (IgG: ○, IgA: □) and LPS (IgG: ◦, IgA: ◇).
the nasal mucosa is likely responsible for the mucosal antibody response in the absence of strong serum antibody as mucosal antigen-presenting cells (APCs), such as dendritic cells, transport vaccine antigens to local lymphoid tissue and present the antigen to resident lymphocytes. During the antigen presentation process, the APC imprints a mucosal homing signal on the lymphocyte so as to direct trafficking to mucosal sites rather than the periphery. The overall result of this process is the induction of mucosal immune responses and moderate or weak systemic antibody responses.

The observed biologic effects also appear to contribute to Invaplex's capacity to augment immune responses to co-administered protein-based and/or DNA-encoded antigens [29]. Additional preclinical evaluation of a highly purified form of S. flexneri 2a Invaplex, consisting primarily of LPS, IpA and IpAC, has demonstrated that the high molecular mass complex of the invasins and LPS are responsible for the protective capacity of parent native Invaplex and enhanced immunogenicity can be achieved through further optimization of IpAC:IpA ratios and LPS quantity relative to the protein content [18].

This study has provided information to advance the clinical development of the candidate Shigella Invaplex vaccine. Ongoing clinical evaluation is directed at optimizing dose selection utilizing an improved nasal delivery device to enhance vaccine uptake and induce a more robust immune response for the monovalent formulation. Future development will target a more highly purified multivalent Shigella vaccine (S. flexneri 1a, S. sonnei, and S. dysenteriae 1), supported by encouraging preclinical results of bivalent and trivalent vaccine candidates [15] and following supportive monovalent efficacy evaluations in challenge studies, leading to large-scale field studies.

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