Mucosal Adjuvant Properties of the *Shigella* Invasin Complex

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The *Shigella* invasin complex (Invaplex) is an effective mucosal vaccine capable of protecting against *Shigella* challenge in animal models. The major antigenic constituents of Invaplex are the Ipa proteins and lipopolysaccharide. The cell-binding capacity of the Ipa proteins prompted the investigation into the adjuvanticity of Invaplex. Using ovalbumin (OVA) as a model antigen, intranasal immunization with OVA combined with Invaplex was found to enhance anti-OVA serum immunoglobulin G (IgG) and IgA responses and induce OVA-specific mucosal antibody responses at sites located both proximal and distal to the immunization site. The immune responses induced with OVA and Invaplex were comparable in both magnitude and duration to the immune responses induced after immunization with OVA and cholera toxin. The OVA-specific immune response was characterized by high levels of serum IgG1 and increased production of interleukin-4 (IL-4), IL-5, or IL-10 from lymphoid cells of immunized animals, suggesting a Th2 response. In addition to enhancing the immunogenicity of OVA, Invaplex-specific immune responses were also induced, indicating the potential for the development of a combination vaccine consisting of Invaplex and other immunogens. Preexisting Invaplex-specific immunity did not interfere with the capacity to enhance the immunogenicity of a second, unrelated vaccine antigen, suggesting that Invaplex could be used as a mucosal adjuvant in multiple vaccine regimens.

Mucosal delivery of subunit vaccines is an ideal way to induce effective local immunity (28). Unfortunately, after delivery of purified protein antigens, the mucosal, antigen-specific responses can be weak and short lived (1). Therefore, several immunity-enhancing strategies have been developed to overcome suboptimal mucosal immune responses to include delivery vehicles such as live bacterial vectors (39), microspheres and liposomes (20), and immunomodulatory substances or mucosal adjuvants (48), such as the heat-labile toxin (LT) of *Escherichia coli* and cholera toxin (CT) from *Vibrio cholerae* (5, 16). Increasingly, Toll-like receptor (TLR) agonists such as CpG DNA, microbial lipopeptides, or synthetic molecules like lipid A analogues and muramyl dipeptide analogues (19) are being recognized as potent mucosal adjuvants, especially in conjunction with the dendritic cell (DC) system (37). The mucosal delivery vehicles and adjuvants are largely designed to preserve antigenic structure and effectively deliver the antigen in an immunologically competent manner. Mechanistically, the activity of adjuvants is not readily defined, but the mucosal immunogenicity and adjuvant activity of LT and CT have been partially linked to the toxin B subunit's targeting of GM1-expressing cells and the ADP ribosylation activity of the A1 subunit (10). Other molecules with similar membrane binding capabilities, such as plant lectins (21), are also potent mucosal immunogens and enhance the immunogenicity of coadministered antigens.

Shigella pathogenesis and immunogenicity is largely a result of the pathogen's capacity to invade the colonic epithelium and stimulate a pronounced inflammatory and specific immune

response (18). The Ipa proteins, encoded by a 180- to 230-kDa virulence plasmid and the effectors of the *Shigella* type three secretion system, are essential for the invasive phenotype of *Shigella* spp., allowing the bacterium to invade host cells and escape endosomal vesicles (14). The Ipa protein's capacity to stimulate internalization by the host cell is also effective at enhancing the uptake of latex beads (27), noninvasive strains of *E. coli* (43), and plasmid-cured *Shigella flexneri* (26) by host cells. Recently, a macromolecular invasin complex, isolated from invasive shigellae and containing IpaB, IpaC, and lipopolysaccharide (LPS), has been used as an intranasal vaccine in small-animal models (33, 45). The invasin complex (Invaplex) vaccine was immunogenic and efficacious and did not require an adjuvant. The main antigenic constituents of the Invaplex are the invasins IpaB and IpaC and LPS.

The Ipa proteins' role in internalization and the immunomodulatory capacity of LPS may be crucial to the efficient and effective immunogenicity of Invaplex. Binding cell surface receptors and cellular uptake are properties also inherent to substances previously identified as potent mucosal adjuvants (36). It is conceivable that Invaplex, which is internalized by host cells in an Ipa protein- and actin-dependent process (R. W. Kaminski, K. R. Turbyfill, E. V. Oaks, Abstr. 104th General Meeting, Am. Soc. Microbiol. 2004, abstr. 1815, 2004) could also enhance the uptake and presentation of heterologous vaccine antigens to mucosal immune cells. Simultaneously, or in concert with the Ipa protein activity, the LPS component of Invaplex, a previously identified immunostimulator (29), may be capable of binding Toll-like receptor 4 (TLR-4) and stimulating cells of the innate immune system to release proinflammatory cytokines, which in turn influence adaptive immune responses.

Since relatively few mucosal adjuvants have been developed for human mucosal vaccines and the toxicity of both LT and CT have restricted their clinical use (23), the ability of Invaplex

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to function as a mucosal adjuvant when coadministered with protein antigens was evaluated. The immune response elicited to ovalbumin (OVA) was evaluated in mice intranasally immunized with OVA alone, OVA coadministered with Invaplex, or OVA combined with CT. Furthermore, as preexisting immunity may alter the effectiveness of mucosal adjuvants (3, 15), the adjuvanticity of Invaplex was also evaluated in mice with Invaplex-specific immunity. The presented studies indicate that Invaplex enhances the immune response to coadministered antigens, suggesting that Invaplex may be useful as both a *Shigella* vaccine and as a mucosal adjuvant.

MATERIALS AND METHODS

Purification of *Shigella* invasin complex. Invaplex was isolated as previously described (45) from water-extracted proteins of virulent *S. flexneri* 2a (2457T). In separate purification schemes, Invaplex was also isolated from *S. sonnei* (Mosley) using a protocol similar to that described for *S. flexneri* 2a (33). Invaplex from both *Shigella* spp. was prepared by applying the water extract to an anion-exchange column (HiTrap Q; Pharmacia) and collecting the peaks eluting at 0.24 M NaCl and 0.5 M NaCl (Invaplex 24 and Invaplex 50, respectively). Invaplex preparations were stored at -80°C . Invaplex 50 isolated from *S. sonnei* (Invaplex_{SS}) and *S. flexneri* 2a (Invaplex_{SF}) was used for this work. Invaplex 50 contains IpaB, IpaC, IpaD, and LPS, a truncated form of the 120-kDa protein VirG (VirG*), and 84-kDa and 72-kDa proteins (45). The *S. flexneri* 2a Invaplex 50 preparation contained 21 μg IpaB/mg total protein, 27 μg IpaC/mg total protein, and 100 μg LPS/mg total protein. The *S. sonnei* Invaplex 50 preparation contained 4 μg IpaB/mg total protein, 25 μg IpaC/mg total protein, and 324 μg LPS/mg total protein.

Animals and intranasal immunizations. Female BALB/cByJ mice (Jackson Laboratories, Bar Harbor, ME), age 6 to 8 weeks, were randomly separated into groups (five mice/group). Mice were intranasally immunized on days 0, 14, and 28 with chicken egg albumin (OVA) alone (5 $\mu\text{g}/\text{dose}$; grade II; Sigma, St. Louis, MO) or OVA (5 μg) plus Invaplex_{SS} (delivered as 1, 5, 10, or 50 μg Invaplex_{SS}/dose) to assess the ability of Invaplex_{SS} to enhance the OVA-specific immune responses. Control groups of animals were immunized with 0.9% saline (Abbott Laboratories, North Chicago, IL) or with OVA (5 μg) plus CT (5 $\mu\text{g}/\text{dose}$; Swiss Serum and Vaccine Institute, Auberg, Switzerland). A separate series of experiments assessed the adjuvanticity of Invaplex_{SF}, indicated in the table included in Fig. 3 as the OVA immunization series. A single amount was evaluated based on previous work evaluating the immunogenicity of Invaplex_{SF} (45). The animals used to assess the adjuvanticity of Invaplex_{SF} were also used to determine the impact of preexisting Invaplex_{SF}-specific immune responses on the adjuvanticity of Invaplex_{SF} by utilizing the protective antigen (PA) from *Bacillus anthracis* (5 $\mu\text{g}/\text{dose}$; List Biological Laboratories, Campbell, CA) as a second model antigen (see Fig. 3, PA immunization series). All vaccine formulations were delivered intranasally by placing 5 to 6 drops, totaling 25 μl , on the external nares of the animals. Prior to intranasal immunization, mice were anesthetized with a mixture of ketamine hydrochloride (40 mg/kg of body weight) (Fort Dodge Laboratories, Inc., Fort Dodge, IL) and xylazine (12 mg/kg) (Bayer Corp., Shawnee Mission, KS) administered intramuscularly. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals, and it adhered to principles stated in the Guide for the Care and Use of Laboratory Animals (NRC Publication, 1996 ed.).

Blood collection technique and schedule. Animals were bled by tail snip on days 0, 28, 35, and 42. Additional blood was collected on days 49, 98, and 112 to assess the impact of Invaplex_{SF}-specific immune responses on the adjuvanticity of Invaplex_{SF}. Blood was collected onto Whatman #1 filter paper (13), allowed to dry, and stored in sealed bags at 4°C until assayed. Blood samples were eluted from the filter paper with 2% casein in Tris-buffered saline.

Mucosal wash collections and processing. After the last immunization, on day 42 or 112, lung and intestinal lavages were collected from the animals as described previously (24). The collected lung and intestinal lavage fluid was spun at $800 \times g$ for 10 min at 4°C , and the supernatant was stored at -30°C . Lung and intestinal lavages were screened for occult blood contamination using a Hemocult assay (Beckman Coulter) per the manufacturer's directions.

Antigen-specific IgG and IgA. Antigen-specific antibody responses were assessed in the mucosal washes and serum samples by an enzyme-linked immunosorbent assay (ELISA) as previously described (45). Coating concentrations of the various antigens were the following: OVA, 1 $\mu\text{g}/\text{well}$; PA, 0.5 $\mu\text{g}/\text{well}$; and

Invaplex_{SF} and Invaplex_{SS}, 0.05 $\mu\text{g}/\text{well}$. Antigen-specific antibody was detected using alkaline phosphatase (AP)-conjugated anti-mouse immunoglobulin G (IgG) or anti-mouse IgA (Kirkegaard & Perry Laboratories, Gaithersburg, MD). AP activity was measured with *para*-nitrophenyl phosphate (1 mg/ml) at an optical density at 405 nm (OD₄₀₅) on a microtiter plate reader (Molecular Devices, Menlo Park, CA). The same methods were used to measure antigen-specific IgG1 and IgG2a titers using subclass-specific anti-mouse IgG to detect bound antibody (Pharmingen, San Diego, CA). ELISA data are expressed as either the mean OD₄₀₅ for each group ± 1 standard error of the mean (SEM) (serum IgG subclass) or as endpoint titers. Endpoint titers either were determined for individual animals as the reciprocal maximum dilution at which the mean OD₄₀₅ of duplicate wells was greater than twice the mean plus two standard deviations of values from preimmune sera or mucosal wash from naive animals or were set at 0.2, whichever was higher.

Total IgG and IgA concentrations in mucosal samples were determined by use of an ELISA (46), using purified mouse IgG (Chemicon, Temecula, CA) and IgA (Bethyl Laboratories, Montgomery, TX) as standards. Antigen-specific activities in mucosal washes were calculated by dividing the reciprocal endpoint titer for each individual mucosal sample by the total Ig concentration of the same isotype within that sample (2).

Collection and processing of murine cervical lymph nodes and spleens to assess antigen-specific proliferation. Cervical (superficial and central) lymph nodes (CLN) (44) were aseptically removed and placed in complete medium (cRPMI) composed of RPMI 1640 (Gibco, Carlsbad, CA) supplemented with 2 mM L-glutamine (Gibco), 100 U/ml penicillin G (Gibco), 100 $\mu\text{g}/\text{ml}$ streptomycin (Gibco), and 10% heat-inactivated fetal calf serum (Gibco). Single-cell suspensions of CLN were washed three times in cRPMI and suspended in cRPMI supplemented with 5 μM 2-mercaptoethanol (2-ME) (Sigma) at a final concentration of 1×10^6 cells/ml. Single-cell suspensions of individual spleens were treated with ammonium chloride to lyse the red blood cells, washed, counted, and suspended in cRPMI and 5 μM 2-ME to a final concentration of 1×10^6 cells/ml.

Antigen-specific proliferation assays. Splenocytes and cells from CLNs were evaluated for antigen-specific proliferation by culturing lymphoid cells (1×10^5 cells/well) in triplicate with either OVA (10 $\mu\text{g}/\text{ml}$) or Invaplex_{SS} (5 $\mu\text{g}/\text{ml}$). Additional cells were incubated with concanavalin A (5 $\mu\text{g}/\text{ml}$; ConA; Sigma) as a positive control. Negative controls for the assay included immune cells incubated with medium alone and cells from naive mice stimulated with antigen. Cells were incubated at 37°C (7% CO₂, 95% humidity) for 3 days (ConA) or 5 days (OVA and Invaplex). At the time of harvest, plates were centrifuged for 10 min at $250 \times g$ to pellet the cells, and 100 μl of the supernatant was transferred to a new plate and stored at -30°C until assayed for cytokine content (see below). Proliferation was assessed using a nonradioactive cell proliferation assay (MTS-PMS; Promega, Madison, WI) per the manufacturer's directions. Stimulation indices (SI) were calculated by dividing the mean OD₄₉₀ of antigen-stimulated cells by the mean OD₄₉₀ of cells from the same animal cultured with cRPMI but without antigen. Data are expressed as the mean SI for each group of mice ($n = 5$).

Analysis of secreted cytokines in supernatants of antigen-stimulated splenocytes. Th1-like (interleukin-2 [IL-2], gamma interferon [IFN- γ], and IL-12) and Th2-like (IL-4, IL-5, and IL-10) cytokines were measured in cell culture supernatants of antigen-stimulated cells using a multiplexed immunobead assay (BioSource, Camarillo, CA). Culture supernatants were incubated for 2 h at 25°C with a fluorochrome-labeled multiplexed bead set with specificity for IL-2, IL-4, IL-5, IL-10, IL-12, or IFN- γ . Each population was individually distinguishable by fluorophore and linked with monoclonal antibodies specific for one of the six different cytokines. Bound cytokines from the supernatants were detected with a cocktail of biotinylated detection monoclonal antibodies (BioSource) followed with streptavidin-phycoerythrin (BioSource). The fluorescent intensity of 100 beads from each bead population was collected and reported as the mean fluorescent intensity (MFI ± 1 SEM), corrected for the MFI of samples treated with culture media.

Statistical analysis. The Statview 5 computer program (Abacus Concepts, Berkeley, CA) was used for calculations and statistical comparisons. Comparisons between group arithmetic means were calculated using unpaired *t* tests. Comparisons between preimmunization and postimmunization antigen-specific immune responses in the same animal were calculated using paired *t* tests.

RESULTS

Antigen-specific serum IgG and IgA responses in mice after intranasal immunization. Groups of mice were intranasally

FIG. 1. Serum antibody responses in mice intranasally immunized with saline, OVA alone, OVA plus Invaplex_{SS}, or OVA plus CT. Groups of mice were intranasally immunized on days 0, 14, and 28 with saline (◇), OVA alone (○), OVA plus CT (△), or OVA plus Invaplex_{SS} (1 μg [◆], 5 μg [●], 10 μg [■], or 50 μg [▲]). Days of immunization are indicated with arrows. Mice were bled on days 0, 28, 35, and 42. The anti-OVA and anti-Invaplex serum IgG and IgA responses were determined by ELISA. Each point represents the geometric mean titer of each group of mice ($n = 5$).

immunized with OVA alone or with OVA and various amounts of Invaplex_{SS} to assess the ability of Invaplex to function as a mucosal adjuvant. Serum collected before immunization (day 0) from all groups of mice did not have detectable levels of OVA-specific serum IgG or IgA (Fig. 1). Intranasal immunization with saline did not induce OVA-specific immunity, and immunization with OVA alone induced low levels of anti-OVA IgG after three immunizations but failed to induce detectable OVA-specific IgA responses (Fig. 1) at any time point assayed. In contrast, intranasal immunization with OVA plus Invaplex_{SS} (1, 5, 10, or 50 μg) resulted in OVA-specific serum IgG responses that were 16- to 64-fold higher ($P \leq 0.02$) than those induced with OVA alone. The antibody response induced by immunization with OVA plus Invaplex_{SS} (5 μg) was of similar magnitude to that induced after immunization with OVA plus CT. In addition, immunization with OVA plus Invaplex_{SS} (1, 5, 10, or 50 μg) induced significantly higher ($P \leq 0.01$) anti-OVA serum IgA responses compared to immunization with OVA alone (Fig. 1). The augmentation of the OVA-specific IgG and IgA immune responses afforded by Invaplex_{SS} were not dose dependent within the range of 1 to 50 μg, suggesting that this dose range was sufficient for adjuvant activity in the mouse model.

Although the focus of the current project was to investigate the adjuvanticity of Invaplex, induction of *Shigella*-specific immunity is important when considering the potential for combination vaccines. Therefore, anti-Invaplex_{SS} serum antibody responses were also assessed to determine if *Shigella*-specific immune responses were also induced after immunization with OVA plus Invaplex_{SS}. Invaplex_{SS}-specific serum IgG and IgA responses were not detected in serum collected before immunization or in samples collected from animals immunized with saline, OVA alone, or OVA plus CT. Immunization with OVA plus Invaplex_{SS} elicited Invaplex-specific serum IgG responses after two immunizations (Fig. 1), with increasingly higher titers present 1 and 2 weeks after the final immunization. In contrast to the serum IgG and IgA responses elicited to OVA, the Invaplex_{SS}-specific serum IgG responses more closely followed a dose-dependent curve, with larger amounts of Invaplex_{SS} inducing higher Invaplex_{SS}-specific endpoint titers. Anti-Invaplex_{SS} serum IgA responses were also induced after immunization with Invaplex_{SS}, with the 5- and 10-μg doses of Invaplex_{SS} resulting in the highest level of Invaplex-specific immunity. The Invaplex_{SS}-specific serum IgG and IgA responses induced after immunization with OVA plus Invaplex_{SS}

FIG. 2. Antigen-specific serum IgG subclass responses in mice intranasally immunized with saline, OVA alone, OVA plus Invaplex_{SS}, or OVA plus CT. Sera collected 2 weeks after the third intranasal immunization (day 42) were assayed by ELISA for antigen-specific serum IgG subclass responses to OVA (top panels) and Invaplex_{SS} (bottom panels). Values represent the mean OD₄₀₅ $\pm$ 1 SEM at a 1:180 dilution for each group of mice ($n = 5$). Treatments for each group are indicated below the x axis. IVP, Invaplex_{SS}.

(5 μ g) were comparable in magnitude to those elicited after immunization with Invaplex_{SS} alone (data not shown).

Antigen-specific serum IgG subclass responses. Murine serum IgG subclass responses have been used to assess the type of immune response elicited with immunization, with IgG1 indicative of a Th2-type response, and with IgG2a indicative of a Th1-type response (42). Intranasal immunization with OVA plus Invaplex_{SS} (1, 5, 10, or 50 μ g) elicited a predominant anti-OVA IgG1 response (Fig. 2, top panels). The OVA-specific immune response induced with OVA plus Invaplex_{SS} (1, 5, or 10 μ g) was similar to the immune response in mice immunized with OVA plus CT, with high levels of anti-OVA IgG1 and minimal amounts of IgG2a, indicating an antigen-specific Th2-type response. Interestingly, whereas immunization with OVA plus Invaplex_{SS} (50 μ g) elicited a predominant anti-OVA IgG1 response, a detectable IgG2a response was also measured that was significantly higher ($P \leq 0.002$) than that of all other groups assayed, indicating that larger amounts of Invaplex_{SS} may be capable of inducing an immune response with an antigen-specific Th1 component. Further experimentation is required to address that possibility.

The Invaplex_{SS}-specific serum IgG subclass responses were also determined in each of the study groups (Fig. 2, bottom panels). The anti-Invaplex serum IgG subclass responses indicate that immunization with OVA plus Invaplex_{SS} results in predominantly IgG1, indicative of a Th2 response. Immunization with Invaplex_{SS} alone also induces a predominant anti-Invaplex serum IgG1 response (data not shown). Mice immunized with OVA plus Invaplex_{SS} (50 μ g) had increased levels of anti-Invaplex_{SS} serum IgG2a, suggesting that higher doses of Invaplex may elicit a Th1 component in addition to the predominant Invaplex-specific Th2 response.

Antigen-specific IgG and IgA antibody responses in mucosal washes. Lung and intestinal washes were collected from each animal 2 weeks after the third immunization (day 42) and assayed for OVA-specific IgG and IgA by ELISA to determine the adjuvant effect of Invaplex_{SS} on the antigen-specific mucosal immune responses (Table 1). The mean total IgG and IgA concentrations in the mucosal washes were the following (in mean micrograms per milliliter $\pm$ 1 standard deviation): intestinal IgG, 0.25 ± 0.30 ; intestinal IgA, 18.0 ± 8.7 ; lung IgG, 1.2 ± 1.0 ; and lung IgA, 0.69 ± 0.49 . Intranasal administration

TABLE 1. Specific activities of anti-OVA and anti-Invaplex_{SS} IgG and IgA in mucosal washes from mice immunized intranasally with OVA alone or in combination with CT or Invaplex_{SS}

Vaccine group	OVA-specific mucosal responses in:				Invaplex-specific mucosal responses in:			
	Intestine		Lung		Intestine		Lung	
	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA
Saline	36 ± 11	0.18 ± 0.02	11 ± 5	6 ± 6	12 ± 6	0.37 ± 0.05	22 ± 10	10 ± 13
OVA	10 ± 6	0.17 ± 0.06	10 ± 4	2 ± 0	10 ± 6	0.34 ± 0.12	20 ± 9	4 ± 0.3
OVA + CT	3,969 ± 1,633 ^a	0.83 ± 0.18 ^b	628 ± 296 ^b	317 ± 104 ^b	7 ± 2	0.21 ± 0.05	2 ± 0.2	4 ± 2
OVA + Invaplex _{SS} (1 µg)	358 ± 179 ^b	0.35 ± 0.28	1,456 ± 948 ^b	761 ± 289 ^b	60 ± 32 ^c	11 ± 9 ^c	302 ± 323 ^c	241 ± 217 ^c
OVA + Invaplex _{SS} (5 µg)	151 ± 32 ^b	0.41 ± 0.46	407 ± 252 ^b	554 ± 373 ^b	46 ± 35	10 ± 4 ^c	156 ± 107 ^c	121 ± 53 ^c
OVA + Invaplex _{SS} (10 µg)	342 ± 142 ^b	0.47 ± 0.21 ^b	814 ± 751 ^b	265 ± 117 ^b	106 ± 63 ^c	21 ± 15 ^c	257 ± 174 ^c	104 ± 70 ^c
OVA + Invaplex _{SS} (50 µg)	350 ± 225 ^b	0.44 ± 0.38	421 ± 240 ^b	301 ± 225 ^b	42 ± 44	8 ± 5 ^c	100 ± 50 ^c	55 ± 11 ^c

^a Mucosal washes were collected 2 weeks after the third intranasal immunization. Specific activities were calculated by dividing the specific OVA or Invaplex endpoint titer by the total IgG or IgA concentration (in micrograms/milliliter) within each mucosal wash (2).

^b $P < 0.05$ compared to groups of mice immunized intranasally with OVA alone.

^c $P < 0.05$ compared to groups of mice immunized intranasally with saline alone.

of saline or OVA alone did not induce a detectable anti-OVA mucosal IgG or IgA response in either mucosal wash assayed (Table 1). As expected, immunization with OVA plus CT induced lung anti-OVA IgG and IgA responses. Intranasal immunization with OVA plus Invaplex_{SS} induced significantly higher anti-OVA IgG and IgA in lung washes compared to immunization with OVA alone ($P \leq 0.04$) or with saline ($P \leq 0.03$) and comparable anti-OVA responses to those responses induced with OVA plus CT. The anti-OVA IgG and IgA in lung washes from OVA plus Invaplex_{SS}-immunized mice were induced in groups of mice immunized with OVA and increasing amounts of Invaplex_{SS}, with the smallest amount of Invaplex_{SS} (1 µg) inducing the highest level of anti-OVA lung IgG and IgA.

Stimulation of the common mucosal immune system (CMIS) and trafficking of immune cells between inductive sites and effector sites on mucosal surfaces makes it possible for a mucosal immune response to occur at sites distal to the immunization site (28). Stimulation of the CMIS was assessed in intestinal lavage fluid collected from intranasally immunized animals by assaying for OVA-specific IgG and IgA by ELISA (Table 1). Immunization with OVA plus Invaplex_{SS} (10 µg) or OVA plus CT induced anti-OVA IgA in intestinal washes ($P < 0.01$). Immunization with OVA plus 1, 5, or 50 µg of Invaplex_{SS} also induced anti-OVA IgA responses in the intestine of at least 60% of the animals in each group that were higher than the anti-OVA IgA responses in animals immunized with OVA alone. Anti-OVA intestinal IgG responses were also detected after immunization with OVA plus 1, 5, 10, or 50 µg of Invaplex_{SS}.

The Invaplex-specific mucosal antibody responses were also measured in the lung and intestinal washes (Table 1). Mice immunized with saline, OVA alone, or OVA plus CT did not mount a detectable Invaplex-specific IgG or IgA response in the lung or intestinal compartment. Anti-Invaplex IgG and IgA were present in lung secretions of mice immunized with OVA plus Invaplex_{SS} (1, 5, 10, or 50 µg). Invaplex-specific IgG and IgA responses were also detected in intestinal washes collected from animals immunized with OVA plus Invaplex_{SS}, indicating that intranasal immunization with Invaplex_{SS} stimulates the CMIS. In both the lung and intestinal washes there was an

opposite trend in the Invaplex-specific immune responses compared with responses in serum, in that increases in the amount of Invaplex used for immunization resulted in a decrease in the Invaplex-specific mucosal response (Table 1).

Antigen-specific proliferation after in vitro stimulation of splenocytes and CLN cells of immunized animals. The cell-mediated immune response elicited after intranasal immunization was measured in cells collected from cervical lymph nodes (CLN) and spleens 2 weeks after the third immunization. After in vitro stimulation with OVA, splenocytes harvested from animals immunized with OVA plus Invaplex_{SS} (5, 10, or 50 µg) or OVA plus CT proliferated to higher levels than splenocytes from mice immunized with OVA alone or with saline (Table 2). Splenocytes from animals immunized with OVA plus Invaplex_{SS} (1 µg) did not proliferate when cultured with OVA. Splenocytes from all groups of immunized animals showed weak or minimal response to stimulation with Invaplex_{SS} (Table 2), except for mice immunized with OVA plus Invaplex_{SS} (50 µg), which had an SI value of 2.39.

Proliferation of CLN cells from immunized animals was also determined after in vitro stimulation with OVA (Table 2). OVA-specific proliferative responses were not detectable in CLN cells from animals immunized with saline, OVA alone, or OVA plus Invaplex_{SS} (1 µg). However, after stimulation with OVA, CLN cells from animals immunized with OVA plus CT or OVA plus Invaplex_{SS} (5, 10, or 50 µg) proliferated to high levels, indicating an OVA-specific cell-mediated immune response. In contrast to the minimal Invaplex-specific proliferative responses measured in splenocytes, high levels of Invaplex-specific proliferation were detected in the CLN cells collected from animals immunized with OVA plus Invaplex_{SS} (5, 10, or 50 µg). Cells from animals immunized with saline, OVA alone, or OVA plus CT did not respond to stimulation with Invaplex, indicating that Invaplex_{SS} did not induce nonspecific cellular proliferation in CLN cells from naïve animals.

Secreted cytokine levels in supernatants of immune mouse lymphoid cells stimulated in vitro with OVA. The immune response elicited after immunization was further characterized by assessing the cytokines secreted during the proliferation of lymphoid cells after in vitro antigenic stimulation. Culture supernatants from OVA-stimulated spleen cells collected from

TABLE 2. Lymphocyte proliferation in spleen and CLN lymphocytes from mice immunized intranasally with saline, OVA alone, OVA plus Invaplex_{SS}, or OVA plus CT^a

Vaccine formulation	Mean stimulation index after in vitro stimulation of cells isolated from:			
	Spleen		CLN	
	OVA stimulation	Invaplex _{SS} stimulation	OVA stimulation	Invaplex _{SS} stimulation
Saline	1.17 ± 0.39	1.01 ± 0.25	0.85 ± 0.28	0.96 ± 0.11
OVA	0.99 ± 0.19	1.02 ± 0.46	0.98 ± 0.03	1.13 ± 0.32
OVA + CT	2.90 ± 0.49 ^b	1.22 ± 0.21	8.43 ± 6.28 ^b	1.23 ± 0.34
OVA + Invaplex _{SS} (1 µg)	1.16 ± 0.21	1.25 ± 0.21	0.92 ± 0.27	1.13 ± 0.15
OVA + Invaplex _{SS} (5 µg)	5.23 ± 1.21 ^b	1.18 ± 0.26	10.97 ± 7.22 ^b	14.77 ± 10.92 ^c
OVA + Invaplex _{SS} (10 µg)	5.13 ± 0.96 ^b	1.40 ± 0.15	11.91 ± 5.08 ^b	9.04 ± 3.65 ^c
OVA + Invaplex _{SS} (50 µg)	4.76 ± 1.41 ^b	2.39 ± 0.89 ^c	6.69 ± 3.27 ^b	14.66 ± 8.47 ^c

^a Splenocytes or CLN cells of immunized mice ($n = 5$ mice/group) were collected 2 weeks after the third intranasal immunization, and proliferative responses were assessed after stimulation with OVA or Invaplex_{SS}. Data represent the mean stimulation index ± 1 standard deviation. Splenocytes and CLN cells stimulated with ConA resulted in high levels of proliferation (mean SI ± 1 SD = 18.34 ± 5.17) comparable among the different vaccine groups, demonstrating that viable lymphocytes were present (data not shown).

^b $P < 0.05$ compared to groups of mice immunized intranasally with OVA alone.

^c $P < 0.05$ compared to groups of mice immunized intranasally with saline.

immunized mice were assessed for IFN- γ , IL-2, IL-12, IL-4, IL-5, and IL-10 using a multiplexed immunobead assay (Table 3). Splenocytes from mice immunized with OVA plus CT and stimulated in vitro with OVA produced high levels of IL-4, IL-5, and IL-10, indicative of cytokine production that promotes a Th2-like immune response, in agreement with previously published findings (4). Elevated levels of IL-4 or IL-5 were also detected in the supernatants of splenocytes stimulated with OVA from animals immunized with OVA plus Invaplex_{SS} (1, 5, or 10 µg), supportive of a Th2-like response. The same cell populations, from animals immunized with OVA plus CT or OVA plus Invaplex_{SS}, also secreted high levels of the Th1 cytokine, IFN- γ , with the absence of appreciable amounts of IL-2 and IL-12. Minimal IFN- γ production was detected in supernatants from cells of animals immunized with saline or OVA alone.

Invaplex from *S. flexneri* 2a also functions as a mucosal adjuvant. The adjuvanticity of Invaplex isolated from *S. flexneri* 2a (Invaplex_{SF}) was also investigated in a separate series of experiments. Immunization with OVA plus Invaplex_{SF} resulted in higher levels of anti-OVA serum IgG and IgA compared to immunization with OVA alone (Fig. 3, top panels), demonstrating the adjuvanticity of Invaplex_{SF}. The anti-OVA serum IgG and IgA enhancement afforded by Invaplex_{SF} was comparable to the immune responses enhanced by Invaplex_{SS}

described in Fig. 1 and were of a magnitude similar to those induced with OVA plus CT.

Preexisting Invaplex-specific immunity does not abrogate the adjuvanticity of Invaplex. Reports have demonstrated that anti-adjuvant immunity may have an inhibitory effect on antigen-specific antibody responses when immunogenic adjuvants are used in a vaccine formulation at certain concentrations or after repeated immunizations (3, 15). An extension of the Invaplex_{SF} adjuvanticity study described above was designed to assess the impact of preexisting, Invaplex-specific immunity on the adjuvanticity of Invaplex_{SF}. Mice previously immunized with OVA plus Invaplex_{SF} were rested for 5 weeks and immunized with a three-dose regimen of PA plus Invaplex_{SF}. After immunization with PA plus Invaplex_{SF}, anti-PA serum IgG and IgA responses were induced at higher levels than those after immunization with PA alone and were of magnitudes comparable to those of the PA-specific responses induced after immunization with PA plus CT (Fig. 3, middle panels), regardless of prior Invaplex-specific immune responses (Fig. 3, lower two panels). In addition to OVA-specific and PA-specific serum responses, anti-Invaplex_{SF} serum responses were also assessed. The anti-Invaplex_{SF} serum IgG and IgA responses induced after immunization with OVA plus Invaplex_{SF} (5 µg) were comparable in both magnitude and duration (Fig. 3, lower panels) to the responses induced with OVA plus Invaplex_{SS}

TABLE 3. Cytokine levels in supernatants of splenocytes isolated from intranasally immunized animals and stimulated in vitro with OVA

Vaccine formulation	Mean fluorescent intensity (mean ± SEM) ^a for:					
	Th1 cytokines			Th2 cytokines		
	IFN- γ	IL-2	IL-12	IL-4	IL-5	IL-10
Saline	498 ± 204	38 ± 11	55 ± 23	47 ± 19	1,159 ± 480	10 ± 4
OVA	491 ± 201	35 ± 14	60 ± 25	91 ± 5	1,232 ± 531	23 ± 2
CT-OVA	6,965 ± 625 ^b	60 ± 2	58 ± 4	416 ± 121 ^b	7,716 ± 484 ^b	105 ± 19 ^b
OVA + Invaplex _{SS} (1 µg)	6,957 ± 446 ^b	55 ± 3	78 ± 9	240 ± 83 ^b	2,938 ± 478 ^b	68 ± 10 ^b
OVA + Invaplex _{SS} (5 µg)	7,975 ± 94 ^b	53 ± 3	81 ± 7	129 ± 19	4,116 ± 229 ^b	36 ± 6
OVA + Invaplex _{SS} (10 µg)	7,352 ± 557 ^b	52 ± 2	92 ± 8	150 ± 15 ^b	1,611 ± 383	25 ± 3
OVA + Invaplex _{SS} (50 µg)	5,701 ± 501 ^b	45 ± 4	79 ± 8	144 ± 29	778 ± 354	17 ± 3

^a Cytokine levels were measured with a multiplexed bead assay using supernatants from splenocytes stimulated in vitro with OVA for 5 days. The cells were collected from individual mice ($n = 5$ mice/group) 2 weeks after the third intranasal immunization.

^b $P < 0.05$ compared to supernatants from splenocytes of mice immunized intranasally with OVA alone.

Grp #	OVA Immunization Series (day 0, 14, 28)	PA Immunization Series (day 49, 63, 77)	OVA + PA Immunization (day 91)	Group Symbol
1	Invaplex _{SF}	PA + Invaplex _{SF}	OVA + PA + Invaplex _{SF}	—●—
2	OVA + Invaplex _{SF}	PA + Invaplex _{SF}	OVA + PA + Invaplex _{SF}	- - ● - -
3	OVA + CT	PA + CT	OVA + PA + CT	- - ▲ - -
4	OVA alone	PA alone	OVA + PA	- - ■ - -
5	saline	saline	saline	- - ◆ - -

FIG. 3. Adjuvanticity and immunogenicity of Invaplex_{SF} when intranasally administered to mice: anti-OVA, anti-PA, and anti-Invaplex serum IgG and IgA responses. Groups of mice were intranasally immunized as outlined in the table at the bottom of the figure. The OVA and PA immunization series are identified on the horizontal axis. Blood was collected on days 0, 28, 42, 49, 91, and 112 from immunized groups of animals and assayed by ELISA for anti-OVA (top panels), anti-PA (middle panels), and anti-Invaplex (bottom panels) serum IgG and IgA responses. Mean ($n = 5$ mice/group) endpoint titers were determined as the reciprocal of the last dilution of sample with an OD₄₀₅ of ≥ 0.2 after a 30-min (IgG) or overnight (IgA) incubation with substrate.

FIG. 4. Enhanced OVA- and PA-specific mucosal IgA responses after intranasal immunization of mice using Invaplex_{SF} as an adjuvant. Two weeks after the final immunization, intestinal and lung washes were collected and assayed for anti-OVA (top two panels), anti-PA (middle two panels), and anti-Invaplex (bottom two panels) IgA responses by ELISA. Values represent the mean endpoint titers $\pm$ 1 standard deviation for each group of mice ($n = 5$ mice/group). Treatments for each group are indicated in the legend to Fig. 3. Endpoint titers significantly higher ($P < 0.05$) than those of OVA-plus-PA-immunized or saline-immunized groups are indicated with an asterisk.

(Fig. 1). Additional immunizations with Invaplex_{SF}-containing vaccine induced increasingly higher Invaplex_{SF}-specific serum IgG and IgA responses (Fig. 3, lower panel, day 49 and day 98).

Induction of OVA-specific and PA-specific mucosal IgA responses after immunization of mice with Invaplex_{SF} as an adjuvant. In addition to measuring antigen-specific antibodies in systemic circulation, the induction of antigen-specific mucosal immunity was also assessed in the intestine and lung after immunization with Invaplex formulated with OVA or PA. Mucosal lavages were collected 2 weeks after the final immunization (day 112) and assayed for OVA-specific, PA-specific, and Invaplex_{SF}-specific IgG and IgA responses by ELISA (Fig. 4). Mice intranasally immunized three times with OVA alone followed by three immunizations with PA alone (group 4) or mice immunized with saline (group 5) did not have detectable levels of anti-OVA, anti-PA, or anti-Invaplex IgG (data not shown) or IgA in the lung or intestine (Fig. 4). In contrast, immuni-

zation with OVA plus Invaplex_{SF} followed by immunization with PA plus Invaplex_{SF} (group 2) induced anti-PA, anti-OVA, and anti-Invaplex IgA (Fig. 4) and IgG (data not shown) in the lung. The anti-OVA and anti-PA lung IgA responses induced after immunization with OVA plus Invaplex_{SF} and PA plus Invaplex_{SF} were comparable to those induced after immunization with OVA plus CT followed by PA plus CT ($P = 0.07$). Preexisting Invaplex_{SF}-specific immunity did not abolish the adjuvanticity of Invaplex, since mucosal immune responses in the lung, directed to a second immunogen (PA), were significantly higher after immunization with PA plus Invaplex_{SF} (group 1 and 2) than the PA-specific response induced after immunization with PA alone (group 4).

Anti-OVA intestinal IgG (data not shown) and IgA responses (Fig. 4) after immunization with OVA plus Invaplex_{SF} (group 2) were comparable to the OVA-specific responses induced after immunization with OVA plus CT. However,

immunization with PA plus Invaplex_{SF} or PA plus CT did not elicit detectable PA-specific intestinal IgA. Immunization of mice with Invaplex_{SF}-containing vaccines also induced Invaplex-specific IgA responses in the intestine.

DISCUSSION

Mucosal immunity is an effective mechanism for neutralization of potential pathogens of the respiratory, gastrointestinal, and genital tracts. Vaccines that stimulate protective mucosal immune responses often need an adjuvant for proper delivery and presentation to the mucosal immune tissue such as the nasal mucosa-associated lymphoid tissue, gut-associated lymphoid tissue, and bronchus-associated lymphoid tissue. Successful mucosal adjuvants often stimulate a Th2 T-cell response, characterized by increased secretory IgA, high proportions of antigen-specific serum IgG1, and the stimulation and synthesis of IL-4, IL-5, and IL-10. One class of highly effective and well-characterized mucosal adjuvants is the enterotoxin proteins CT and LT (5, 16). The pronounced enhancement of the mucosal immune response to antigens code-livered with CT or LT sets a standard by which many new mucosal adjuvants are measured (36). The research described in this report has evaluated the *Shigella* invasin complex as a possible mucosal adjuvant that exploits the inherent uptake-stimulatory properties of IpaB and IpaC and the immunomodulatory capacity of LPS.

The potential use of *Shigella* Invaplex as a mucosal adjuvant was assessed in the current study by evaluating its adjuvant activity with two different antigens, OVA and PA. Invaplex 50, isolated from either *S. flexneri* 2a or *S. sonnei* and admixed with protein antigens and delivered by the intranasal route, promoted enhanced antigen-specific mucosal and systemic immunity while also eliciting Invaplex-specific immune responses. Invaplex 24, isolated from *S. flexneri* 2a, also functions as a mucosal adjuvant with immune enhancement comparable to that achieved with *S. flexneri* 2a Invaplex 50 (data not shown). Invaplex 24, isolated from *S. sonnei*, is deficient in IpaB and LPS and is not protective in animal challenge models (33); therefore, it has not been used for adjuvant activity studies. Increased amounts of Invaplex used for immunization increased the IgG2a or Th1 component of the immune response and may have resulted in a reduction in the predominant antigen-specific Th2 response, which is primarily responsible for mucosal antibody production. Further experimentation is required to address the possibility.

Antigen-specific IgG was detected in lung and intestinal lavages of immunized mice. IgG transport across intestinal epithelial barriers has been demonstrated via an IgG binding receptor, FcRn (11). In addition to active transport mechanisms, monomeric serum IgG can also gain access to mucosal surfaces by passive diffusion or transudation, which is thought to be the mechanism by which IgG gains access to the pulmonary mucosa (31). Both mechanisms rely on the generation of high levels of antigen-specific immune responses in the serum, a capacity demonstrated with Invaplex formulation. Several studies have advanced the concept that mucosal IgA is primarily involved in protection against infection in the upper respiratory tract, whereas serum IgG antibodies are predominantly involved in protection in the lower respiratory tract (8, 30).

Invaplex-mediated induction of antigen-specific IgA in the lung may confer protection against microbial infection in the upper respiratory tract and antigen-specific serum IgG that may be important in providing protection in the lower respiratory tract.

The T helper cell response plays a substantial role in the ensuing immune response and may differentiate between protective and nonprotective immunity, with Th1-like responses generally required to combat intracellular pathogens and Th2-like responses for extracellular pathogens and mucosal pathogens (32). Previous studies have shown that CT enhances antigen-specific responses by inducing CD4⁺ T cells secreting IL-4, IL-5, IL-6, and IL-10, correlating directly with serum IgG1 subclass responses and mucosal IgA responses typical of Th2 responses (25). Immunization with OVA plus Invaplex resulted in a predominant Th2-like response, with high levels of OVA-specific serum IgG1 and mucosal IgA and secretion of IL-4, IL-5, or IL-10 after in vitro antigenic stimulation of splenocytes from immunized animals. Interestingly, spleen cells from mice immunized with OVA combined with either CT or Invaplex also released high levels of IFN- γ in the presence of a predominant OVA-specific IgG1 response. The apparent inconsistency between IgG subclass response and cytokine profile may be due to immune cells other than T helper cells present in the bulk splenocyte cultures. It is likely that antigen-presenting cells (APCs), such as macrophages or dendritic cells (DCs), specifically CD8 α ⁺ DCs, may contribute to the high levels of IFN- γ (35) in culture supernatants. The lack of IFN- γ in supernatants of mice immunized with saline or OVA alone may reflect the lack of mature DCs present in the splenocyte cultures, which may be present after immunization with OVA combined with CT or Invaplex. Future studies utilizing purified T-cell subsets and donor APCs will be used to further investigate the nature of the T helper response.

In addition to the predominant Th2-like response after immunization, data suggest that increases in the amount of Invaplex used for immunization may contribute to a mixed Th1-Th2 response. For example, the OVA-specific and Invaplex-specific Th2-like response was reduced when the amount of Invaplex used for immunization was increased to 50 μ g, with a more balanced level of antigen-specific serum IgG1 and IgG2a and a decrease in antigen-specific mucosal IgA. The increase in the Th1 component of the Invaplex-specific and OVA-specific immune response after immunization with Invaplex (50 μ g) compared to lower amounts of Invaplex (1, 5, or 10 μ g) may have been partially due to increases in the amount of LPS in the Invaplex preparation used for immunization. It is estimated, based on 2-keto-3-deoxyoctulosonic acid analysis, that 50 μ g of Invaplex contains approximately 15 μ g of *Shigella* LPS, whereas 1 μ g of Invaplex contains only 0.3 μ g of LPS (E. V. Oaks and K. R. Turbyfill, unpublished results). It has been reported that increases in the LPS dose (from 0.01 μ g to 100 μ g) delivered intranasally resulted in a shift from antigen-specific Th2 responses to Th1 responses (12). The amount of Invaplex-associated LPS used during intranasal inoculation may therefore play a role in the nature of the antigen-specific immune response. Additionally, the genetic predisposition of a BALB/c mouse strain to develop predominantly Th2-based immune responses may have also influenced the effect of Invaplex on the type of T helper antigen-specific responses in-

duced after vaccination (17). The more balanced Th1-Th2 OVA-specific immune response induced with larger amounts of Invaplex is currently under further investigation utilizing additional mouse strains.

Immunization with increased amounts of Invaplex in combination with OVA resulted in increased Invaplex-specific serum IgG responses. One concern of immunogenic adjuvants or carriers is that the adjuvant-directed immune response may reduce or nullify the adjuvant action of adjuvants and carriers after repeated exposures or at highly immunogenic doses (3, 15). In the current study, increases in the number of vaccinations or in the amount of Invaplex used for immunization did not significantly decrease the adjuvant effect of Invaplex, over a range of 1 to 50 μ g or up to seven intranasal immunizations with Invaplex-containing vaccines. Mice intranasally immunized with OVA plus Invaplex could be immunized after a relatively short rest period, with PA plus Invaplex resulting in substantial increases in the PA-specific immune response. The immune responses elicited after PA plus Invaplex were of a magnitude comparable to the those of responses elicited with PA plus CT (Fig. 3) and responses elicited after immunization with PA plus Invaplex in the absence of Invaplex priming (data not shown), suggesting retention of potent adjuvant activity despite Invaplex-specific immunity. This point is crucial when considering which adjuvant or delivery mechanism will be employed to deliver a poorly immunogenic antigen, since prior vaccination or natural infection with a bacterial (47) or viral vector (40) used to deliver heterologous antigens may negatively impact antigen expression and the immunogenicity of the vaccine construct. In addition, the immune responses elicited after immunization with OVA plus Invaplex or PA plus Invaplex were directed to both the heterologous protein antigen and Invaplex. The Invaplex-specific response after immunization with OVA plus Invaplex was comparable to the antibody responses elicited with Invaplex alone. Immunization with a similar amount (5 μ g) of Invaplex alone has been shown previously to be protective in a pulmonary mouse model of *Shigella* infection (45), suggesting that *Shigella*-specific protective immunity may be induced when Invaplex is administered with a heterologous antigen.

The exact mechanism(s) underlying the effectiveness of Invaplex to promote the induction of secretory antibodies as well as enhanced systemic immunity to coadministered protein antigens is not completely understood. It is likely that Invaplex employs several modes of adjuvant action due to the diversity of functions established for the individual components contained within the complex. Two of the key proteins within Invaplex that may contribute to its adjuvant activity are IpaB and IpaC. These proteins possess the ability to interact with host cell receptors, such as CD44 (41) and $\alpha 5\beta 1$ integrins (49), which may facilitate the entry and transcytosis of Invaplex and coadministered vaccine antigens across mucosal epithelial barriers. The Ipa proteins also interact with and can disrupt cellular membranes (9), which may also facilitate presentation of vaccine antigens through intracellular release mechanisms. Recently, we have demonstrated that Invaplex is internalized into epithelial and fibroblast cells and induces the uptake of heterologous proteins and plasmid DNA admixed with Invaplex (R. W. Kaminski, K. R. Turbyfill, and E. V. Oaks, Abstr. 104th Gen. Meet. Am. Soc. Microbiol., abstr. 3711, 2004). Invaplex-

induced uptake of heterologous molecules is dependent on the Ipa proteins, proteins that are also intimately involved in uptake of wild-type *Shigella* (14). Invaplex-mediated uptake of vaccine antigens and subsequent transport across mucosal epithelial barriers may be one mechanism by which Invaplex enhances antigen presentation to cells of the immune system.

In addition to the Ipa proteins, LPS is also a component of Invaplex and likely contributes to the adjuvant activity of the complex. LPS is a major stimulant of the innate immune system through interactions with TLR-4, and it often exhibits potent adjuvant properties (29, 34). Previously established immunological mechanisms involved with adjuvant function of LPS include the production of proinflammatory or immunoregulatory cytokines (7), the stimulation of antigen-presenting cells (APCs) to upregulate the expression of costimulatory molecules (6), and the control of dendritic cell migration (38). By signaling through TLR-4, LPS contained within Invaplex may stimulate a cascade of events culminating in the release of proinflammatory cytokines and the increased expression of costimulatory molecules on APCs, which directly augment the migration of antigen-stimulated immune effector cells and the antigen presentation of vaccine antigens. APC populations also express higher levels of major histocompatibility complex and cell surface costimulatory molecules following LPS stimulation, resulting in increased efficiency of interactions with T and B lymphocytes (22).

The immunogenicity and adjuvant activity of Invaplex is likely due to contributions from both the Ipa protein and LPS components of the invasin complex. Research is currently under way to better understand the mechanisms by which Invaplex functions as both a vaccine and as a mucosal adjuvant. *Shigella* Invaplex is a potent immunogen when administered intranasally, is safe and protective in small-animal *Shigella* infection models (45), and is currently being evaluated in phase 1 clinical trials. Future work will directly investigate the protective capacity of Invaplex-adjuvanted vaccines against *Shigella* infection to develop combination vaccines capable of protecting against *Shigella* and other pathogenic microbes.

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