



Assembly, Biochemical Characterization, Immunogenicity, Adjuvanticity, and Efficacy of *Shigella* Artificial Invaplex

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ABSTRACT The native Invaplex (Invaplex_{NAT}) vaccine and adjuvant is an ion exchange-purified product derived from the water extract of virulent *Shigella* species. The key component of Invaplex_{NAT} is a high-molecular-mass complex (HMMC) consisting of the *Shigella* lipopolysaccharide (LPS) and the invasin proteins IpaB and IpaC. To improve product purity and immunogenicity, artificial Invaplex (Invaplex_{AR}) was developed using recombinant IpaB and IpaC proteins and purified *Shigella* LPS to assemble an HMMC consisting of all three components. Characterization of Invaplex_{AR} by various methods demonstrated similar characteristics as the previously reported HMMC in Invaplex_{NAT}. The well-defined Invaplex_{AR} vaccine consistently contained greater quantities of IpaB, IpaC, and LPS than Invaplex_{NAT}. Invaplex_{AR} and Invaplex_{NAT} immunogenicities were compared in mouse and guinea pig dose escalation studies. In both models, immunization induced antibody responses specific for Invaplex_{NAT} and LPS while Invaplex_{AR} induced markedly higher anti-IpaB and -IpaC serum IgG and IgA endpoint titers. In the murine model, homologous protection was achieved with 10-fold less Invaplex_{AR} than Invaplex_{NAT} and mice receiving Invaplex_{AR} lost significantly less weight than mice receiving the same amount of Invaplex_{NAT}. Moreover, mice immunized with Invaplex_{AR} were protected from challenge with both homologous and heterologous *Shigella* serotypes. Guinea pigs receiving approximately 5-fold less Invaplex_{AR} compared to cohorts immunized with Invaplex_{NAT} were protected from ocular challenge. Furthermore, adjuvanticity previously attributed to Invaplex_{NAT} was retained with Invaplex_{AR}. The second-generation *Shigella* Invaplex vaccine, Invaplex_{AR}, offers significant advantages over Invaplex_{NAT} in reproducibility, flexible yet defined composition, immunogenicity, and protective efficacy.

IMPORTANCE *Shigella* species are bacteria that cause severe diarrheal disease worldwide, primarily in young children. Treatment of shigellosis includes oral fluids and antibiotics, but the high burden of disease, increasing prevalence of antibiotic resistance, and long-term health consequences clearly warrant the development of an effective vaccine. One *Shigella* vaccine under development is termed the invasin complex or Invaplex and is designed to drive an immune response to specific antigens of the bacteria in an effort to protect an individual from infection. The work presented here describes the production and evaluation of a new generation of Invaplex. The improved vaccine stimulates the production of antibodies in immunized mice and guinea pigs and protects these animals from *Shigella* infection. The next step in the product's development will be to test the safety and immune response induced in humans immunized with Invaplex.

KEYWORDS *Shigella*, adjuvant, immunology, vaccine

Shigellosis is a leading cause of diarrheal disease worldwide, particularly in developing countries (1), and is a continuing problem for civilian and military travelers visiting regions of endemicity (2–5). Vaccine development remains a high priority given

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the disease burden, increasing antibiotic resistance, and increasing appreciation of the postinfectious sequelae associated with shigellosis (6, 7). Vaccine efforts are directed at the most prevalent serogroups, such as *Shigella flexneri* and *Shigella sonnei*, which together account for approximately 89% of shigellosis cases in developing regions (8).

Humans and higher primates infected with *Shigella* develop robust immune responses to several invasion plasmid antigens (Ipa), which are key effectors secreted by the type III secretion system (TTSS) involved in the ability of shigellae to invade the colonic mucosa. The Ipa proteins are highly conserved among all four *Shigella* species and share functional and structural homology with TTSS effector proteins of other Gram-negative bacteria, e.g., *Salmonella* and *Yersinia* (9). Although the prominent immune response to the Ipa proteins has been noted for decades (10, 11), the role of the Ipa proteins in protective immunity in humans is not clear. In contrast, immunity to shigellosis is strongly associated with antibodies to the O antigen of lipopolysaccharide (LPS) and is serotype specific (12, 13). Progressing from antigen identification to clinical trials and licensure has been difficult for the many *Shigella* vaccine approaches that are in various stages of development. To date, all vaccines in clinical trials are focused primarily on stimulating a response to the O antigen of the LPS in attempts to stimulate serotype-specific immunity and the potential of serogroup cross-reactivity (14).

The prototype Invaplex vaccine, native Invaplex (Invaplex_{NAT}), purified from virulent *Shigella*, contains the conserved invasin proteins IpaB and IpaC, serotype-specific LPS, and other proteins of undefined function and limited to no immunogenicity (15, 16). Intranasal immunization of small animals with Invaplex_{NAT} drives a pronounced immune response to LPS, IpaB, and IpaC that mimics that observed after natural infection with virulent *Shigella*. Protection with Invaplex_{NAT} has been achieved in both the mouse and guinea pig models (15, 17), prompting the clinical evaluation of the vaccine in two phase 1 safety and immunogenicity studies (18, 19). The current good manufacturing practice (cGMP)-manufactured *S. flexneri* 2a Invaplex_{NAT} vaccine was found to be safe, well tolerated, and immunogenic in humans. The efficacy results of a follow-on phase 2b study were inconclusive due to the product being less immunogenic, likely due to product instability upon prolonged storage and a lower-than-expected attack rate in placebo controls (M. S. Riddle and R. W. Kaminski, unpublished data).

Further purification of Invaplex_{NAT} by size-exclusion chromatography (SEC) yielded a highly purified (HP) Invaplex_{NAT} with a high molecular mass of >669 kDa. This high-molecular-mass complex (HMMC) consisted of only the principal antigens IpaB, IpaC, and LPS and showed enhanced immunogenicity and protection against infection (16). Using the molar ratios of the components present in HP Invaplex_{NAT}, a synthetic Invaplex, termed artificial Invaplex or Invaplex_{AR}, has been assembled with purified *Shigella* LPS and purified recombinant IpaB and IpaC proteins. Here, the first-generation Invaplex_{NAT} vaccine is compared with the second-generation Invaplex_{AR} vaccine in tests of biochemical composition, immunogenicity, adjuvanticity, and protective efficacy. The studies indicate that not only is the Invaplex_{AR} a better-defined product biochemically, making it more compliant with FDA guidelines on chemistry, manufacturing, and controls, but it exhibits enhanced immunogenicity and protection compared to Invaplex_{NAT}.

RESULTS

Assembly and characterization of artificial Invaplex. In order to evaluate and accurately compare the immunogenicities and protective efficacies of Invaplex_{AR} and Invaplex_{NAT} produced from wild-type shigellae, a sufficient amount of the *Shigella* Invaplex_{AR} products to immunize groups of both mice and guinea pigs with ranging vaccine doses was required. The Invaplex_{AR} was purified from the reaction mixture by ion-exchange chromatography. Two dominant peaks were observed at both 50% B (~0.5 M NaCl, 0.02 M Tris-HCl, pH 9.0) and 100% B. Spot blot analysis and total protein measurements showed the presence of IpaB, IpaC, and LPS within the 50% B elution peak but did not identify significant amounts of IpaB or IpaC within the 100% B peak. The 50% B peak elution fractions were combined and were defined as the Invaplex_{AR}.

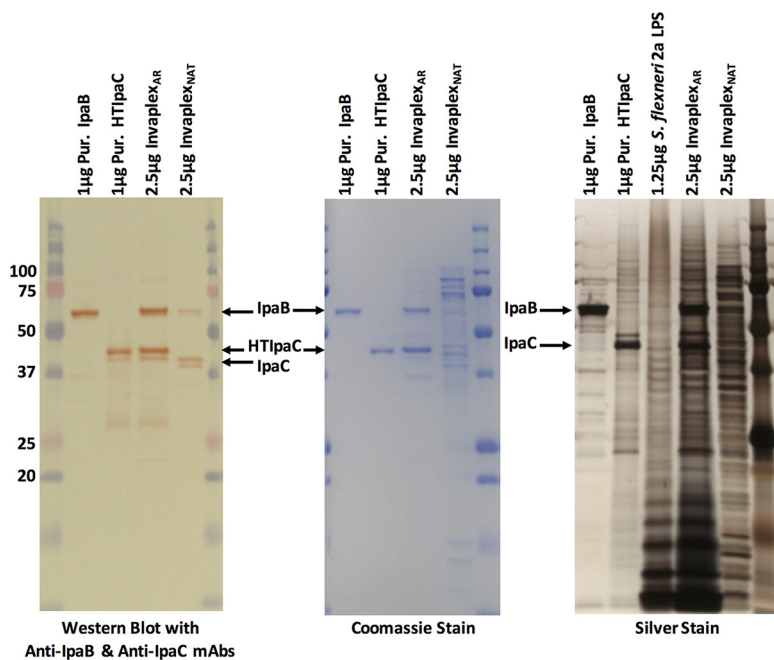


FIG 1 SDS-PAGE analysis of Invaplex_{AR} and Invaplex_{NAT}. Samples loaded were *S. flexneri* 2a Invaplex_{AR} (2.5 µg), Invaplex_{NAT} (2.5 µg), IpaB (1 µg), HTIpaC (1 µg), and purified *S. flexneri* 2a LPS (1.25 µg). Samples were separated by SDS-PAGE and blotted to nitrocellulose (left panel) or stained with Coomassie blue (middle panel) or silver (right panel). Nitrocellulose blots were probed with MAbs specific for IpaB (2F1) and IpaC (2G2). Silver-stained gels detected both proteins and LPS. Molecular mass standards are in the extreme left (Western blot and Coomassie blue) and right (Western blot and Coomassie blue and silver stain lanes) of the gels. Numbers at far left are kilodaltons.

product. Additional experiments using *S. flexneri* 1a and *Shigella dysenteriae* 1 LPS also resulted in Invaplex_{AR} products purified within the 50% B elution peak of anion chromatography (data not shown).

The yield efficiency of the Invaplex_{AR} production was calculated by dividing the total protein yield of the product by the total protein amount of the reactants and then multiplying by 100 and ranged from 8 to 22% yield.

Comparison of *S. flexneri* 2a Invaplex_{AR} and Invaplex_{NAT} by SDS-PAGE. Equal amounts, based on protein content, of the *S. flexneri* 2a Invaplex_{AR} product and Invaplex_{NAT} product were separated by SDS-PAGE followed by Western blotting or staining with either silver or Coomassie blue (Fig. 1). Western blotting and probing with the anti-IpaB specific monoclonal antibody (MAb) 2F1 and the anti-IpaC specific MAb 2G2 (20) identified IpaB and IpaC, respectively, in both Invaplex_{AR} and Invaplex_{NAT} (Fig. 1, left panel), although the antibody reactivity with the proteins in the Invaplex_{NAT} product was weaker, suggesting that IpaB and IpaC are in lower concentrations within the total protein makeup of this product.

The IpaC protein band in the Invaplex_{AR} product, which was produced using histidine-tagged IpaC (HTIpaC), migrated to the same position as the purified HTIpaC, while the IpaC band identified in the Invaplex_{NAT} product migrated slightly lower. The higher apparent molecular mass of the HTIpaC band is attributed to the polyhistidine tag.

Coomassie blue staining (Fig. 1, center panel) of the Invaplex products clearly demonstrated the IpaB and IpaC proteins within the Invaplex_{AR} product but did not detect these bands definitively within the Invaplex_{NAT} product, instead showing multiple bands of various molecular weights that stained with various intensities. The Coomassie blue staining suggests that the proportion of the Ipa proteins in the total protein composition is lower within the less-defined Invaplex_{NAT} product than in the Invaplex_{AR} product, which contains only two proteins, IpaB and IpaC. The

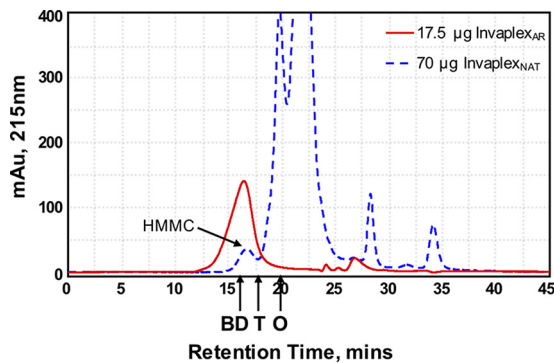


FIG 2 SEC-HPLC of Invaplex_{NAT} and Invaplex_{AR}. A TSK-Gel G5000PW_{XL} column was calibrated as described in Materials and Methods. Retention times of blue dextran (BD; 2 MDa), thyroglobulin (T; 669 kDa), and ovalbumin (O; 43 kDa) are indicated. The UV (215-nm) traces of *S. flexneri* 2a Invaplex_{AR} (17.5 µg, red line) and *S. flexneri* 2a Invaplex_{NAT} (70 µg, blue dashed line) are shown. The higher total protein load for Invaplex_{NAT} was necessary to reveal the high-molecular-mass complex (HMMC) peak (run time of approximately 16.8 min) in the Invaplex_{NAT} sample. mAu, milliabsorbance units.

approximate antigen amounts in a 1-µg dose of Invaplex_{AR} are 370 ng IpaB, 600 ng IpaC, and 953 ng LPS.

The more sensitive silver staining technique detects both LPS and proteins. Many more polypeptide bands within the two Invaplex products were detected along with the *S. flexneri* 2a LPS “ladder” (Fig. 1, right panel). In addition to the full-sized IpaB and HTIpaC proteins, lower-molecular-weight bands were identified by silver staining within the purified IpaB and HTIpaC components and were likely degradation products of the antigens. The same lower-molecular-mass bands were also present in the Invaplex_{AR} product. The LPS ladder was prominent within the Invaplex_{AR} product compared to the Invaplex_{NAT} product, which suggests a greater proportion of the *S. flexneri* 2a LPS component within the Invaplex_{AR} product.

Comparison of native and artificial Invaplex products by SEC-HPLC and dynamic light scattering (DLS). The *S. flexneri* 2a Invaplex_{NAT} and Invaplex_{AR} products were separated by size-exclusion chromatography–high-pressure liquid chromatography (SEC-HPLC). Chromatograms were recorded at 215 nm in order to detect the Invaplex_{AR} product as both the IpaB and IpaC proteins have a low proportion of aromatic amino acids (Fig. 2). The Invaplex_{AR} product (17.5 µg total protein loaded) revealed a single dominant symmetric peak with a retention time of 16.5 min (Fig. 2, red trace) which is resolved before thyroglobulin (669 kDa; 18.5 min) and is therefore interpreted as being larger than thyroglobulin. The Invaplex_{NAT} product (70 µg total protein loaded) resolved as several lower-molecular-mass peaks with retention times after thyroglobulin (Fig. 2, dashed blue trace) and one small peak at a retention time of 16.8 min that meets the definition of the previously reported high-molecular-mass complex (16). SEC-HPLC demonstrates that the production of an Invaplex_{AR} product from individual components yields a slightly higher-molecular-mass product that is more homogeneous than Invaplex_{NAT} prepared from wild-type *Shigella* water extract.

The *S. flexneri* 2a Invaplex_{NAT} and Invaplex_{AR} were also analyzed by dynamic light scattering (DLS) to determine their size distribution. Both Invaplex products displayed a polydisperse profile containing molecular species with different sizes. After controlling for the relative mass of the peaks, a major peak for each of the Invaplex products was identified (Fig. 3). The major peaks of the Invaplex products measured an average hydrodynamic radius of 18.6 ± 3.9 nm for Invaplex_{NAT} and 27 ± 11.5 nm for Invaplex_{AR}, although the majority of the Invaplex_{NAT} product is smaller species of shorter hydrodynamic radii. The hydrodynamic radius of Invaplex_{AR} is greater than the reported hydrodynamic radius of 8.5 nm for thyroglobulin and thus supports the results achieved using SEC-HPLC (21).

Immunogenicity and protective efficacy of native and artificial Invaplex vaccines. The mouse pulmonary challenge (22) and the guinea pig keratoconjunctivitis

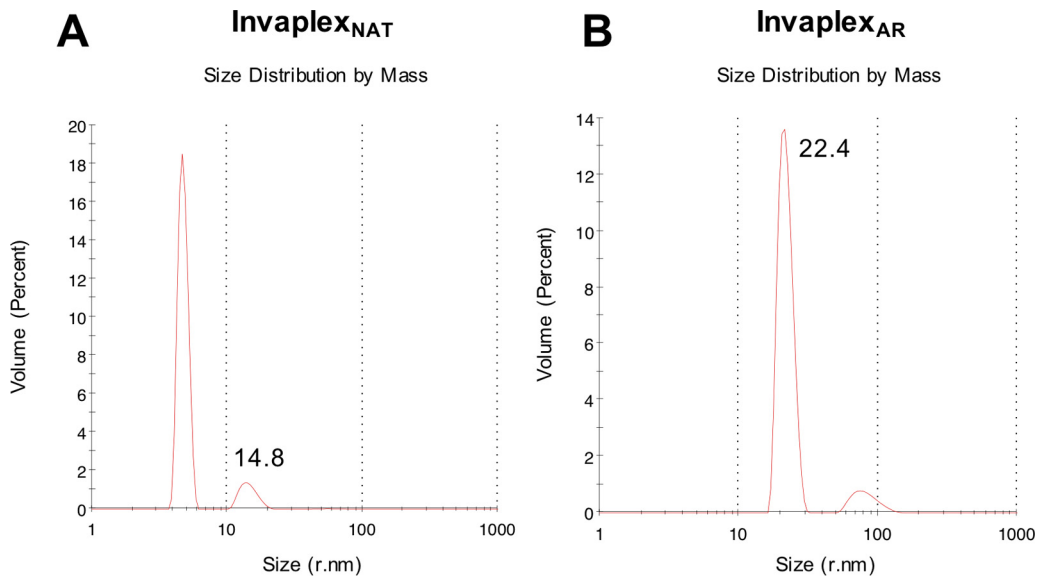


FIG 3 Dynamic light scattering (DLS) analysis of *S. flexneri* 2a Invaplex_{NAT} (A) and Invaplex_{AR} (B). Fifteen microliters of each Invaplex product was added to the cuvette with a 2- μ l window of a Malvern Zetasizer μ V DLS unit. Each graph is representative data for the specific analyte from 1 of 5 separate runs, with each run consisting of 11 to 13 separate scans, all of which were performed at 4°C. The annotated radius numbers are derived from the intensity graph (data not shown), while the graphs presented show the relative quantities of these species in solution.

(23) models were used to determine whether intranasal immunization with *S. flexneri* 2a Invaplex_{AR} stimulates a protective immune response that is effective against homologous challenge with wild-type *S. flexneri* 2a.

In mouse experiments utilizing a dose escalation study design, intranasal immunization with *S. flexneri* 2a Invaplex_{AR} induced serum IgG and IgA antibodies directed to *S. flexneri* 2a LPS, IpaB, and IpaC (Fig. 4) that were either significantly higher than or comparable to the antibody responses induced after immunization with similar dose amounts of *S. flexneri* 2a Invaplex_{NAT}. In particular, the antibody responses directed to IpaB (IgA and IgG) and IpaC (IgG) were significantly higher after immunization with Invaplex_{AR} than after immunization with Invaplex_{NAT} at comparable dose amounts. Bronchial lavage fluid IgA responses showed increased IpaC titers in groups immunized with Invaplex_{NAT} and Invaplex_{AR}, with higher responses observed in the Invaplex_{AR} groups. Furthermore, intranasal immunization of mice with ≥ 1 μ g of Invaplex_{NAT} or ≥ 0.1 μ g of Invaplex_{AR} resulted in significant protection against lethal challenge with *S. flexneri* 2a strain 2457T (Table 1). A delay in death was evident in all immunized mice. The day on which 27% (4 of 15) mice died occurred on day 5 in the saline control group, day 9 in the 0.1- μ g and 1.0- μ g Invaplex_{NAT} groups, and day 11 in the 0.1- μ g Invaplex_{AR} group. In all other groups, 4 or more mice did not die during the study. Moreover, animals immunized with 1 μ g (Fig. 5) or 2.5 μ g Invaplex_{AR} lost significantly less weight ($P < 0.02$) after challenge than did mice immunized with Invaplex_{NAT}. In contrast, mice immunized with 100% protective 5- μ g doses of either vaccine exhibited similar weight losses, each of which was significantly less than that of the saline control group (Invaplex_{AR}, $P = 0.0005$; Invaplex_{NAT}, $P = 0.041$). Mice immunized with the lowest dose (0.1 μ g) of either vaccine lost weight that was comparable to that observed in the saline control group.

To better understand the immune responses and protective efficacy afforded after immunization with Invaplex_{NAT} and Invaplex_{AR}, the 50% protective dose (PD₅₀) and 50% immunizing dose (ImmD₅₀) were calculated using an adaptation of the Reed-Muench 50% lethal dose (LD₅₀) formula (24). The PD₅₀ for Invaplex_{NAT} was determined to be 0.5 μ g, which was approximately 7-fold higher than the calculated PD₅₀ for Invaplex_{AR} (Table 2). Using similar calculations, it was determined that the dose of Invaplex_{AR} required to induce antibody responses in 50% of the immunized animals

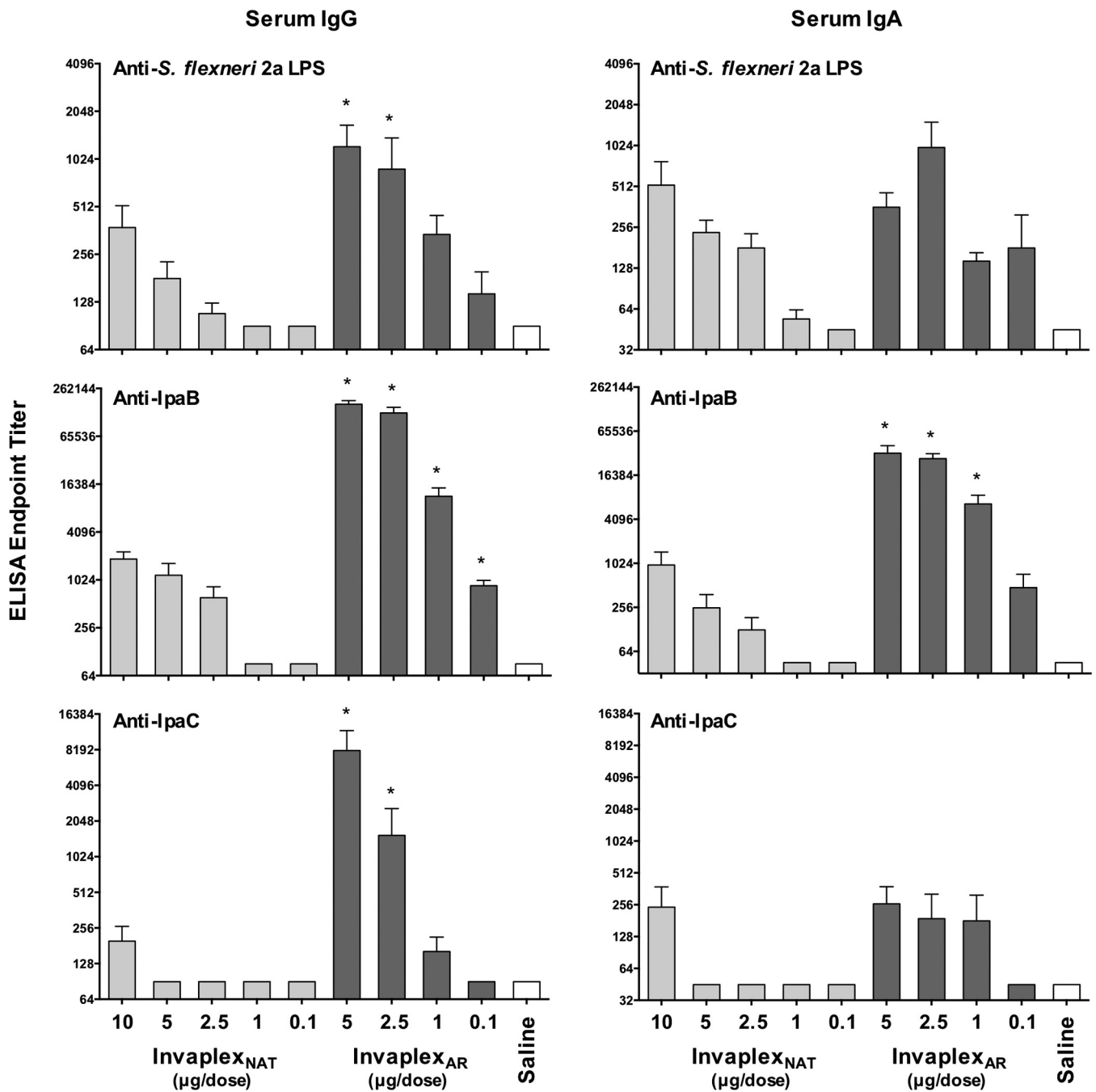


FIG 4 *Shigella*-specific serum IgG and IgA endpoint titers in mice immunized intranasally with *S. flexneri* 2a Invaplex_{NAT} or *S. flexneri* 2a Invaplex_{AR}. Groups of mice ($n = 15$ /group) were immunized intranasally on days 0, 14, and 28 with either *S. flexneri* 2a Invaplex_{NAT} (0.1, 1, 2.5, 5, or 10 μg), *S. flexneri* 2a Invaplex_{AR} (0.1, 1, 2.5, or 5 μg), or saline and challenged on day 49 ($n = 15$ mice/group) with *S. flexneri* 2a strain 2457T. Blood was collected on days 0, 28, 42, and 63 ($n = 5$ mice/group) and analyzed by ELISA for anti-*S. flexneri* 2a LPS (top panels), IpaB (middle panels), or IpaC (bottom panels) serum IgG (left panels) and IgA (right panels) endpoint titers. The mean endpoint titers + 1 standard error of the mean on day 42 are plotted, and significant differences (two-way analysis of variance; $P < 0.05$) between dose-matched groups are indicated with an asterisk.

(ImmD₅₀) to Invaplex, LPS, IpaB, or IpaC was 2.7- to >21-fold lower than the dose of Invaplex_{NAT} required to induce antigen-specific immune responses, demonstrating that Invaplex_{AR} is a more potent immunogen than Invaplex_{NAT}.

Using a similar dose escalation study design, groups of guinea pigs were intranasally immunized with *S. flexneri* 2a Invaplex_{NAT} or Invaplex_{AR}. Control groups were inoculated with saline. Blood collected on days 0, 28, and 40 was assayed by enzyme-linked immunosorbent assay (ELISA) for titers of antibodies directed to LPS, IpaB, and IpaC.

TABLE 1 Protective efficacy in the mouse pulmonary infection and guinea pig keratoconjunctivitis models after intranasal immunization with various doses of *S. flexneri* 2a Invaplex_{NAT} or Invaplex_{AR}

Model and treatment	Dose (μg)	No. of animals ^a		% protection ^b	P value ^c
		Protected	Unprotected		
Mouse lung (n = 15)					
<i>S. flexneri</i> 2a Invaplex _{NAT}	10	15	0	100	<0.001
	5	15	0	100	<0.001
	2.5	12	3	75	0.003
	1	10	5	59	0.025
	0.1	9	6	50	0.06
<i>S. flexneri</i> 2a Invaplex _{AR}	5	15	0	100	<0.001
	2.5	15	0	100	<0.001
	1	13	2	84	0.001
	0.1	10	5	59	0.025
Saline	None	3	12	N/A ^d	N/A
Guinea pig keratoconjunctivitis (n = 12)					
<i>S. flexneri</i> 2a Invaplex _{NAT}	25	9	3	75	0.0005
	5	3	9	25	0.22
	1	0	12	0	1.00
<i>S. flexneri</i> 2a Invaplex _{AR}	23	7	5	59	0.0053
	5	7	5	59	0.0053
	1	4	8	33	0.09
Saline	None	0	10	N/A	N/A

^a"Protected" indicates number of survivors in the mouse lung model and number of animals with eyes protected in the keratoconjunctivitis model. "Unprotected" indicates number of dead animals in the mouse lung model and number of animals with unprotected eyes in the keratoconjunctivitis model.

^bPercent protection = (AR for controls – AR for immunized)/AR for controls \times 100, where the attack rate (AR) for a group is determined by (number of diseased animals/total number of animals) \times 100.

^cFisher exact test.

^dN/A, not applicable.

Similar to the results in mice, immunization of guinea pigs with Invaplex_{AR} promoted antibody responses directed to LPS, IpaB, and IpaC at similar or higher titers than those for animals immunized with Invaplex_{NAT} (Fig. 6). Guinea pigs ocularly challenged with *S. flexneri* 2a strain 2457T were significantly protected after three immunizations with 25 μg of Invaplex_{NAT} (75% protection; $P = 0.0005$), but immunization with lower doses

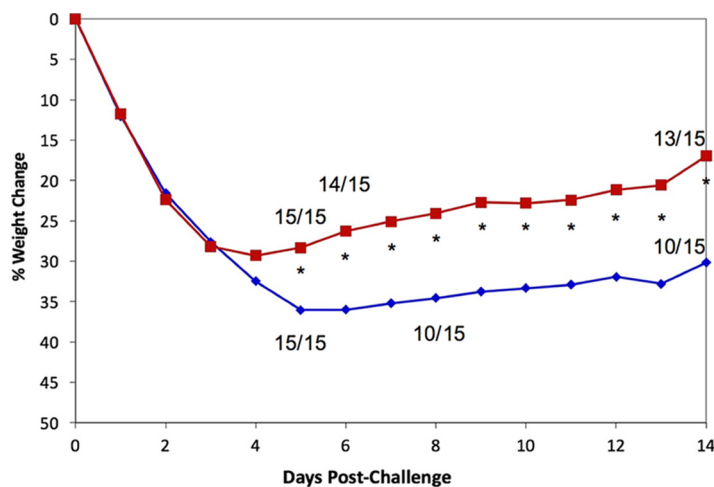


FIG 5 Mice immunized with 1 μg Invaplex_{AR} experience less weight loss and recover more rapidly than those immunized with Invaplex_{NAT}. Mice immunized with *S. flexneri* 2a Invaplex_{AR} (red) or Invaplex_{NAT} (blue) were challenged intranasally with a lethal dose of *S. flexneri* 2a strain 2457T. Mice were weighed on the day of challenge and each day for 14 days postchallenge. The average weight for each group was calculated, and the difference was expressed as a percentage of the change in weight from the prechallenge values. The numbers of surviving animals/animals in groups immunized with Invaplex_{NAT} and Invaplex_{AR} are shown. While protection was significant for both products compared to saline controls, a significant difference in weight was observed for the 1- μg Invaplex_{AR} group compared to the 1- μg Invaplex_{NAT} group (*, $P = 0.0126$).

TABLE 2 Intranasal immunization with *S. flexneri* 2a Invaplex_{AR} provides protection and drives an immune response to *Shigella*-specific antigens at lower doses than *S. flexneri* 2a Invaplex_{NAT} in mice and guinea pigs^a

Animal model	Treatment	PD ₅₀ (μg)	ImmD ₅₀ (<i>Shigella</i> antigen-specific serum IgG) (μg)			
			<i>S. flexneri</i> 2a			
			Invaplex	<i>S. flexneri</i> 2a LPS	IpaB	IpaC
Mouse	Invaplex _{NAT}	0.5	1.9	>10	2.1	>10
	Invaplex _{AR}	0.07	0.7	0.9	<0.1	1.8
	Fold difference	7.1	2.7	>11.1	>21	>5.6
Guinea pig	Invaplex _{NAT}	9.3	2.6	9	2.6	13.1
	Invaplex _{AR}	4.5	0.8	3.8	0.6	0.6
	Fold difference	2.1	3.2	2.4	4.3	21.8

^aThe PD₅₀ and ImmD₅₀ were calculated as described in Materials and Methods. The quantities of LPS, IpaB, and IpaC for each Invaplex product were determined as described in Materials and Methods.

of Invaplex_{NAT} did not result in significant protection (Table 1). In contrast, groups immunized with 5 or 23 μg of Invaplex_{AR} were significantly protected against challenge (59% protection; $P = 0.0053$). The Reed-Muench formula was again used to calculate the PD₅₀ and ImmD₅₀ for the guinea pig model, and similar trends were achieved as obtained for the mouse model in that a higher Invaplex_{NAT} dose than Invaplex_{AR} dose was necessary to provide protection and elicit a *Shigella*-specific immune response in 50% of the animals immunized with Invaplex_{NAT} (Table 2).

Homologous and heterologous protection afforded after immunization with *S. sonnei* Invaplex_{AR}. Naturally acquired immunity against shigellosis is primarily serotype specific, and therefore, a multivalent vaccine containing multiple O-antigen types will likely be required to achieve broadly protective efficacy. Consequently, a vaccine that is capable of inducing protective immunity against multiple serogroups or serotypes would be highly advantageous. Immunization of mice with *S. sonnei* Invaplex_{NAT} can provide cross protection against challenge with *S. flexneri* 2a strain 2457T (25). The ability of Invaplex_{AR} to induce protective immunity against heterologous species or serotypes was also tested in the mouse pulmonary lung model. Mice were immunized intranasally with 2.5 μg of *S. sonnei* Invaplex_{AR} and divided into two subgroups: one subgroup was challenged with *S. sonnei* strain Moseley, the homologous serotype to the vaccine, and the other subgroup was challenged with *S. flexneri* 2a strain 2457T. Controls for the study included animals immunized with saline and challenged with either *S. sonnei* strain Moseley or *S. flexneri* 2a strain 2457T (negative controls) and animals immunized with 5 μg of either *S. sonnei* Invaplex_{NAT} or *S. flexneri* 2a Invaplex_{NAT} and challenged with the homologous serotype (positive controls). None of the animals immunized with saline survived the challenge (Fig. 7), and all lost >20% of their prechallenge weight prior to death. Mice immunized with the native Invaplex vaccines and challenged with the homologous serotype all survived challenge (100% protection). As expected, all animals immunized with *S. sonnei* Invaplex_{AR} and challenged with *S. sonnei* strain Moseley survived challenge and exhibited <10% weight loss. Interestingly, all animals immunized with *S. sonnei* Invaplex_{AR} and challenged with *S. flexneri* 2a strain 2457T survived challenge and followed a similar percent weight loss curve as animals immunized with *S. flexneri* 2a Invaplex_{NAT}. Mice immunized with *S. sonnei* Invaplex_{AR} and protected against *S. flexneri* 2a produced serum IgG antibodies (geometric mean titer [GMT]) to *S. sonnei* LPS (136), IpaB (5,760), and IpaC (1,091) but weak to undetectable levels against *S. flexneri* 2a LPS (GMT of 90). These results demonstrate the ability of the *Shigella* Invaplex_{AR} to provide a level of protective immunity against a heterologous *Shigella* species challenge similar to that of the Invaplex_{NAT} product (17, 25).

Adjuvanticity of *S. flexneri* 2a Invaplex_{AR}. In addition to inducing *Shigella*-specific immune responses, Invaplex_{NAT} also functions as an adjuvant, capable of enhancing the

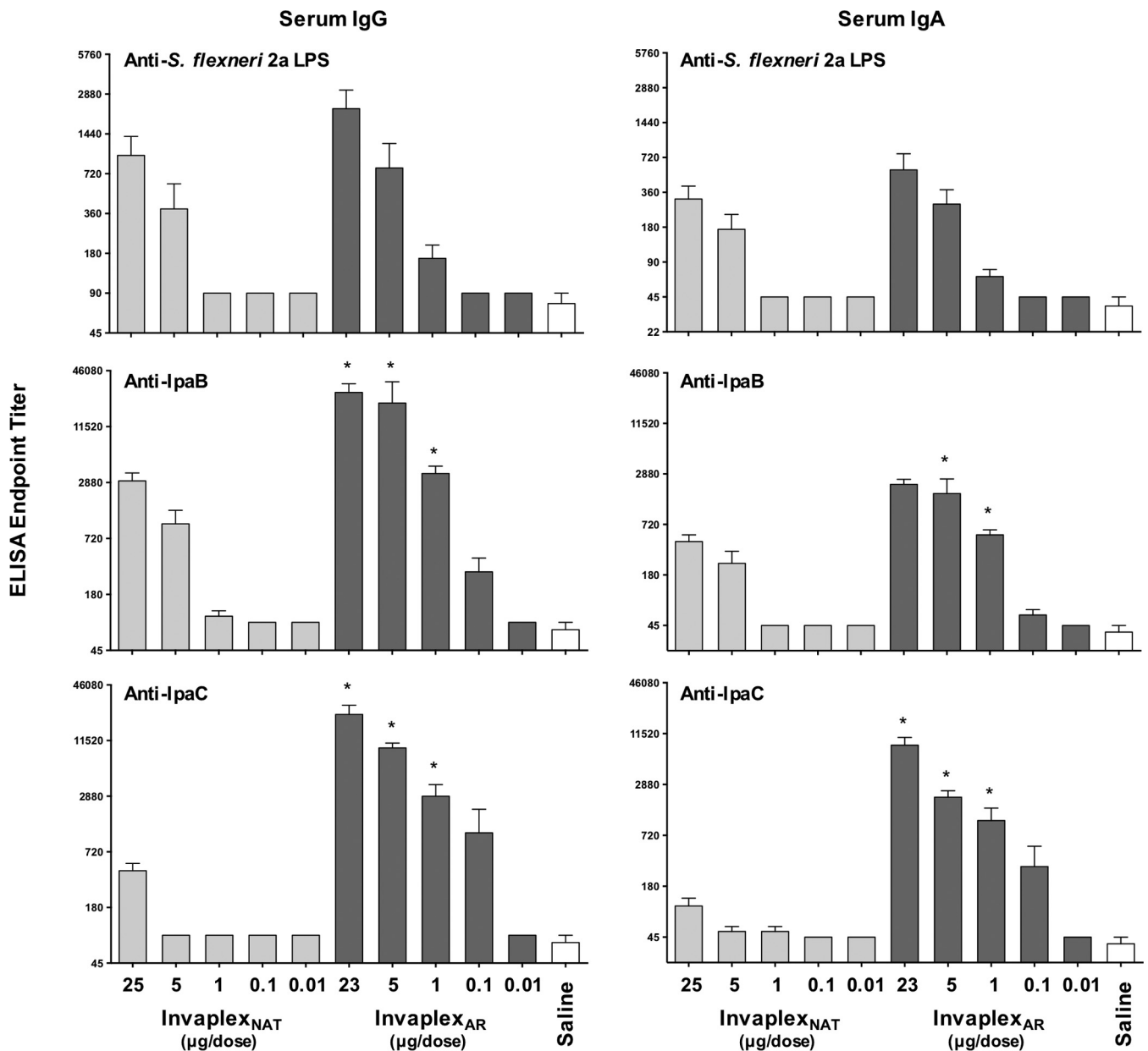


FIG 6 *Shigella*-specific serum IgG and IgA endpoint titers in guinea pigs immunized intranasally with *S. flexneri* 2a Invaplex_{NAT} or Invaplex_{AR}. Groups of guinea pigs ($n = 6$ /group) were immunized intranasally on days 0, 14, and 28. Dose amounts of *S. flexneri* 2a Invaplex_{NAT} and *S. flexneri* 2a Invaplex_{AR} are indicated on the x axis. Guinea pigs were challenged on day 49 with *S. flexneri* 2a strain 2457T. Blood was collected on days 0, 28, 40, and 63 and analyzed by ELISA for anti-*S. flexneri* 2a LPS (top panels) or IpaB (middle panels) or IpaC (bottom panels) serum IgG (left panels) and IgA (right panels) endpoint titers. The mean endpoint titer + 1 standard error of the mean on day 40 is plotted, and significant differences (two-way analysis of variance; $P < 0.05$) between dose-matched groups are indicated with an asterisk.

magnitude of the immune response directed to coadministered protein antigens (26) or antigens encoded on plasmid DNA (27). To determine if adjuvanticity was retained with Invaplex_{AR}, groups of mice were intranasally immunized with either ovalbumin (OVA) alone or in combination with *S. flexneri* 2a Invaplex_{NAT}, Invaplex_{AR}, or cholera toxin (CT), a known mucosal adjuvant. Comparable levels of OVA-specific serum IgG (Fig. 8) and IgA titers (data not shown) were detected on days 28 and 42 after immunization with OVA mixed with Invaplex_{NAT}, Invaplex_{AR}, and cholera toxin. All responses were significantly higher ($P < 0.001$) than the OVA-specific immune response induced after immunization with OVA alone. In addition to enhancing the OVA-specific immune response, *Shigella*-specific immunity was induced in the groups immunized

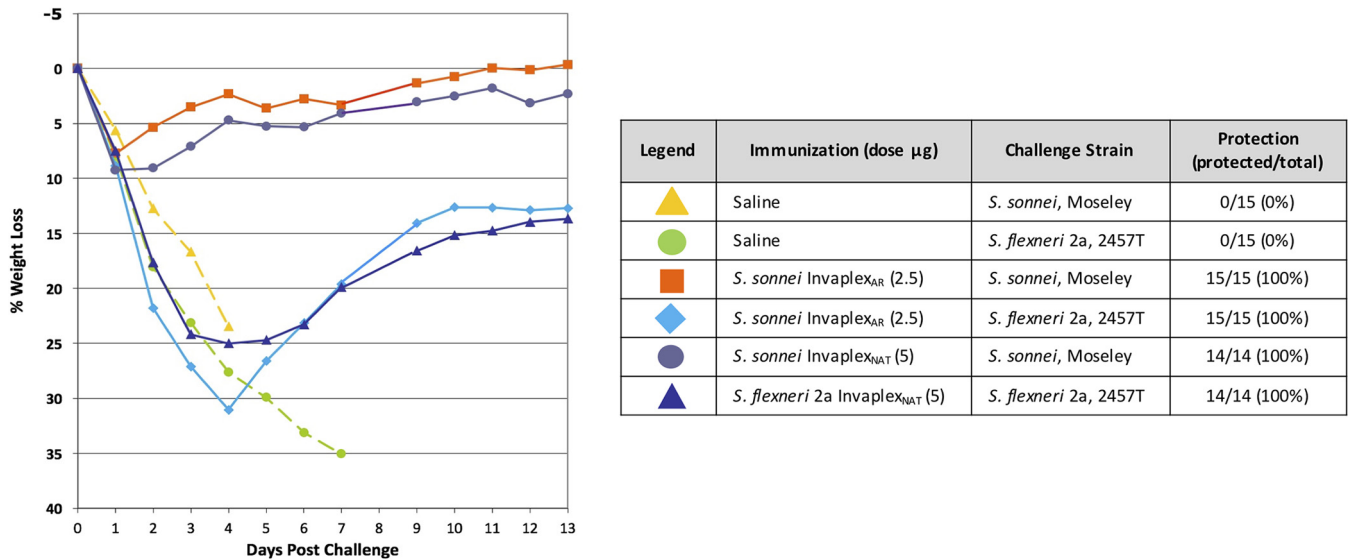


FIG 7 Intranasal immunization of mice with *S. sonnei* Invaplex_{AR} induces protective immune responses against *S. sonnei* and *S. flexneri* 2a. Mice were immunized intranasally on days 0, 14, and 28 with *S. sonnei* Invaplex_{AR}, *S. sonnei* Invaplex_{NAT}, or *S. flexneri* 2a Invaplex_{NAT} and challenged using the pulmonary mouse model on day 49 with either *S. flexneri* 2a strain 2457T or *S. sonnei* strain Moseley. Mice were weighed on the day of challenge and each day for 14 days postchallenge. The average weight for each group was calculated, and the difference was expressed as a percentage of the change in weight from the prechallenge values.

with OVA combined with Invaplex_{NAT} or Invaplex_{AR} (Fig. 8), indicating that Invaplex_{AR} could be used in a combination vaccine strategy as both an adjuvant and an immunogen.

Antigen-specific antibodies in lung washes collected 2 weeks after the third immunization (day 42) were also assessed by ELISA to investigate the mucosal immune responses induced after immunization (Fig. 8). Similar levels of OVA-specific lung IgA were induced after immunization with OVA mixed with Invaplex_{AR}, Invaplex_{NAT}, or CT, and these levels were significantly higher ($P < 0.001$) than those induced after immunization with OVA alone (Fig. 8). Immunization with OVA mixed with Invaplex_{AR} induced similar levels of LPS-specific (data not shown) and Invaplex_{NAT}-specific IgA in lung washes as those after immunization with OVA mixed with Invaplex_{NAT} and higher levels than those induced after immunization with OVA alone or OVA mixed with CT (Fig. 8).

DISCUSSION

Analysis of the immune response generated after natural infection may guide the rational design of a *Shigella* vaccine capable of stimulating a protective immune response comparable to that induced postinfection. Several studies in humans (13, 28, 29) have demonstrated that individuals orally infected with *Shigella* are resistant to subsequent infections with homologous or closely related *Shigella* serotypes but not heterologous serotypes. In nonhuman primates (10), infected animals producing serum antibodies to homologous LPS and the Ipa proteins are resistant to homologous challenge but not challenge with a heterologous serotype. The results of these studies combined with epidemiological data (30) suggest that serotype-based immunity is prevalent after natural infection. *Shigella* infection induces a potent immune response to LPS with the O-specific polysaccharide (O-SP) generating serotype-specific immunity in both humans and nonhuman primates (10, 21). In addition, a pronounced antibody response to the protein effectors of the TTSS, specifically IpaB, IpaC, and IpaD, which is characterized by cross-reactivity with all serotypes and species of *Shigella*, is induced (10–12, 20, 31, 32). Other protein antigens such as VirG (10, 12), OmpA (33), or pan-*Shigella* surface protein 1 (PSSP-1) (34) may also stimulate cross-reactive immune responses. However, in some cases chromosomally encoded protein antigens, such as major outer membrane proteins, are broadly conserved among enteric Gram-negative

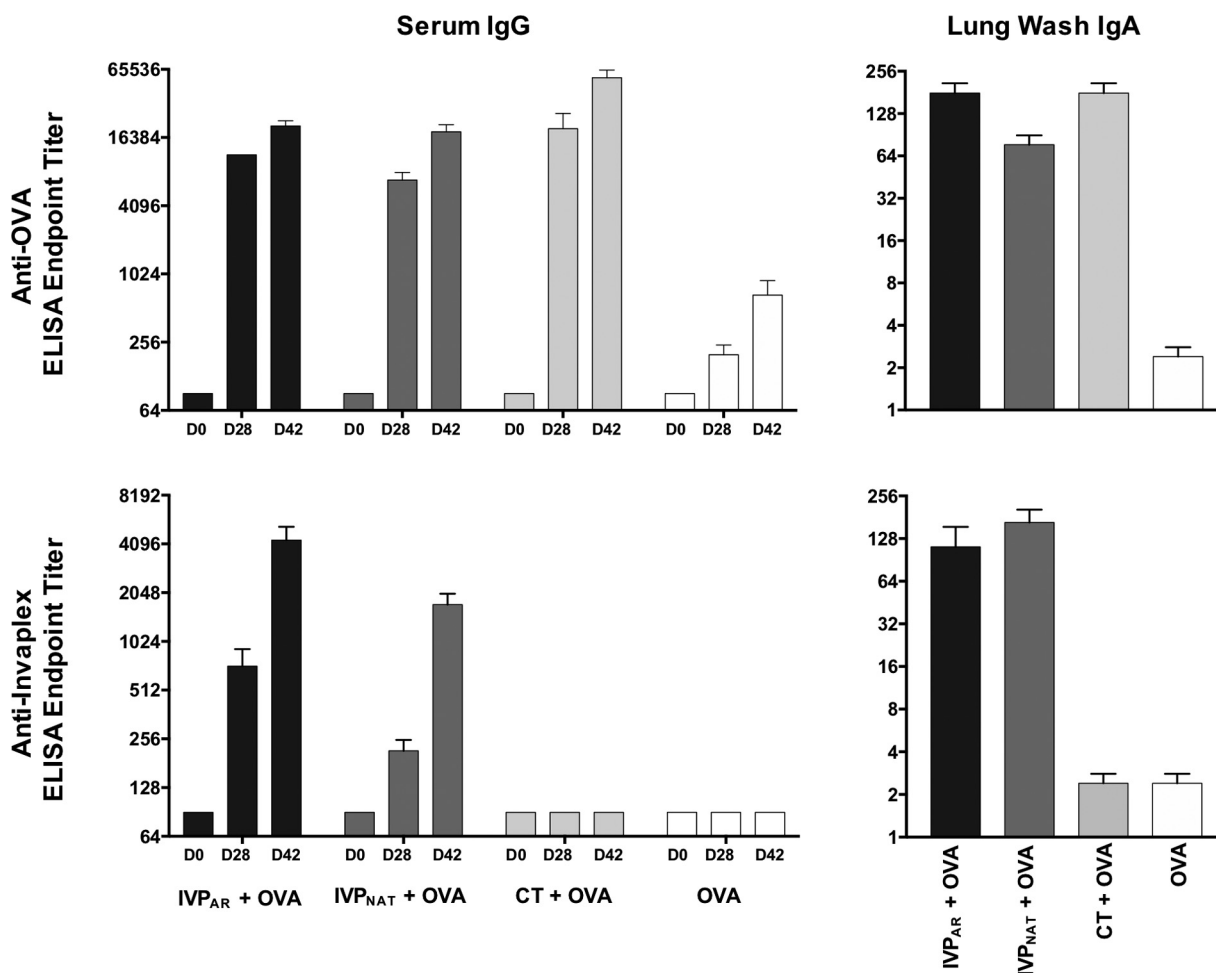


FIG 8 OVA-specific and Invaplex_{NAT}-specific serum IgG endpoint titers in mice after intranasal immunization with OVA alone or OVA combined with Invaplex_{AR}, Invaplex_{NAT}, or cholera toxin (CT). Groups of mice were intranasally immunized on days 0, 14, and 28 with OVA or OVA combined with either *S. flexneri* 2a Invaplex_{AR} or Invaplex_{NAT} or CT. Blood collected on days 0, 28, and 42 was analyzed by ELISA for anti-OVA and anti-Invaplex_{NAT} serum IgG endpoint titers. Lung washes collected on day 42 were analyzed by ELISA for anti-OVA and anti-Invaplex_{NAT} IgA endpoint titers. Data represent the mean ($n = 5$ mice/group) endpoint titer ± 1 standard error of the mean. Significant differences between group mean log-transformed titers were determined by two-way analysis of variance ($P < 0.05$).

bacteria and it is often difficult to identify specific infection-induced signals over the abundance of background cross-reactivity due to the structural similarity.

Vaccines that stimulate an immune response that mimics the natural response in magnitude, specificity, and functionality hold promise for inducing protective immunity in humans. Candidate *Shigella* vaccines with this capability include live attenuated *Shigella* (32, 35), inactivated whole cells (36–38), and the Generalized Modules for Membrane Antigens (GMMA) product, although the last is deficient in the invasins (39). Vaccines focused primarily on only O-SP or only conserved proteins are promising but may not stimulate the comprehensive, fully protective immune response observed after infection. The *Shigella* Invaplex vaccine has been developed to mimic both the predominant serotype-specific aspect and pan-*Shigella* (protein-specific) immune responses induced after natural infection by presenting the LPS, IpaB, and IpaC antigens in a manner or context that induces a functional and effective immune response.

The first-generation *Shigella* Invaplex vaccines were isolated from water extracts of wild-type, virulent organisms (15) and have been given the nomenclature of “native” Invaplex. The prototype Invaplex_{NAT} vaccines were evaluated in a series of studies that demonstrated the immunogenicity and efficacy of the product in preclinical studies (15,

17), the feasibility of cGMP manufacture, and the safety and immunogenicity of the product in a series of phase 1 clinical trials (18, 19). The Invaplex_{NAT} product was safe and immunogenic when delivered intranasally to over 100 human volunteers. Although promising in early clinical trials, potential instability upon long-term storage, resulting in lower than previously achieved immunogenicity, combined with a lower than expected attack rate, hampered efforts to demonstrate proof-of-concept efficacy in a phase 2b trial (R. W. Kaminski and M. S. Riddle, unpublished results). The composition of Invaplex_{NAT} includes the target major *Shigella* antigens (LPS, IpaB, and IpaC) and many other proteins, including IpaD, elongation factor G (EF-G), and DnaK, along with many other proteins with limited to no immunogenicity (see list of proteins in Table S1 in the supplemental material). The lack of product homogeneity and definition may have been associated with the instability and lower immune response induced by Invaplex_{NAT} products stored for a prolonged period of time and suggested that a better understanding of the active components in Invaplex_{NAT} was warranted. For this reason, efforts were undertaken to improve the product's homogeneity, manufacturing yield, and immunogenicity, and these efforts have led to the development of a second-generation Invaplex product.

Research conducted on a highly purified Invaplex_{NAT} product clearly demonstrated that LPS, IpaB, and IpaC were combined in an HMMC that was essential for the product's immunogenicity and protective efficacy (16). Furthermore, the molar ratios of the IpaB and IpaC proteins and the protein-to-lipopolysaccharide ratios in the HMMC provided a blueprint for the design and construction of second-generation Invaplex_{AR}. The methodology used to assemble LPS, IpaB, and IpaC into a synthetic or artificial high-molecular-mass complex has inherent flexibility that allows the three constituents to be assembled in ratios that optimize immunogenicity, protective efficacy, and maintenance of component solubility. The resultant Invaplex_{AR} product is much more defined than Invaplex_{NAT} and can be scaled up more readily than the process used to manufacture Invaplex_{NAT}.

The customization of Invaplex_{AR} is evidenced in the ease of switching from one *Shigella* serotype/species to another serotype by simply substituting the LPS used during the assembly process. Research-grade lots of Invaplex_{AR} have been manufactured for *S. flexneri* 2a and *S. sonnei* as well as *S. flexneri* 3a, *S. flexneri* 1a, and *S. dysenteriae* 1 (data not shown), in which the same IpaB and IpaC lots were used with the various *Shigella* LPSs. Indeed, the LPS component was not only key as an antigen but was also essential for the solubility of the Invaplex_{AR} product as, in experiments in which only IpaB and IpaC were mixed, the proteins precipitated out of solution. The presence of LPS prevented the precipitation of IpaC (data not shown), similar to the use of a detergent (40). The insolubility of purified IpaC has hampered subunit vaccine efforts including this critical virulence protein (40). IpaB and IpaC contain substantial regions of hydrophobicity (40–42), and both IpaC and IpaB have been reported to insert themselves into red blood cell and mammalian cell membranes (40, 43–45). The amphiphilic nature of LPS likely provides a favorable environment to facilitate the hydrophobic collapse or folding of the Ipa proteins in a soluble and potentially active state in a manner similar to the folding and membrane insertion of *Escherichia coli* OmpA protein (46) and *E. coli* OmpT protein (47, 48). Once the Invaplex_{AR} is formed, the complex is stable without evidence of precipitation or insolubility of any of the components or the complex. It is unknown if the complex represents a structure naturally found in *Shigella*, but as an LPS/IpaB/IpaC structure is also found in Invaplex_{NAT} and is of similar size and composition and capable of inducing uptake in mammalian cells, we speculate that the Invaplex_{AR} represents the active complex or HMMC found in Invaplex_{NAT}. The slightly earlier SEC-HPLC retention time for the Invaplex_{AR} product may be the result of the increased mass of the product compared to Invaplex_{NAT} in part due to the use of a His-tagged IpaC product in the manufacture of the second-generation vaccine. Investigations to better define and characterize the structure of Invaplex_{AR} are under way. The amount of lipopolysaccharide required in the assembly process to achieve levels of LPS-specific immunity which correlate with protection

across multiple *Shigella* serotypes is also under active investigation. Studies optimizing the LPS content for new *Shigella* serotypes will permit the manufacture of a multivalent *Shigella* Invaplex_{AR} product targeting the most predominant serotypes.

The immunogenicity and protective efficacy of the Invaplex_{NAT} and Invaplex_{AR} products were compared directly in the mouse and guinea pig models. Both models have been used extensively to evaluate potential *Shigella* vaccines, with the mouse model used primarily for early-stage vaccine candidates and the guinea pig conjunctivitis model applied to more advanced vaccine candidates immediately prior to initial studies in humans (19, 22, 23) as it depends largely upon a mucosal immune response for protection (36). In both models, Invaplex_{AR} was comparable or superior to Invaplex_{NAT} in the magnitude of the immune response directed to *Shigella* LPS, IpaB, and IpaC as well as affording significant protection against a lethal pulmonary or keratoconjunctivitis challenge. Neither product required an adjuvant to induce a protective immune response. Furthermore, the total protein dosage of Invaplex_{AR} was 2- to 10-fold lower than that of Invaplex_{NAT} for both immunogenicity and protective efficacy in both animal models. Solid protection (less weight loss and increased number of survivors) was achieved with 1 μ g of Invaplex_{AR}. This dose amount contains 370 ng IpaB, 600 ng IpaC, and 953 ng LPS. Other reports (49) using IpaB as an antigen indicate that more than 2.5 μ g of IpaB is required for protection in mice, suggesting that less IpaB is required when presented in the context of Invaplex_{AR}. The improved potency of Invaplex_{AR} will not only conserve product but may open the possibility of including adjuvants to the formulation that may further enhance the immunogenicity of the product.

Recent epidemiological data (8) suggest that to achieve broad protection, a *Shigella* vaccine would need to provide coverage against *S. sonnei*, *S. flexneri* 2a, *S. flexneri* 3a, and potentially *S. flexneri* 6, with the idea that all LPS serotypes and serogroups, defined as group (3)4, group 6, and group 7(8), would be represented and provide expanded coverage (14). The Invaplex_{AR} vaccine approach is amenable to multivalency by admixing successful monovalent vaccines. Bivalent formulations of Invaplex_{NAT} have successfully demonstrated the feasibility of this approach (17). In addition, as indicated in our study, the ability of monovalent Invaplex_{AR} vaccines to provide cross protection against multiple *Shigella* serotypes could be leveraged to broaden coverage. The LPS component of Invaplex_{AR} provides the serotype and serogroup specificity of the vaccine. A quadrivalent Invaplex_{AR} vaccine directed at the 4 prevalent serotypes would theoretically provide immunity against $\geq 80\%$ of the *Shigella* isolates that cause global shigellosis (8). Furthermore, as the Ipa proteins are highly conserved across all *Shigella* serotypes, they add an attractive advantage of Invaplex_{AR} for use in a pan-*Shigella* vaccine. Studies in mice using IpaB and IpaD (31) have demonstrated that a protein-only-based vaccine is capable of protecting against challenge with other *Shigella* serotypes in the murine pulmonary model. Using a live attenuated *S. flexneri* 2a vaccine overexpressing TTSS antigens, broad protection has been observed in guinea pigs (32). However, other studies in guinea pigs immunized with only purified Ipa proteins have not demonstrated similar protective effects; when LPS was delivered in combination with IpaB and IpaC, the vaccine formulation generally resulted in significant protection (R. W. Kaminski and E. V. Oaks, unpublished data).

Another attribute of Invaplex_{NAT} is the ability of the complex to function as a mucosal adjuvant. Studies conducted with protein antigens (ovalbumin, protective antigen from *Bacillus anthracis*, CFA/I antigen from enterotoxigenic *E. coli*, and FlaA from *Campylobacter jejuni*) have demonstrated that Invaplex_{NAT} increases the magnitude of the humoral and cellular immune response directed to the coadministered antigen (26). Furthermore, Invaplex_{NAT} also enhanced the immune response directed to vaccine antigens encoded on DNA plasmids (27). The adjuvanticity of Invaplex has been partially attributed to its inherent ability to induce cellular uptake of coadministered antigens as well as providing the necessary Toll-like receptor 4 (TLR-4) agonist that stimulates robust immune responses. The ability to induce cellular uptake is likely due to the biological activity associated with the Ipa proteins, which as TTSS effectors are

actively involved in host cell invasion by *Shigella* spp. (9). Invaplex_{AR} retains similar biological functions and can induce cellular uptake in normally nonphagocytic epithelial and fibroblast cell lines (50). The slightly larger size of Invaplex_{AR} than Invaplex_{NAT} may improve its interactions with antigen-presenting cells (APCs) and enhance its immunogenicity and adjuvanticity. The retention of the biological activity combined with inclusion of LPS allows Invaplex_{AR} to also function as a mucosal adjuvant when codelivered with heterologous antigens such as ovalbumin (27). The adjuvanticity of Invaplex_{AR} is very appealing when considering the development of a combination vaccine, such as an anti-diarrheal vaccine to protect against enterotoxigenic *Escherichia coli* (ETEC) and *Shigella* infection.

Collectively, the comparisons between the first-generation Invaplex_{NAT} vaccine and the second-generation Invaplex_{AR} vaccine have demonstrated Invaplex_{AR} to have comparable and enhanced properties in relation to Invaplex_{NAT}. The Invaplex_{AR} products have improved biochemical consistency and potency and offer the increased formulation flexibility necessary for a multivalent *Shigella* vaccine approach. Research to transition the recombinant IpaC protein to a non-histidine-tagged version and improved levels of recombinant protein expression, as well as efforts to increase the production yield efficiency of the mixing process, is actively under way in order to make a consistent product that is suitable for cGMP manufacturing and transition to clinical evaluations to evaluate the safety and immunogenicity of the second-generation product.

MATERIALS AND METHODS

Bacterial strains and growth. *Shigella flexneri* 2a strain BS103 and *Shigella sonnei* strain Moseley, both grown in brain heart infusion (BHI) broth, served as the source of purified LPS, while Invaplex_{NAT} was isolated from virulent *S. flexneri* 2a strain 2457T grown in a modified animal-product-free formulation based on antibiotic medium number 3 (Becton, Dickinson) as previously described (18).

Recombinant clones expressing the Ipa of interest were kindly provided by Bill Picking of the University of Kansas (51–53). *Escherichia coli* strain BL21(DE3)pLysS harboring the IpaB pCAYC/HTIpgC pET15b or HTIpaC pET15b plasmid construct was grown in LB broth (Lennox) with appropriate antibiotics. Both recombinant strains were induced at an optical density at 600 nm (OD₆₀₀) of 0.5 with isopropyl- β -D-thiogalactopyranoside (IPTG), followed by continued incubation at 37°C for 3 h, and then harvested for further processing (see below).

Purification of IpaB and HTIpaC (IpaC). Clarified supernatants of sonicated cells containing IpaB/His-tagged IpgC (HTIpgC) complexes were applied to a 20-ml HisPrep FF 16/10 nickel Sepharose (GE Healthcare) column in binding buffer (20 mM Tris-HCl, 500 mM NaCl, 5 mM imidazole, pH 7.9) (51). Bound IpaB/HTIpgC complexes were eluted with 20 mM Tris-HCl, 500 mM NaCl, 50 mM EDTA, pH 7.4. Fractions containing both IpaB and IpgC were pooled and dialyzed followed by addition of *N*-octyl-oligoxyethylene (OPOE; 1% final concentration; Alexis Biochemicals) (51) and incubated at room temperature with gentle swirling for 30 min. The OPOE-treated sample was applied to a 25-ml XK 16/10 nickel Sepharose HP (GE Healthcare) column. Fractions were collected during the loading and initial wash buffers. These void volume fractions, containing predominantly IpaB, were pooled, the OPOE concentration was made 1%, and the pools were incubated at room temperature for 30 min and then reappplied to the column according to the steps above to remove residual HTIpgC. The final purified IpaB product was stored at –80°C.

His-tagged IpaC (HTIpaC) was released from induced recombinant bacteria by suspension in binding buffer containing 6 M urea (52). The treated cells were sonicated and centrifuged, and the supernatant was stored frozen at –80°C prior to application to a 60-ml XK 26/10 nickel Sepharose HP (GE Healthcare) column previously washed and equilibrated in binding buffer containing 6 M urea (53). After washing, HTIpaC was eluted from the resin with elution buffer (20 mM Tris-HCl, 0.5 M NaCl, 1 M imidazole, 6 M urea, pH 7.9). The final sample was dialyzed against 20 mM Tris-HCl, 0.5 M NaCl, 6 M urea, pH 7.9, and the resulting purified HTIpaC was stored at –80°C.

LPS purification. LPS was extracted from *Shigella flexneri* 2a strain BS103 and *S. sonnei* strain Moseley by the procedure of Westphal and Jann (54). Purified LPS was lyophilized and analyzed by SDS-PAGE (silver stained) and by ELISA with serotype-specific monoclonal antibodies. Residual protein was accessed by the Bradford total protein assay (55). Protein levels were less than 0.15% by weight.

Purification of Invaplex_{NAT}. Native Invaplex (Invaplex_{NAT}) was purified by anion-exchange chromatography of water extracts of virulent shigellae as previously described (15). The final Invaplex_{NAT} product contained LPS, IpaB, IpaC, and many other proteins in low concentrations (see Table S1 in the supplemental material).

Production of artificial Invaplex (Invaplex_{AR}). Using the IpaC:IpaB molar ratio previously described (16), HTIpaC (8 μ M) was mixed with IpaB (1 μ M) and diluted in a buffer containing 20 mM Tris-HCl, 500 mM NaCl, 5 M urea, pH 7.9. Reaction mixtures that contained a final urea concentration less than 5 M were prone to precipitation as evidenced by marked turbidity of the solution. Reaction volumes of 5 to 25 ml were used. The IpaB-HTIpaC mixture was slowly added to a vessel containing dry *Shigella* LPS

(*S. flexneri* 2a strain BS103 LPS or *S. sonnei* strain Moseley LPS) at a ratio of 0.56 (mass/mass) of the total protein content of the final reaction mixture. After gentle swirling to solubilize the LPS, the vessel containing the “reaction mixture” was placed at 37°C and incubated with shaking at 200 rpm for 2 h. The reaction mixture was diluted with 4 volumes of 20 mM Tris-HCl, pH 7.9, at 37°C and applied to a 5-ml HiTrap Q HP column (GE Healthcare). The Invaplex product was eluted using the Invaplex_{NAT} chromatography purification strategy (15). A step gradient of 0%, 24%, 50%, and 100% buffer B (1 M NaCl in 20 mM Tris-HCl, pH 9.0) was used to elute fractions. Invaplex_{NAT} elutes in the 24% and 50% fractions. Elution fractions were analyzed for total protein by the bicinchoninic acid (BCA) assay (Pierce), and the presence of IpaB, IpaC, and LPS was analyzed by spot blotting. Fractions containing all three antigens were consistent with the Invaplex product and eluted in the 50% B step gradient (i.e., 500 mM NaCl). The resulting Invaplex_{AR} product was further characterized by SDS-PAGE, quantitative ELISA, and *Limulus* amoebocyte lysate (LAL) assay.

Electrophoresis, Western blotting assays, and ELISA. Polyacrylamide gel electrophoresis with Coomassie blue and silver staining and Western blotting assays were performed as previously described (10, 15). ELISAs were used to measure levels of antibodies to *S. flexneri* 2a LPS, IpaB, IpaC, and ovalbumin (OVA; Sigma-Aldrich). Primary antibodies were diluted in 2% casein and were incubated with the antigen for 2 h. After washing in phosphate-buffered saline (PBS)–Tween 20, plates were probed with anti-IgG or –IgA conjugated with alkaline phosphatase (mouse studies) or probed with anti-IgG conjugated with alkaline phosphatase or anti-IgA conjugated with horseradish peroxidase (guinea pig studies). The substrate used for assays utilizing alkaline phosphatase was *p*-nitrophenylphosphate (1 mg/ml in diethanolamine), and the optical density was measured at 405 nm. The substrate used for assays utilizing horseradish peroxidase was 3,3',5,5'-tetramethylbenzidine (TMB), the 30-min reaction was stopped with 1 N HCl, and optical density was measured at 450 nm.

Positive, negative, and background controls were assayed on each plate, and strict acceptability criteria (acceptability ranges) were employed to reduce assay variability. Geometric mean titers (GMTs) were determined for each group using the individual titers within that group at a specific time point. To calculate GMT, titers less than the limit of detection (LOD) were assigned a value of 1/2 LOD. A 4-fold increase in the GMT over baseline samples was considered seroconversion.

SEC-HPLC. A TSK-Gel G5000PW_{XL} 7.8-mm by 30-cm column (Tosoh Bioscience) with a 10- μ m particle size and an exclusion limit of 1×10^7 Da was calibrated using blue dextran (2 MDa), thyroglobulin (669 kDa), catalase (232 kDa), ovalbumin (43 kDa), and RNase A (13.7 kDa) (all from GE Healthcare) in 0.02 M Tris-HCl, 0.5 M NaCl, pH 9.0 (Invaplex_{AR} buffer), connected to a Shimadzu 10ADvp HPLC system. *S. flexneri* 2a Invaplex_{AR} (17.5 μ g) and Invaplex_{NAT} (70 μ g) were applied in separate runs under the same conditions at a flow rate of 0.5 ml/min and 400-lb/in² maximum pressure. Chromatographic traces were recorded both at 280 nm using an SPD-10Avp UV detector and at 215 nm using the SPD-M10Avp photodiode array.

DLS. Dynamic light scattering (DLS) experiments were performed using a Malvern Zetasizer μ V system using the Zetasizer software version 7.11 for the analysis of data. Fifteen microliters of each sample (Invaplex_{NAT} at 1.8 mg/ml in 20 mM Tris-HCl, 250 mM NaCl, pH 9.0, and Invaplex_{AR} at 234 μ g/ml in 20 mM Tris-HCl, 500 mM NaCl, pH 9.0) was transferred to a 2- μ l-window Hellma Analytics quartz cuvette. The sample chamber was cooled to 4°C, the laser power and attenuation were automatically adjusted for each run so that they were consistent for each scan, and the results for each run are the averages from 11 to 13 individual scans. The final size of the product is reported as the hydrodynamic radius and is the average from 5 runs with 1 standard deviation. The reported results represent the HMMC of Invaplex_{NAT} compared to the predominant species present in the Invaplex_{AR} sample as determined by mass.

Quantitative IpaB, IpaC, and LPS assays. The concentration of IpaB and IpaC in HP Invaplex fractions was determined using a modified ELISA procedure as previously described (16). The amount of *S. flexneri* 2a LPS in each Invaplex product was determined using the kinetic colorimetric EndoSafe-PTS device and EndoSafe cartridges from 0.1 to 10 endotoxin units (EU)/ml (Charles River, Inc.).

Immunogenicity and protective efficacy of Invaplex in animal models. The ability of Invaplex_{NAT} and Invaplex_{AR} to promote an immune response in BALB/cByJ mice (Jackson Laboratories) was tested in groups of 15 mice. Mice were immunized intranasally with either *S. flexneri* 2a Invaplex_{AR} (5, 2.5, 1, and 0.1 μ g) or Invaplex_{NAT} (10, 5, 2.5, 1, and 0.1 μ g) or saline on days 0, 14, and 28. A total antigen volume of 25 μ l was delivered in small drops applied to the external nares with a micropipette. Mice were bled on days 0, 28, 42, and 63. Three weeks after the final immunization (day 49), mice were challenged intranasally with a lethal dose of *S. flexneri* 2a strain 2457T (1.6×10^7 CFU/30 μ l) as described previously (15, 22). Prior to intranasal immunization or challenge, mice were anesthetized with a mixture of ketamine hydrochloride (40 mg/kg of body weight) and xylazine (12 mg/kg). After challenge, mice were monitored daily for weight loss and death for 14 days.

The ability of *S. sonnei* Invaplex_{AR} to protect against a homologous challenge with *S. sonnei* strain Moseley and a heterologous challenge with *S. flexneri* 2a strain 2457T was assessed by immunizing BALB/cByJ mice intranasally on days 0, 14, and 28 and challenging them on day 49 using a similar study design as outlined above. Groups of mice were immunized with *S. flexneri* 2a Invaplex_{AR} (2.5 μ g) or *S. sonnei* Invaplex_{AR} (2.5 μ g). Control groups were immunized with saline (negative control), and the positive-control groups were immunized with *S. flexneri* 2a Invaplex_{NAT} (5 μ g) or *S. sonnei* Invaplex_{NAT} (5 μ g). On day 49, mice were challenged with either the homologous challenge organism (1.6×10^7 CFU *S. flexneri* 2a in 30 μ l) or the heterologous challenge organism (8×10^6 CFU *S. sonnei* in 30 μ l).

Guinea pigs, Hartley strain (6 per group), were immunized intranasally with 25 (23, for Invaplex_{AR}), 5, 1, 0.1, or 0.01 μ g/dose of *S. flexneri* 2a Invaplex_{NAT} or Invaplex_{AR} or with saline. Prior to intranasal

immunization, guinea pigs were anesthetized with ketamine-xylazine. The antigen was delivered with a micropipette, 50 μ l per nostril. Guinea pigs were immunized on days 0, 14, and 28 and were bled on days 0, 28, 40, and 63. Four weeks after the third immunization, guinea pigs were challenged intraocularly with 3×10^8 CFU of *S. flexneri* 2a strain 2457T in 25 μ l and observed daily for 5 days for the occurrence of keratoconjunctivitis (23). Research was conducted under Walter Reed Army Institute of Research (WRAIR) IACUC-approved protocols 11-BRD-27 (mouse) and IB03-10 (guinea pig) in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the *Guide for the Care and Use of Laboratory Animals* (56).

PD₅₀ and ImmD₅₀. The dose of Invaplex necessary to achieve 50% protection (PD₅₀) and the dose of Invaplex necessary to seroconvert 50% of immunized animals (ImmD₅₀) were calculated for the mouse and guinea pig models using an adaptation of the Reed-Muench LD₅₀ formula (24). To determine the PD₅₀ in the mouse model, animals surviving challenge were scored as protected. In the guinea pig model, an eye was scored “negative” if the eye score was 0/1 and positive if the eye score was 2/3 on day 3 postinfection. To calculate the ImmD₅₀, a 4-fold or higher increase in the antigen-specific serum IgG endpoint titer over baseline was scored as positive in both animal models.

Adjuvant effect of *S. flexneri* 2a Invaplex_{AR}. The ability of Invaplex_{AR} to function as a mucosal adjuvant was determined in mice using OVA as a model protein antigen. BALB/cByJ mice (5 mice/group) were intranasally immunized on days 0, 14, and 28 with OVA (5 μ g) alone or OVA (5 μ g) combined with either Invaplex_{AR} (2.5 μ g), Invaplex_{NAT} (5 μ g), or cholera toxin (CT; 5 μ g). Blood was collected on days 0, 28, and 42. Lung washes were collected on day 42. Serum endpoint titers on days 0, 28, and 42 with specificity for Invaplex_{NAT} and OVA were determined by ELISA. *Shigella* antigen-specific antibodies in mucosal washes were also assessed.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/mSphere.00583-17>.

TABLE S1, DOCX file, 0.03 MB.

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