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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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14. ABSTRACT

Shigellosis is a leading cause of diarrhea worldwide prompting vaccine development The Shigel/ajlexneri lnvaplex 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 J.Lg). 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 :>:SOJ.Lg with the highest lgG ASC, Invaplex 50 (100%) and S.jlexneri 2a LPS (71%), as well as. serologic responses (43%) occurring with the 240 J.Lg dose. FecallgA responses, Invaplex 50 (38.5%) and LPS (30.8%) were observed at doses ;::240 J.Lg. The lnvaplex 50 nasal vaccine was safe with encouraging mucosal immune responses. Follow-on studies will optimize dose, delivery mechanism and assess efficacy in aS. jlexneri 2a challenge study.

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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.

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/seasonal rhinitis, 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 rhinoplasty, 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 clean 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 exam, 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 1 week after each dose), post-vaccination laboratory (hematology and serum chemistry) screens (1 week after each dose), and post-vaccination nasal cytology examinations (1 week prior to first dose and 24 h after each dose). Subject diaries were completed 4 h after each immunization and at 7 a.m. and 7 p.m. each day to assess interval change.

Solicited symptoms included malaise, headache, rhinorrhea, nasal congestion, nasal burning, nasal itching, sore throat, post-nasal drip, cough, sinus pain, sneezing, itching eyes, epistaxis and fever. Symptom severity grading was as follows: absent (Grade 0), minimal (Grade 1: barely noticeable), mild (Grade 2: noticeable, but not interfering with daily activities), moderate (Grade 3: interfering with daily activities), and severe (Grade 4: preventing daily activities). Serial standardized physical examinations were conducted by trained physicians, primarily by one allergist/immunologist (MN) assessing for nasal mucosa hyperemia, nasal discharge, nasal edema, pharyngeal erythema, sinus tenderness, lymphadenopathy, conjunctival injection, tearing, epistaxis, abnormal lung exam finding, or abnormal cranial nerve finding using a standardized grading (0–4) criteria. The degree of relatedness to the vaccination was determined by the principal investigator based on considerations of temporality and alternative explanations.

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 mucosa for 7–10 s. Nasal swabs were stored at −70 °C in preservative until tested for total and vaccine-specific IgA response. Calgiswabs™ 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 (NuLYTELY®, Braintree Laboratories, Braintree, 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 (WRAIR).

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

Table 1
Properties of *S. flexneri* 2a Invaplex 50 lot 0994 and lot 0808.

Property	Lot 0994	Lot 0808
Manufacturer	WRAIR PBF	WRAIR PBF
Appearance	Clear, colorless, homogenous, sterile	Clear, colorless, homogenous, sterile
pH	8.8	8.9
Protein concentration	1.2 mg/mL	1.2 mg/mL
Antigen content	IpaB, IpaC, *S. flexneri* 2a LPS	IpaB, IpaC, *S. flexneri* 2a LPS
Endotoxin (LPS)	1.2×10^6 EU/mL	0.6×10^6 EU/mL
	100 μg LPS/mg protein	50 μg LPS/mg protein
IpaB	8.4 μg/mg total protein	22.6 μg/mg total protein
IpaC	18.1 μg/mg total protein	32.2 μg/mg total protein
Immunogenicity		
Mice ($n = 5$)	≥16-fold increase[a]	≥8-fold increase[a]
Guinea pigs ($n = 5$)	≥8-fold increase[a]	≥16-fold increase[a]
Protective efficacy		
Mice ($n = 15$)	92.3% ($p < 0.001$)[b]	78%, $p < 0.001$)[b]
Guinea pigs ($n = 5$)	100%, $p = 0.003$[b]	78%, $p = 0.005$[b]
Preclinical safety		
GST[c]	Passed	Passed
Pyrogenicity (intranasal)[d]	Passed	Passed
Toxicology (GLP)	No significant histopathology in nasal cavity	ND[e]
Stability	No loss of antigen content and immunogenicity at time of study	No loss of antigen content and immunogenicity at time of study

[a] Immunogenicity against Invaplex 50 antigen [minimum fold increase in titer in all animals ($n = 5$ or 6)].
[b] p value determined by Fisher's exact test (immunized vs. saline treated animals).
[c] GST: General Safety Test; no weight loss or significant effects 7 days after intraperitoneal injection with 500 μg of Invaplex.
[d] Pyrogenicity: intranasal pyrogenicity in rabbits. No rise in rectal temperature was observed 3 h post-vaccination with 100 μg of Invaplex.
[e] ND: not done.

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 ($20{,}000 \times g$ 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 ($10{,}000 \times g$, 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 sIgA (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-T_{20}), HRP-conjugated goat antihuman IgA (0.25 μg/mL in 1% BSA-T_{20}) was added and incubated for an additional 2 h. The bound antibody was detected using ABTS-H_2O_2 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 periods. 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 10^6 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 analysis, 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

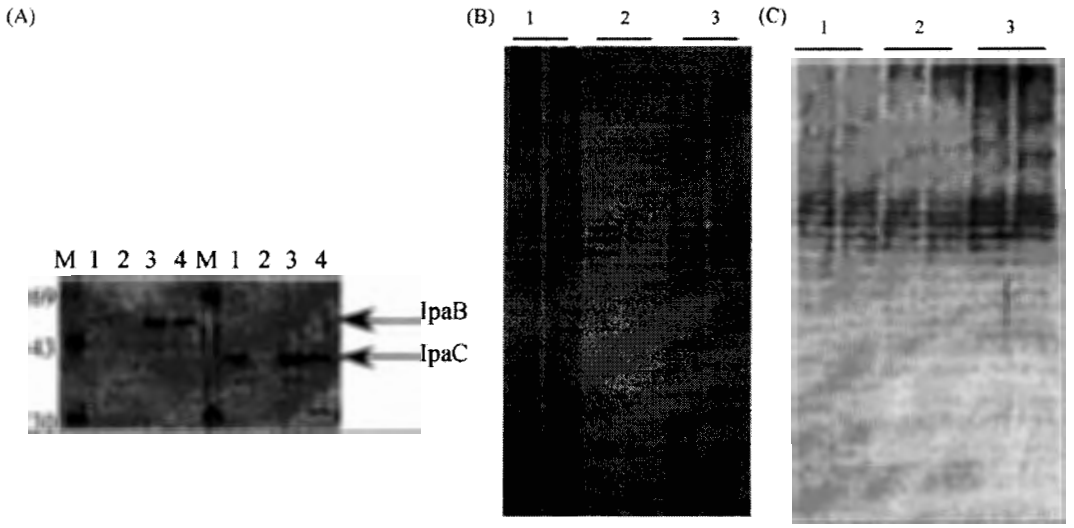

Fig. 1. Panel A: Western blot analysis of cGMP *S. flexneri* 2a Invaplex 50 using monoclonal antibodies to IpaB and IpaC. Whole cell lysates (WCL) of virulent *S. flexneri* 5 (M90T-W) and virulence-plasmid free *S. flexneri* (M90T-55) are in lanes 1 and 2, respectively. Research grade *S. flexneri* 2a Invaplex 50 (20 μg/lane) is in lane 3; cGMP *S. flexneri* 2a Invaplex 50 Lot 0994 (20 μg/lane) is in lane 4. Molecular weight standards are in lanes "M". The blot on the left was incubated with anti-IpaB mAb 2F1 and the blot on the right was incubated with anti-IpaC mAb 2G2. The IpaB and IpaC proteins are indicated. Panel B: LPS analysis by silver stain of SDS-PAGE gels of proteinase K-treated *S. flexneri* 2a Invaplex 50. Lanes marked "1" contain purified *S. flexneri* 2a LPS (5 μg). CGMP *S. flexneri* 2a Invaplex 50 (10 μg protein per lane) lots 0808 and 0994 are in duplicate lanes under "2" and "3", respectively. Panel C. Western blot analysis of *S. flexneri* 2a Invaplex 50 lot 0994 LPS with mAb specific for *S. flexneri* 2a LPS. Gel lanes contain: lane 1 is purified *S. flexneri* 2a LPS (5ug); lane 2 is Invaplex 50 *S. flexneri* 2a, Lot 0808; lane 3 is Invaplex 50 *S. flexneri* 2a, Lot 0994. The blot was probed with mAb 2E8 which is specific for *S. flexneri* 2a LPS. All lanes were run in duplicate.

fashion using alpha = 0.05; no adjustments were made for multiple comparisons. Statistical analyses were performed using SAS v. 8.2 for Windows (SAS Institute, Cary, NC).

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 30 L (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 0808 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 GMP 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 2
Baseline characteristics of S. flexneri 2a Invaplex 50 study participants by study group.

Group (N = 8/group)	Group A 10 μg	Group B 50 μg	Group C 240 μg	Group D 480 μg
Mean age (SD)	28.8 (5.3)	24.3 (3.7)	26.5 (5.5)	30.0 (7.7)
Gender (% male)	62.5	75	62.5	87.5
Ethnicity (%)				
African-American	50	75	50	50
Caucasian	50	12.5	12.5	12.5
Hispanic	0	12.5	12.5	0
Other	0	0	25	12.5
Complete 3-dose series (%)[a]	6 (80)	7 (87.5)	6 (80)	8 (100)
Serum antibody – geometric mean titer (SD)				
Invaplex 50 IgG[b]	835 (3.6)	1181 (2.6)	3200 (3.4)	542 (3.2)
Invaplex 50 IgA	55 (1.3)	65 (1.4)	57 (1.4)	50 (1.0)
S. flexneri 2a LPS IgG	1131 (2.0)	1671 (2.6)	1181 (2.0)	617 (2.7)
S. flexneri 2a LPS IgA	84 (1.3)	109 (2.4)	55 (1.3)	81 (2.0)
Fecal IgA – geometric mean titer (SD)				
Invaplex 50	5.0 (1.5)	13.1 (4.5)	7.8 (3.0)	6.0 (2.1)
S. flexneri 2a LPS	4.6 (1.4)	17.9 (4.4)	9.2 (3.1)	13.1 (3.7)

[a] 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 intercurrent 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.

[b] Statistically significant difference in baseline Invaplex 50 IgG titers across the 4 study groups (ANOVA p-value = 0.03). All groups contained 8 subjects at baseline. Baseline titers (log-transformed geometric means and standard deviations) are reported as an average of two pre-vaccination time points when available.

Table 3
Frequency of signs and symptoms (%) referable to local (nasal) adverse events.

Vaccine group	Dose/follow-up period	Reported symptoms				Physical exam findings		
		Rhinorrhea	Nasal congestion	Postnasal drip	Sneezing	Nasal hyperemia	Nasal discharge	Inferior turbinate edema
Group A 10 μg	Pre-vaccine	0	0	0	0	0	13	25
	Dose 1 – 1st 24 h	13	25	25	0	0	25	50
	Dose 1 – days 2–7	13	0	0	13	0	29	43
	Dose 2 – 1st 24 h	14	14	14	0	29	57	71
	Dose 2 – days 2–7	14	14	14	14	0	29	43
	Dose 3 – 1st 24 h	0	17	0	0	0	33	33
	Dose 3 – days 2–7	0	0	0	0	0	33	17
	Any – post-vaccine	38	50	25	50	25	63	88
Group B 50 μg	Pre-vaccine	13	0	0	0	0	0	13
	Dose 1 – 1st 24 h	25	0	0	0	25	38	25
	Dose 1 – days 2–7	38	25	0	13	38	13	63
	Dose 2 – 1st 24 h	0	0	0	0	38	13	88
	Dose 2 – days 2–7	13	0	13	0	13	0	75
	Dose 3 – 1st 24 h	29	0	0	14	0	43	57
	Dose 3 – days 2–7	0	14	0	0	0	14	71
	Any – post-vaccine	50	50	13	25	63	100	100
Group C 240 μg	Pre-vaccine	13	13	13	13	13	63	75
	Dose 1 – 1st 24 h	38	25	38	25	0	38	88
	Dose 1 – days 2–7	25	25	13	25	0	43	88
	Dose 2 – 1st 24 h	0	14	14	0	0	100	88
	Dose 2–days 2–7	0	0	0	0	0	57	72
	Dose 3 – 1st 24 h	0	0	0	0	0	33	83
	Dose 3 – days 2–7	0	0	0	17	0	67	100
	Any – post-vaccine	63	50	75	50	0	100	88
Group D 480 μg	Pre-vaccine	25	13	13	0	38	63	63
	Dose 1 – 1st 24 h	50	38	38	38	0	88	63
	Dose 1 – days 2–7	25	25	0	25	38	63	75
	Dose 2 – 1st 24 h	13	38	38	13	50	88	75
	Dose 2 – days 2–7	13	13	0	13	38	88	75
	Dose 3 – 1st 24 h	38	25	13	13	25	63	100
	Dose 3 – days 2–7	50	50	25	25	38	38	88
	Any – post-vaccine	88	88	63	38	50	100	100

safety (GST), 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 year [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 (6% vs. 22%), and cough (13% vs. 13%). A few additional symptoms, not in the serial survey, were reported: excess salivation ($n=1$), throat burning ($n=1$), medicinal/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 hematology and serum chemistry results showed no significant changes from baseline among any of the study groups.

Table 4
Immune response summary following intranasal vaccination with *S. flexneri* 2a Invaplex 50.

Antigen	Assay	%Responders in study groups[a]			
		A 10 μg	B 50 μg	C 240 μg	D 480 μg
Invaplex 50	ASC[b] (IgA)	0 (n/a)	25 (20)	43 (31)	25 (23)
	ASC[b] (IgG)	0 (n/a)	25 (21)	100 (23)	50 (28.5)
	IgA (serum)[c]	0 (n/a)	13 (4)	14 (4)	0 (n/a)
	IgG (serum)[c]	0 (n/a)	0 (n/a)	14 (4)	0 (n/a)
	IgA (feces)[c]	0 (n/a)	13 (6)	29 (12)	38 (6)
	IgA (intestinal lavage)[c]	–	–	–	0 (n/a)
	IgA (nose swab)[c]	0 (n/a)	25 (4)	0 (n/a)	25 (4)
	IgA (nasal lavage)[c]	–	–	–	50 (6)
LPS	ASC[b] (IgA)	0 (n/a)	13 (39)	43 (26)	25 (20)
	ASC[b] (IgG)	0 (n/a)	13 (71)	71 (19)	38 (37)
	IgA (serum)[c]	0 (n/a)	13 (8)	0 (n/a)	0 (n/a)
	IgG (serum)[c]	0 (n/a)	0 (n/a)	29 (4)	0 (n/a)
	IgA (feces)[c]	0 (n/a)	13 (9)	29 (9)	50 (5.3)
	IgA (intestinal lavage)[c]	–	–	–	0 (n/a)
	IgA (nose swab)[c]	0 (n/a)	13 (7.5)	0 (n/a)	25 (4)
	IgA (nasal lavage)[c]	–	–	–	63 (8)
Invaplex 24	IgA (serum)[c]	0 (n/a)	38 (4)	29 (4)	0 (n/a)
	IgG (serum)[c]	14 (4)	13 (4)	14 (4)	13 (4)
Water extract (vir+)	IgA (serum)[c]	0 (n/a)	0 (n/a)	14 (4)	0 (n/a)
	IgG (serum)[c]	0 (n/a)	0 (n/a)	14 (4)	0 (n/a)
Water extract (vir−)	IgA (serum)[c]	0 (n/a)	0 (n/a)	0 (n/a)	0 (n/a)
	IgG (serum)[c]	0 (n/a)	0 (n/a)	29 (4)	0 (n/a)
IpaB	IgG (serum)[c]	0 (n/a)	0 (n/a)	0 (n/a)	0 (n/a)
IpaC	IgG (serum)[c]	14 (4)	0 (n/a)	29 (4)	25 (4)

n/a: not calculated.
[a] Response rates are denoted by the percentage of volunteers meeting the responder definition. There were 7 subjects in groups A and C and 8 subjects in groups B and D.
[b] ASC responses are summarized as % responders and the median maximum number of ASCs among responders in parentheses.
[c] For ELISA-based assays the number in parentheses is the median peak fold-rise from baseline titer among responders.

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 (25%) subjects with both Invaplex 50 and LPS-specific 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 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).

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].

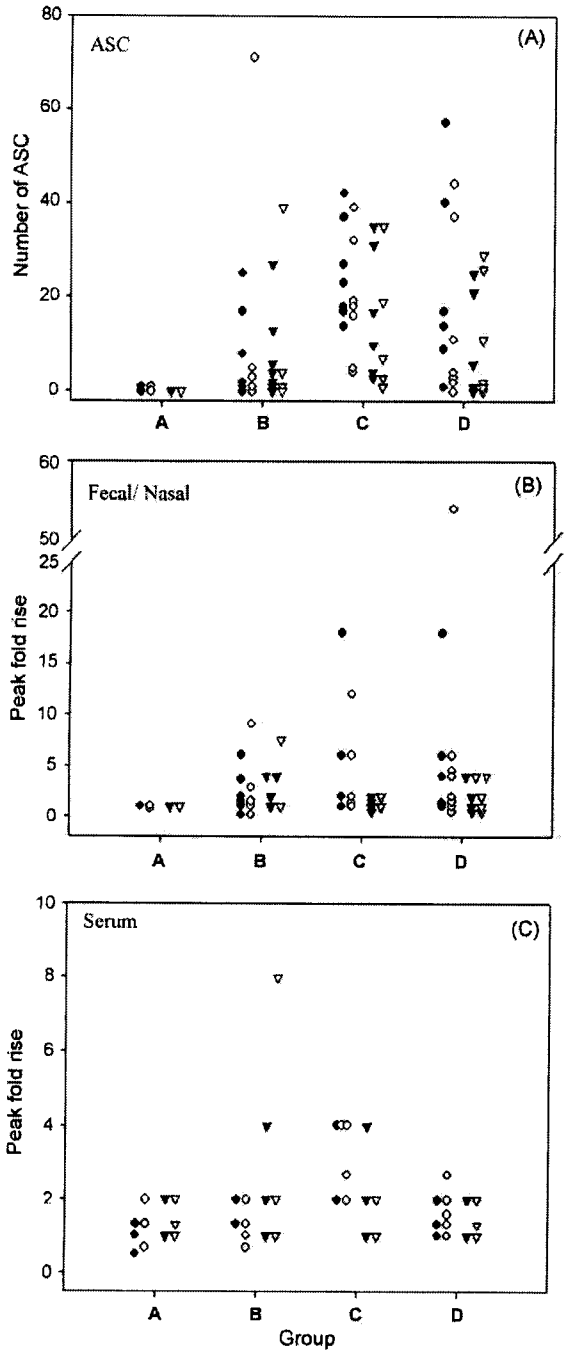

Fig. 2. Peak immune responses following intranasal vaccination with *S. flexneri* 2a Invaplex 50. Blood and mucosal washes (fecal and nasal) collected from individuals immunized with 10, 50, 240, or 480 μg of Invaplex 50 (A–D, on horizontal axis, respectively) were assayed for ASC responses (Panel A), 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 IgA: ○, nasal IgA: ▽). Panel C: Serologic responses to Invaplex 50 (IgG: ●, IgA: ▼) and LPS (IgG: ○, IgA: ▽).

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 adverse events, such as headache, malaise, 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 at Invaplex doses of ≥50 μg with dose–response most apparent with ASC 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 inductive 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

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, IpaB 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:IpaB 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* 2a, *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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