Mucosal Adjuvanticity of a *Shigella* Invasin Complex with DNA-Based Vaccines

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Protection against many infectious diseases may require the induction of cell-mediated and mucosal immunity. Immunization with plasmid DNA-based vaccines has successfully induced cell-mediated immune responses in small animals but is less potent in humans. Therefore, several methods are under investigation to augment DNA vaccine immunogenicity. In the current study, a mucosal adjuvant consisting of an invasin protein-lipopolysaccharide complex (Invaplex) isolated from *Shigella* spp. was evaluated as an adjuvant for DNA-based vaccines. Coadministration of plasmid DNA encoding the Orientia tsutsugamushi r56Karp protein with Invaplex resulted in enhanced cellular and humoral responses in intranasally immunized mice compared to immunization with DNA without adjuvant. Mucosal immunoglobulin A, directed to plasmid-encoded antigen, was detected in lung and intestinal compartments after Invaplex-DNA immunization followed by a protein booster. Moreover, immunization with Invaplex elicited *Shigella*-specific immune responses, highlighting its potential use in a combination vaccine strategy. The capacity of Invaplex to enhance the immunogenicity of plasmid-encoded genes suggested that Invaplex promoted the uptake and expression of the delivered genes. To better understand the native biological activities of Invaplex related to its adjuvanticity, interactions between Invaplex and mammalian cells were characterized. Invaplex rapidly bound to and was internalized by non-phagocytic, eukaryotic cells in an endocytic process dependent on actin polymerization and independent of microtubule formation. Invaplex also mediated transfection with several plasmid DNA constructs, which could be inhibited with monoclonal antibodies specific for IpaB and IpaC or Invaplex-specific polyclonal sera. The cellular binding and transport capabilities of Invaplex likely contribute to the adjuvanticity and immunogenicity of Invaplex.

Immunization with plasmid DNA encoding vaccine antigens holds the promise of inducing cell-mediated and humoral immunity while offering several advantages over traditional immunization technologies, including ease of production, low cost, and the ability to engineer a highly defined product (10). However, several obstacles must be overcome prior to the widespread use of DNA vaccines. For example, although potent cell-mediated and humoral responses have been induced in small animal models, the success of DNA-based vaccines in humans is limited (22). The modest immune responses elicited in humans have highlighted the need for methods to enhance the immunogenicity of DNA-based vaccines. Inducing antigen-specific immune responses after plasmid DNA immunization is dependent on either in vivo transfection of antigen-presenting cells (APCs), expression of intracellular peptide and presentation in major histocompatibility complex (MHC) class I by transfected nonprofessional APCs, or presentation of phagocytosed antigen, exported from transfected cells, in MHC class I or class II by APCs (6). Thus, several approaches have been used to improve the immunogenicity of DNA vaccines.

One effort to enhance DNA-derived immunity is to increase the effects of unmethylated immunostimulatory CpG motifs present in the DNA backbone (42). Other approaches include using either facilitated or targeted DNA delivery to appropriate immune cells or to increase the magnitude or duration of encoded gene expression via plasmid modifications (9). Cytoplasmic delivery of DNA can be achieved using particle bombardment or in vivo electroporation, recombinant bacterial and viral vectors, or admixtures of DNA and vaccine adjuvants (reviewed in reference 10).

Effective delivery of DNA to target cells has been achieved with several adjuvants, including liposomes (18), polymers, and microparticles (5). Since the initiation of immune responses is dependent on costimulation of T cells and can be achieved with toll-like receptor (TLR) signaling, several TLR agonists, such as monophosphoryl lipid A (39) and Hsp-70 (34), have also been investigated as adjuvants for DNA vaccines. Other adjuvants, such as QS-21 (40), alum salts (47), and two classical mucosal adjuvants, cholera toxin (CT) and LT, isolated from *Vibrio cholerae* (20) and the heat-labile toxin (LT) isolated from *Escherichia coli* (16), have also been investigated with limited success. Mucosal administration of DNA vaccines with CT or LT leads to the induction of mucosal antibody responses (20); however, the associated toxicity of both CT and LT suggest the need for additional mucosal adjuvants. Ideally, a mucosal DNA vaccine adjuvant would increase the cellular uptake of plasmid DNA and subsequent expression of encoded antigens while recruiting relevant immune cells to the site of antigen production, facilitating the induction of mucosal antibody-mediated immunity.

The *Shigella* invasin complex, or Invaplex, is a subcellular...
product currently under investigation as a vaccine to prevent shigellosis (46). Invaplex consists of the invasion plasmid antigen (Ipa) proteins IpaB and IpaC, in a native complex with Shigella lipopolysaccharide (LPS). After intranasal immunization of mice (46) or guinea pigs (31) with Invaplex, mucosal and systemic immune responses directed to the Ipa proteins and LPS are induced (15) and the resulting immune response is protective against challenge with virulent shigellae in animal models (31). Furthermore, phase 1 clinical studies in humans indicate that Invaplex is both safe and immunogenic, inducing a potent mucosal immune response (R. W. Kaminski and E. V. Oaks, unpublished results).

Invaplex also has inherent adjuvant properties (15), which are likely due to the native biological activities of the major antigenic components. The IpaB and IpaC proteins are used by wild-type shigellae to induce actin polymerization, phagocytosis, and eventual escape from phagolysosomes (reviewed in reference 43). The Ipa proteins induce uptake of latex beads (24) and noninvasive shigellae (23), although induced uptake of traditional vaccine antigens (proteins, polysaccharides, or plasmid DNA) has not been demonstrated. In addition, the LPS component of the complex likely engages TLR-4 on APCs, such as resident macrophages, beginning a cascade of events culminating in the release of chemokines and cytokines and eventual recruitment of additional APCs to the site of immunization. By potentially functioning as both an immunomodulator and as a vaccine delivery system, Invaplex has augmented the immunogenicity of several codelivered heterologous protein antigens, including ovalbumin and the protective antigen from Bacillus anthracis (15), colonization factors from enterotoxigenic E. coli (32), and a recombinant Campylobacter sp. FlaA protein (14).

The primary objective of this study was to determine if Invaplex can be used as a mucosal adjuvant to enhance the immunogenicity of DNA-encoded antigens. A candidate DNA vaccine (pKarp56) encoding the Sta56 gene from the Karp strain of Orientia tsutsugamushi induces protective immune responses in mice (29) and has been used as a model plasmid DNA vaccine. Studies in mice using the pKarp56 DNA vaccine and Invaplex as an adjuvant demonstrated enhanced cell-mediated and humoral immunity, with the induction of mucosal immunoglobulin A (IgA) directed to both Shigella and O. tsutsugamushi antigens. Using in vitro tissue culture models, it was determined that Invaplex is a capable transporter of plasmid DNA using an actin-dependent internalization mechanism.

MATERIALS AND METHODS

Purification of Shigella invasion complex (Invaplex). Invaplex 24 and Invaplex 50 were isolated from water extracts of virulent Shigella flexneri 2a (2457T) as previously described (46).

Mucosal delivery of plasmid DNA-based vaccine constructs with Invaplex. The ability of Invaplex 50 to enhance the immunogenicity of a plasmid DNA-based vaccine was evaluated in mice. Plasmid DNA (pKarp56) containing the Sta56 gene (30) from the Karp strain of O. tsutsugamushi linked to a cytomegalovirus (CMV) promoter was used as a model plasmid DNA vaccine construct (29). Female BALB/cByj mice (Jackson Laboratories, Bar Harbor, ME), age 6 to 8 weeks, were randomly separated into groups (10 mice/group) and intranasally immunized on days 0, 14, and 28 with pKarp56 alone (100 µg) or pKarp56 (25 µg or 100 µg) combined with Invaplex50 (15 µg). Controls for the study included groups of mice intranasally immunized with either Invaplex 50 (15 µg), saline, or the empty expression vector (pVR1012; 100 µg) combined with Invaplex 50 (15 µg). Five mice per group were boosted on day 56 with an intranasal immunization of purified recombinant Sta56 protein (r56Karp; 15 µg) combined with Invaplex 50 (5 µg). Vaccine formulations (total volume, 25 µl) were delivered intranasally as previously described (15). Research was conducted 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 National Research Council’s 1996 Guide for the Care and Use of Laboratory Animals (28).

Blood, mucosal wash, and spleen collection schedule. Animals were bled on days 0, 28, 35, and 42. Mice boosted with Invaplex-purified r56Karp protein were also bled on day 56 before immunization and on days 63 and 70. Lung and intestinal washes were collected on days 42 and 70 as previously described (15). Spleens were aseptically removed on day 42 (after DNA immunization) or day 70 (after Invaplex-r56Karp protein boost). Single-cell suspensions of individual spleens were treated with ammonium chloride to lyse the red blood cells, washed, counted, and suspended in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 200 mM l-glutamine, and 5 µM 2-mercaptoethanol (crPME) to a final concentration of 1 × 10^6 cells/ml.

Assessment of antigen-specific serum IgG and IgA responses and cellular proliferation. Antigen-specific antibody responses were assessed in serum and mucosal samples by an enzyme-linked immunosorbant assay (ELISA) as previously described (46). Coating concentrations of the various antigens plated at 50 µl/well were Invaplex 50 at 1 µg/ml and the r56Karp protein at 3 µg/ml. Splenocytes were evaluated for antigen-specific proliferation as previously described (15) by culturing lymphoid cells (1 × 10^6 cells/ml) in triplicate with either the r56Karp protein (20 µg/ml) or Invaplex 24 (5 µg/ml). Additional cells were incubated with concanavalin A (ConA; 5 µg/ml; Sigma). Stimulation indices (SI) were calculated by dividing the mean optical density at 490 nm (OD_{490}) of antigen-stimulated cells by the mean OD_{490} of cells from the same animal cultured with crPME without antigen. Data are expressed as the mean SI for each group of mice (n = 5) ± 1 standard error from the mean (SEM). Cytokine concentrations in culture supernatants from cells collected on day 70 from immunized animals (n = 5/group) after ex vivo stimulation with r56Karp were determined using a multiplexed Luminex-based assay as previously described (15).

Cell culture conditions and cytotoxicity assays. The baby hamster kidney fibroblast cell line (BHK-21; CCL-10) was obtained from the American Type Culture Collection (Manassas, VA) and cultured at 37°C in a humidified atmosphere of 95% air and 7% CO2 in minimal essential medium (MEM; Invitrogen, Carlsbad, CA) with 7% FCS (Sigma, St. Louis, MO).

For cytotoxicity, transfection, and internalization experiments (see below), BHK-21 cells (2.5 × 10^5 cells/well) were cultured overnight at 37°C in Lab-Tek II glass chamber slides (Nalge Nunc International, Naperville, IL) resulting in a 70 to 80% confluent monolayer. BHK-21 cells were washed three times with MEM and incubated in triplicate for 2 h at 37°C with an isotonic solution of S. flexneri 2a Invaplex 24 or S. flexneri 2a Invaplex 50 diluted to 100 or 500 µg/ml in MEM. The level of lactate dehydrogenase (LDH) in the culture medium was determined as a measure of cytotoxicity (CytoTox 96; Promega, Madison, WI). The percentage of cells exhibiting cytotoxic effects was calculated as the ratio of experimental LDH release (OD_{570}) divided by the maximum LDH release (induced with Triton X treatment) multiplied by 100. Staining of Invaplex-treated cells with trypan blue was used as an additional measure of cellular toxicity (17, 50).

Cellular internalization of Shigella Invaplex. Subconfluent BHK-21 cells were washed twice with MEM and incubated with S. flexneri 2a Invaplex 24 or Invaplex 50 (100 µg/ml in MEM) at 37°C for 1, 5, 15, 30, or 60 min. Controls consisted of BHK-21 monolayers incubated with MEM for the same time periods. After incubation, the monolayers were washed twice with phosphate-buffered saline (PBS) and fixed for 10 min with 10% formalin at 23°C. Cell-associated Invaplex was detected as detailed below.

Invaplex-mediated transfection of mammalian cells with plasmid DNA. The ability of Invaplex to mediate the transfection of cells with plasmid DNA was assessed by incubating duplicate BHK monolayers for 4 h at 37°C with 2.5 µg/ml of plasmid DNA encoding gfp linked to a CMV promoter (pEgfp-N1; Gene Therapy Systems), plasmid DNA encoding lacZ linked to a CMV promoter (gWIZ β-galactosidase [β-Gal] plasmid; Gene Therapy Systems), or plasmid DNA encoding the Sta56 gene (pKarp56) (29), and either Invaplex 24 or Invaplex 50 diluted to either 10, 100, or 500 µg/ml. The FCS concentration was adjusted to 10%, after which the cells were incubated for an additional 18 h at 37°C. Monolayers incubated with MEM alone or with 2.5 µg/ml of plasmid DNA and 10% of Geneporter (Gene Therapy Systems) served as negative and positive controls, respectively. After overnight incubation, cells were washed three times with PBS, fixed with methanol (gfp and the Sta56 gene) or 10% formalin (β-Gal) for 10 min at 23°C, and examined for protein expression as indicated below.
Detection of intracellular Invaplex, GFP, r56Karp and β-Gal protein. Cells (>200 cells per treatment) located within five randomly chosen fields in each monolayer were scored as being green fluorescent protein (GFP) positive or GFP negative based on the presence or absence of intracellular green fluorescence, respectively, using epifluorescence microscopy. Intracellular β-Gal protein was detected by incubating the fixed monolayers at 23°C for 90 min with 5-bromo-4-chloro-3-indoly-β-D-galactopiranoside staining buffer (Genetic Therapy Systems). Each monolayer was examined by bright-field microscopy and the cells were scored as β-Gal positive (blue color) or β-Gal negative. Intracellular r56Karp protein was detected by indirect immunofluorescence microscopy by incubating treated cells with r56Karp-specific monoclonal antibody (K13F85A) (44) for 2 h at 23°C. After washing with PBS containing 0.1% saponin (PBS-SAP), bound monoclonal antibody (Mab) was detected with goat anti-mouse IgG antibody conjugated to Texas Red (Invitrogen). Intracellular Invaplex was detected in fixed cells by first probing with anti-Shigella flexneri 2a (2457T) mouse sera followed by goat anti-mouse IgG antibody conjugated to Oregon Green 488 (Invitrogen). The Golgi apparatus was stained in some experiments to serve as a cytoplastological landmark with wheat germ agglutinin conjugated to Texas Red (WGA-TR; Molecular Probes) diluted to 10 µg/ml (48) and the nucleus with 4',6-diamidino-2-phenylindole (DAPI, Molecular Probes) diluted to 2 µg/ml. The mean percentage of reporter protein-positive cells of three separate experiments was determined.

Inhibition of Invaplex internalization with MAbs and polyclonal sera. S. flexneri 2a Invaplex 24 (100 µg/ml in MEM) was incubated with either anti-Shigella polyclonal sera or MAbs diluted 1:100 or 10 µg/ml, respectively, for 30 min at 23°C. The polyclonal sera used for inhibition were hyperimmune mouse serum pooled 10 (HMSP 10) and HMSP 8, both with specificity for S. flexneri 2a LPS (12). MAbs used for inhibition included affinity-purified 2F1 (anti-IpaB) (27), 2G2 (anti-IpaC) (27), and 2E8 (anti-S. flexneri 2a LPS) (12). The antibody-Invaplex mixtures were then incubated with duplicate BHK-21 monolayers for 30 min at 37°C. Controls included monolayers treated with mouse sera in the absence of Invaplex and separate monolayers treated with Invaplex pretreated with naïve mouse sera (NMS) or Invaplex alone to establish the level of Invaplex internalization in the absence of blocking antibody. After incubation, the monolayers were washed three times with PBS to remove non-cell-associated Invaplex and fixed with formalin for 10 min at 23°C.

Internalized Invaplex was detected in BHK-21 cells by probing formalin-fixed cells with polyclonal rabbit sera (Rabbit 7) specific for Shigella antigens. After washing with PBS-SAP, bound anti-Invaplex antibodies were detected with antirabbit IgG–Oregon Green 488-labeled antibody (Invitrogen). A combination of bright-field and epifluorescence microscopy was used to score cells in duplicate monolayers (>100 cells per monolayer) as either Invaplex positive or Invaplex negative. The degree of Invaplex uptake inhibited by immune sera was calculated using the following formula: 1 - [(percentage of Invaplex-positive cells after treatment with Shigella-specific antibodies)/(percentage of Invaplex-positive cells after treatment with normal serum)] × 100.

Inhibition of Invaplex-mediated DNA transfection with antibodies. Invaplex 24 (75 µg/ml) or Invaplex 50 (35 µg/ml) was incubated for 30 min with anti-IpaC Mab 2G2 at 0.1, 1.0, or 10 µg/ml, anti-Shigella polyclonal mouse serum (HMSP 10 diluted 1:100, 1:500, or 1:5,000), or normal mouse serum diluted 1:100. Antibody-Invaplex mixtures were then incubated with pEGFP-N1 (2.5 µg/ml) for 30 min prior to incubation with BHK-21 cells. The percentage of GFP-positive cells was determined after each treatment and is expressed as the mean ± standard error from three experiments.

Invaplex internalization in the presence of cytoskeletal inhibitors. BHK-21 cells were incubated with either cytochalasin B (0.1, 1.0, or 10 µg/ml; Sigma) or colchicine (0.1, 1.0, or 10 µg/ml; Sigma), both diluted in MEM, or MEM alone at 30 min at 37°C. After treatment, BHK-21 cells were washed and then incubated (30 min, 23°C) with S. flexneri 2a Invaplex 24 (100 µg/ml) diluted in either MEM or MEM supplemented with colchicine (0.1, 1.0, or 10 µg/ml) or cytochalasin B (0.1, 1.0, or 10 µg/ml). After incubation, the cells were washed with PBS and fixed with 10% formalin. The percentage of cells positive for Invaplex was determined for each treatment as described above. To verify disruption of filamentous actin or microtubule formation, treated cells were probed with either Texas Red-labeled phalloidin (Invitrogen) or with mouse anti-α-tubulin (Sigma) followed by anti-mouse IgG–Oregon Green 488 (Invitrogen) to detect bound anti-α-tubulin antibodies.

Statistical analysis. The Prism 4 for Macintosh computer program (GraphPad Software, Inc., San Diego, CA) was used for calculations and statistical comparisons. Comparisons between group arithmetic means were performed using a one-way analysis of variance with a Bonferroni post hoc test (stimulation indices) or chi-square tests (cellular internalization inhibition assays). Comparisons between group mean serum endpoint titers were accomplished using a two-way analysis of variance with a Bonferroni post hoc test of log-transformed antibody titers.

RESULTS

Invaplex augments systemic antibody responses to DNA-encoded vaccine immunogens. Shigella Invaplex functions as an adjuvant, enhancing systemic and mucosal immune responses to codelivered protein antigens (15). Therefore, it was of interest to investigate the ability of Invaplex to enhance the immunogenicity of DNA vaccines. An investigational plasmid DNA vaccine encoding the 56-kDa outer membrane protein gene from O. tsutsugamushi (pKarp56) confers protection against scrub typhus in a mouse model (29) and was used as a model DNA vaccine for the adjuvanticity studies. The immune responses in mice intranasally immunized with pKarp56 (25 and 100 µg) admixed with Invaplex were compared to mice immunized with plasmid DNA alone, pVR1012 (empty vector) admixed with Invaplex, saline, or Invaplex. Five of the 10 animals in each group were boosted 4 weeks after the final DNA immunization with Invaplex combined with purified r56Karp protein (DNA prime, protein boost regimen). r56Karp-specific serum IgG responses (Fig. 1, top panel) were below detection limits in groups immunized with pKarp56 alone, Invaplex alone, Invaplex combined with pVR1012, or saline at all time points assayed, including on days 63 and 70, after one immunization with r56Karp protein combined with Invaplex. Mice immunized with Invaplex plus pKarp56 (25 or 100 µg) did not have detectable levels of r56Karp-specific serum IgG prior to the Invaplex-r56Karp protein booster immunization. However, mice primed with Invaplex plus pKarp56 (25 or 100 µg) and boosted with r56Karp protein codelivered with Invaplex had detectable r56Karp-specific serum IgG levels 1 week after the protein boost (day 63) and endpoint titers greater than 256 on day 70 that were significantly higher (P < 0.002) than titers in mice immunized with saline or pKarp56 alone and boosted with r56Karp protein plus Invaplex (Fig. 1, top panel).

The serum antibody response to Invaplex was also measured throughout the duration of the study to determine if Shigella-specific immunogenicity was retained. Mice immunized with Invaplex alone, Invaplex plus pVR1012, or Invaplex plus pKarp56 (25 or 100 µg) produced high levels of Invaplex-specific serum IgG after three immunizations, with subsequent increases in titer on day 70, 2 weeks after the booster immunization with r56Karp protein plus Invaplex (Fig. 1, lower panel). As expected, mice that were intranasally administered saline or pKarp56 alone did not mount a detectable Invaplex-specific serum IgG response, even after one immunization with Invaplex combined with r56Karp protein.

Enhancement of antigen-specific cellular proliferation. Antigen-specific proliferative responses after immunization were also measured as an indicator of cell-mediated immunity. Splenocytes from 5 of the 10 mice per group were collected after DNA immunization on day 42 and from the other 5 animals on day 70. Splenocytes were stimulated in vitro with either purified r56Karp protein or Invaplex. Splenocytes from all animals in each treatment group proliferated to comparable levels in response to in vitro stimulation with ConA (data not shown). After DNA immunization (day 42), the r56Karp-specific proliferative response after immunization with Invaplex...
FIG. 1. Anti-r56Karp and anti-Invaplex serum IgG endpoint titers after intranasal immunization of mice with pKarp56 alone or combined with Invaplex. Groups of mice (n = 10/group) were intranasally immunized on days 0, 14, and 28 with plasmid DNA (indicated below the x axis with arrows) encoding the Sta56 gene (pKarp56; 100 μg) or pKarp56 (25 or 100 μg) combined with Invaplex. Control groups were immunized with saline, Invaplex alone, or Invaplex combined with the empty expression vector (pVR1012). A subset of mice (n = 5/group) was boosted on day 56 (indicated with an arrow) with purified r56Karp protein (15 μg) combined with Invaplex (5 μg). Blood collected on days 0, 28, 35, 42, 56, 63, and 70 was assessed by ELISA for r56Karp (top panel) and Invaplex-specific (lower panel) serum IgG endpoint titers. The mean endpoint titers for each group are indicated on the y axis and time (in days) is shown on the x axis. Asterisks indicate statistically significant differences (two-way ANOVA with Bonferroni post hoc; P < 0.05) of log-transformed endpoint titers compared to groups inoculated with saline and pKarp56.
plus pKarp56 (25 and 100 μg) was significantly higher \((P < 0.002)\) than the r56Karp-specific proliferative responses detected after immunization with pKarp56 alone (100 μg) (Fig. 2A). The mean SI in groups of mice immunized with Invaplex plus pKarp56 (25 μg) after in vitro incubation with r56Karp protein was similar \((P = 0.12)\) to the mean SI from mice immunized with Invaplex plus pKarp56 (100 μg). Lower levels of r56Karp-specific proliferation were detected in splenocytes collected on day 70 (Fig. 2C). Invaplex-specific proliferation (Fig. 2B and D) was also detected in splenocytes collected on days 42 and 70 from mice immunized with Invaplex alone, Invaplex plus pVR1012, or Invaplex plus pKarp56 (25 or 100 μg).

**Mucosal antibodies elicited after immunization with Invaplex and pKarp56.** Systemic antibodies and cell-mediated immunity likely contribute to the protection against scrub typhus afforded after immunization with pKarp56 (29). Enhanced levels of both systemic antibody and cell-mediated immune cell proliferation were achieved after immunization with pKarp56 combined with Invaplex compared to immunization with pKarp56 alone. However, protection against mucosal pathogens, such as *Shigella* spp., may require mucosal IgA responses at the initial entry site. In the current study, the mucosal IgA responses in lung and intestinal washes were assessed after DNA vaccination (day 42) and after DNA vaccination followed by a protein booster immunization (day 70) to determine if Invaplex immunization induced *Shigella*-specific mucosal responses. In addition, r56Karp-specific mucosal IgA responses were also studied to determine if mucosal administration of Invaplex with a DNA vaccine could stimulate mucosal responses to DNA-encoded protein. After DNA immunizations, r56Karp-specific IgA responses in the lung and intestinal compartments were below detectable levels on day 42 from all immunized groups (Fig. 3). However, after a single booster immunization with r56Karp protein combined with Invaplex,
r56Karp-specific IgA levels were significantly increased ($P < 0.01$) in mucosal washes from groups immunized with pKarp56 (25 or 100 $\mu$g) combined with Invaplex compared to groups primed with pKarp56, saline, Invaplex, or Invaplex combined with pVR1012 and boosted with Invaplex plus r56Karp (Fig. 3, top panels). In addition, groups of mice intranasally immunized with Invaplex coadministered with empty vector or pKarp56 had similar levels of Invaplex-specific IgA in lung and intestinal washes as mice immunized with Invaplex alone ($P > 0.47$) and significantly higher than those induced after saline administration ($P < 0.05$). After the r56Karp-Invaplex booster immunization, Invaplex-specific mucosal immune responses were also boosted to higher levels than those measured in samples collected on day 42 (Fig. 3, bottom panels).

**Immune response phenotype.** The phenotypes of the immune response directed to r56Karp were also determined using serum IgG subclass and secreted Th1 and Th2-like cytokine profiles. Serum samples collected on day 70 were analyzed by ELISA for r56Karp-specific IgG1 and IgG2a endpoint titers (Fig. 4A). The r56Karp-specific IgG1 and IgG2a endpoint titers in animals immunized with pKarp56 (25 or 100 $\mu$g) combined with Invaplex were of comparable magnitude, indicating a balanced Th1/Th2 response. These observations were confirmed by measuring the concentration of cytokines secreted during ex vivo r56Karp stimulation of lymphocytes from immunized animals (Fig. 4B). Both Th1 cytokines (gamma interferon [IFN-γ] and interleukin-12 [IL-12]) and cytokines secreted from Th2 cell populations (IL-4 and IL-10) were detected in supernatants after lymphocyte stimulation (Fig. 4B).

**Cellular uptake of Invaplex and lack of cytotoxicity.** The adjuvanticity of Invaplex with DNA-based vaccines suggested that Invaplex is able to stimulate the delivery of plasmid DNA into mammalian cells, leading to protein expression. Other adjuvants, such as liposomes, have intracellular delivery capabilities and have been utilized to enhance DNA vaccine immunogenicity (19). As Invaplex contains IpaB and IpaC, which are...
required for *Shigella* entry into mammalian host cells (3, 13, 38), further studies were undertaken to determine if the complex had the capacity to stimulate or enhance uptake of biomolecules by mammalian cells.

Initial studies characterized the interactions of Invaplex with host cells to include toxicity and uptake experiments. Both preparations of Invaplex, Invaplex 24 and Invaplex 50, were found to be nontoxic at concentrations ranging from 100 to 500 μg/ml using LDH release and trypan blue exclusion assays to assess membrane integrity and cytotoxicity (data not shown). To determine if Invaplex retained the cellular internalization capacity attributed to the Ipa proteins, mammalian cells were pulsed with Invaplex, stained, and examined using immunofluorescence microscopy after various incubation times. Similar observations were made for both Invaplex 24 and Invaplex 50. After a brief 1-min incubation, Invaplex-treated monolayers were characterized by Invaplex-specific fluorescence localized to several (2 to 15) regions or patches on the surface of BHK-21 plasma membranes. Overlays of images captured using bright-field and epifluorescence microscopy (Fig. 5A and B) indicated that the Invaplex-specific fluorescence was on the cell surface, in different-sized patches, colocalized with the

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**FIG. 4.** Phenotype of the r56Karp-specific immune response after intranasal immunization of mice. r56Karp-specific serum IgG subclass endpoint titers were determined by ELISA on day 70, after intranasal immunization of mice with various vaccine formulations and a r56Karp-Invaplex booster immunization, to assess the phenotype of the immune response (top two panels). The concentrations of Th1 (IFN-γ and IL-12) and Th2 (IL-4 and IL-10) cytokines secreted into culture supernatants from lymphocytes collected on day 70 from immunized animals after ex vivo stimulation with purified r56Karp were determined using a multiplexed Luminex-based assay.
host cell plasma membrane on approximately 25 to 45% of cells incubated with Invaplex. After 5 min, Invaplex was internalized and located within the cell cytoplasm (Fig. 5C). The Invaplex-specific fluorescent staining pattern was punctate, with a random distribution of the fluorescence throughout the peripheral and perinuclear cytoplasm, generally located closer to the cell plasma membrane than to the nucleus. The Invaplex-specific fluorescent staining pattern detected after a 15-min incubation period was also punctate and located in both the peripheral and perinuclear cytoplasm. However, a strong Invaplex-specific fluorescent signal was juxtaposed with the nucleus and appeared as condensed cytoplasmic vesicles forming a distinct, circular ring around the nucleus (Fig. 4D), differentiating the 15-min observations from the 5-min incubation time point. The Invaplex-specific fluorescent staining pattern after a 30-min incubation was located primarily in the perinuclear cytoplasm, appearing as condensed, punctate structures or patches of fluorescence (Fig. 5E). The majority of the Invaplex-specific fluorescent activity was either localized to one side of the nucleus next to the Golgi apparatus or observed with the Golgi apparatus, stained with Texas Red-labeled WGA. Concomitantly, there was a reduction in the intensity of the Invaplex-specific punctate structures in the peripheral cytoplasm of the cell, suggesting a trafficking of Invaplex from the peripheral cytoplasm to the perinuclear cytoplasm. Observation of the Invaplex-specific fluorescent staining after a 60-min incubation of Invaplex with BHK-21 cells revealed that Invaplex was located in the perinuclear cytoplasm and in close proximity to the Golgi apparatus. In addition, a diffuse Invaplex-specific staining pattern was observed throughout the cytoplasm (Fig. 5F), contrasting with the punctate staining pattern visualized at early time points. The diffuse fluorescent

FIG. 5. Intracellular location of S. flexneri 2a Invaplex 24 after 1, 5, 15, 30, or 60 min of incubation with mammalian cells. BHK-21 fibroblasts were incubated with S. flexneri 2a Invaplex 24 for either 1 (A and B), 5 (C), 15 (D), 30 (E), or 60 (F) min. After treatment, formalin-fixed cells were probed with anti-S. flexneri antibodies which were subsequently detected with fluorophore-labeled species-specific antibodies (green). As a cytological landmark, the Golgi apparatus (5, 15, 30, and 60 min) and nucleus (at only 30 and 60 min) were stained with WGA-TR (red) and DAPI (blue), respectively. Overlays of digitally captured red, blue, and green fluorescent images are presented for each time point. Arrows indicate patches of Invaplex on the host cell surface (A and B).
The cellular uptake and trafficking of Invaplex and the in vivo data demonstrating gene expression suggested that other substances, either physically associated with the complex or in close proximity to Invaplex during the endocytic event, may also be cointernalized by the host cell. Transfection potential was determined by incubating BHK cells with mixtures containing Invaplex 24 or Invaplex 50 (10 to 500 μg/ml) and various plasmid DNA constructs including pKarp56 (Table 1). Incubation of BHK-21 cells with plasmid DNA alone did not result in significant intracellular protein expression, with less than 1% of cells scored as target protein positive after treatment. Expression of protein was detected in 3 to 8% of cells after incubation of cells with plasmid DNA and 10 μg/ml of either Invaplex 24 or Invaplex 50. An increase in the concentration of Invaplex used for transfection from 10 to 100 μg/ml resulted in increases in the number of cells expressing the target protein, with 17% of the cells incubated with pEGFP-N1 and Invaplex 24 (Table 1 and Fig. 6A) and 24% of cells incubated with pEGFP-N1 and Invaplex 50 (Table 1 and Fig. 6B). Moderate levels of transfection were achieved using the pgWIZβ-gal plasmid, with 14 to 28% of treated cells expressing enzymatically active β-galactosidase (Table 1 and Fig. 6D and E). Using the pKarp56 plasmid (Table 1 and Fig. 6G and H), 23% and 30% of cells treated with pKarp56 mixed with either Invaplex 24 or Invaplex 50, respectively, tested positive for r56Karp expression. Fivefold increases in the concentrations of Invaplex (from 100 μg/ml to 500 μg/ml) did not result in equivalent increases in the percentage of cells scored as target protein positive.

**Internalization of Shigella Invaplex in the presence of cytoskeletal inhibitors and inhibition with polyclonal sera or monoclonal antibodies.** Several inhibition experiments were designed to aid in defining which Invaplex components interacted with host cells and resulted in internalization. It was hypothesized that the IpaB and IpaC components of Invaplex would play an essential role in the cellular internalization pro-

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**TABLE 1. Invaplex-mediated plasmid DNA transfection of mammalian cells in vitro**

| Transfection mediator | Concen (μg/ml) | Mean % transfection with indicated plasmid | pEGFP-N1 | pKarp56 | pgWIZβ-gal |
|-----------------------|---------------|-------------------------------------------|---------|---------|------------|
| Invaplex 24           | 10            | 5 ± 2                                     | 8 ± 2   | 6 ± 2   |            |
|                       | 100           | 17 ± 2*                                   | 23 ± 6* | 19 ± 3* |            |
|                       | 500           | 20 ± 1*                                   | 32 ± 9* | 14 ± 2* |            |
| Invaplex 50           | 10            | 3 ± 2                                     | 8 ± 1   | 6 ± 1   |            |
|                       | 100           | 24 ± 6*                                   | 30 ± 6* | 28 ± 1* |            |
|                       | 500           | 23 ± 2*                                   | 29 ± 5* | 20 ± 5* |            |
| Culture medium        | 0             | <1                                        | <1      | <1      |            |
| Geneporter            | 10 μl         | 60 ± 6*                                   | 48 ± 4* | 67 ± 12*|            |

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* The mean percent transfection from three separate experiments ± 1 standard deviation was calculated by dividing the number of cells expressing the indicated protein by the total number of cells counted (≥200). Values significantly different (P < 0.05) from the culture medium controls are indicated with an asterisk.

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**FIG. 6. Invaplex-mediated transfection of mammalian cells with plasmid DNA.** BHK-21 cells were incubated with plasmid DNA encoding either gfp, lacZ, or Sta56 genes in the absence (C, F, and I, respectively) or presence of Invaplex 24 (A, D, and G) or Invaplex 50 (B, E, and H). Protein expression was assessed 18 h posttransfection as described in Materials and Methods. Images of cells were collected at 10× (A through F) or 60× (G, H, and I) magnification.
cess, similar to their role in *Shigella*-induced phagocytosis (24). Biochemicals such as cytochalasin B inhibit actin polymerization and inhibit the induced uptake of *shigellae*, whereas inhibitors of microtubule formation (colchicine, vincristine, and vinblastine) do not affect *shigellae* internalization by mammalian cells (8, 11). BHK-21 cells pretreated with cytochalasin B (0.1 μg/ml) had a 66% decrease (P < 0.001) in the percentage of cells with Invaplex-specific fluorescence compared to Invaplex-treated cells in the absence of cytochalasin B (Table 2). Increases in the concentration of cytochalasin B further reduced the percentage of cells containing internalized Invaplex. The internalization of *Shigella* Invaplex into BHK-21 cells pretreated with colchicine (0.1 or 1 μg/ml) had little effect (Table 2) compared to cells not treated with colchicine.

Specific components of the invasin complex and their role in the internalization process were further investigated using *Shigella*-specific antibodies. In the absence of antibodies, approximately 60% of cells exhibited an Invaplex-specific fluorescent staining pattern after a 30-min incubation with Invaplex. Hyperimmune mouse serum pools (HMSP 8 and 10) inhibited internalization of Invaplex into BHK cells at levels greater than 82% (Table 2). The antigen specificity of the hyperimmune sera included reactivity with IpaB, IpaC, and LPS. Monoclonal antibodies specifically recognizing IpaC (MAb 2G2) also exhibited significant inhibition of Invaplex internalization at levels comparable to the hyperimmune sera. Pretreatment of Invaplex with MAb 2F1, which recognizes IpaB, resulted in 40% inhibition, whereas pretreatment with anti-LPS MAb 2E8 or NMS did not significantly reduce the ability of Invaplex to induce uptake and internalization.

### Inhibition of Invaplex-mediated DNA transfection with antibodies

To determine if inhibitors of Invaplex internalization would also inhibit the transport capacity of the complex, Invaplex was incubated with MAb 2G2 or HMSP 10 and then the antibody-Invaplex combination was used to transfect cells with the pEGFP-N1 plasmid (Fig. 7). Preincubation of Invaplex 24 or Invaplex 50 with MAb 2G2 (1 or 10 μg/ml) reduced (P <...
0.01) the transfection capacity of Invaplex, suggesting a major role of IpaC in the functionality of Invaplex as a DNA transfection agent. Polyclonal serum (HMSP 10), with antibodies specific for IpaB, IpaC, and LPS, also reduced the transfection capacity of Invaplex to levels two to four times lower \((P < 0.01)\) than those achieved in the absence of antibody or in the presence of nonimmune serum (data not shown). In both sets of experiments, increased quantities of antibodies resulted in higher levels of inhibition.

**DISCUSSION**

Vaccine-induced protection against many infectious diseases may require the generation of both antigen-specific cell-mediated immunity and mucosal antibodies, since many intracellular pathogens initially enter the host via mucosal surfaces. DNA-based vaccination has the potential to induce potent cell-mediated immunity, as demonstrated in several animal models; however, the results in humans have been less robust (22). Thus, several strategies have been developed to enhance the immunogenicity of DNA vaccines.

In the current study, the utility of Invaplex as a mucosal adjuvant for protein-based vaccines was expanded to DNA-based vaccines. Intranasal immunization with Invaplex and plasmid DNA constructs induced antigen-specific cell-mediated and humoral immunity. Cell-mediated immunity was induced after immunization with DNA combined with Invaplex, whereas humoral immunity required a booster immunization consisting of purified protein delivered intranasally with Invaplex. The lack of r56Karp-specific antibody responses after three DNA immunizations was not surprising in that DNA vaccines stimulate poor antibody responses (37). However, when DNA vaccines are used to prime followed by a protein antigen boost (DNA prime-protein booster vaccine regimen), higher levels of humoral immunity are achieved, compared to DNA immunization without adjuvants or combined with proteins (16). Similar results were achieved in these studies with antibody responses induced to detectable levels only after r56Karp protein-Invaplex booster immunization. Four intranasal immunizations with pKarp56 (100 \(\mu g\)) previously resulted in 63% protection against scrub typhus infection in a CD-1 outbred mouse model and modest serum antibody responses (29). The differences in serum responses after DNA immunization between the two studies could have been a product of the route of immunization, number of immunizations, or strain of mouse used for the studies.

After one protein booster immunization, antigen-specific mucosal IgA responses were detected in lung and intestinal compartments, indicating that delivery of DNA vaccines with Invaplex via the mucosal route had primed the mucosal responses. Furthermore, the phenotype of the immune response was determined to be a balanced Th1/Th2 response, with similar levels of r56Karp-specific serum IgG1 and IgG2a endpoint titers and the secretion of IFN-\(\gamma\), IL-12, IL-4, and IL-10 from ex vivo-stimulated lymphocytes. Inducing both mucosal IgA responses and cell-mediated immunity with a balanced phenotype may be critical to inducing protective immune responses against many mucosal intracellular bacterial and viral pathogens, such as human immunodeficiency virus and *Mycobacterium tuberculosis*. Cell-mediated and humoral immune responses were directed to both Invaplex antigens and antigens encoded on the plasmid DNA, indicating the potential development of combination vaccine strategies. Moreover, since preexisting immunity to Invaplex does not negate the adjuvant effects of the complex (15), Invaplex could potentially be used in multiple clinical scenarios as either a vaccine to prevent shigellosis or as an adjuvant with protein-based (15) or DNA-based vaccines.

Adjuvant technologies used to enhance DNA vaccines can be loosely categorized into groups that function as immunostimulatory adjuvants, adjuvants that protect DNA from degradation, or adjuvants that induce the cellular uptake of the DNA vaccine (9). TLR-4 agonists, such as monophosphoryl lipid A, which enhances humoral responses directed to DNA-encoded antigens, likely function by increasing proinflammatory cytokine or chemokine secretion by APCs, proving necessary immune stimulation and driving a Th1-biased immune response. Other technologies, such as liposomes, protect DNA from degradation by serum proteins and facilitate the transfer of DNA across cellular membranes through an endocytic process into the cytosol (52). Several approaches to enhance immunogenicity of DNA-derived immunity combine an immunostimulatory adjuvant with a vaccine delivery technology to simultaneously achieve both efficient DNA delivery and immune stimulation, as is the case with DNA-encoded antigens formulated with TLR agonists and liposomes (51). Although the mechanism of adjuvant activity employed by Invaplex is unknown, it is likely that the complex is capable of functioning as both an immunostimulatory adjuvant and as an intracellular delivery transporter. One goal of the current study was to better understand the biological functionality of the complex as it relates to adjuvanticity and immunogenicity. Incubation of nonphagocytic cells with Invaplex resulted in the internalization of Invaplex and the intracellular trafficking of the complex from the host cell membrane to a perinuclear location, evidenced by the differential staining patterns after increasing incubation times. Invaplex also induced the uptake of plasmid DNA constructs in vitro, which resulted in successful transfection of mammalian cells in a nontoxic manner. Invaplex-mediated uptake of DNA may have occurred as a result of interactions between Invaplex and DNA prior to treatment of cells through noncovalent or electrostatic forces that might result in a more compact form of the DNA, as reported for liposome-DNA complexes (7). The ability of Invaplex to induce higher transfection efficiency in vitro compared to plasmid DNA alone likely contributes to the higher levels of immunogenicity after immunization with Invaplex-pKarp56 DNA vaccines compared to pKarp56 DNA vaccine delivered in the absence of an adjuvant.

Previous studies have addressed the essential role of the invasins IpaB and IpaC in the cellular uptake of wild-type shigellae (13, 24). The proteins are stored in the cytoplasm of the bacterial cell, associated with the chaperone IpgC, and secreted through a type III secretion apparatus upon cellular contact (24–26, 33, 49). A major role of the Ipa proteins in *Shigella* pathogenesis is to remodel cellular actin, either through polymerization (45) or destabilization of F-actin (2), creating a filopodium structure on the host cell surface that is used to engulf and internalize the bacterium. In the current study, the cellular internalization of Invaplex was significantly
inhibited by both cytochalasin B, which interferes with actin polymerization, and with MAb directed to IpaB and IpaC, indicating that the Ipa proteins are also involved with the actin-dependent internalization of Invaplex. Furthermore, IpaC-specific MAb inhibited the Invaplex-mediated uptake of plasmid DNA encoding GFP, providing additional evidence that IpaC plays a central role in Invaplex-mediated cellular uptake and transfection.

Once translocated across the cellular membrane, the Invaplex-specific staining pattern was punctate, indicative of containment within endocytic vesicles (36). Presumably, plasmid DNA transported with Invaplex would also be contained within the endocytic vesicles. Since the Invaplex-specific staining pattern after a 60-min incubation was observed as diffuse staining throughout the cytoplasm and reporter proteins encoded on the DNA constructs were expressed, it is likely that both Invaplex and the plasmid DNA were released from the endocytic vesicles after internalization. The mechanism of Invaplex and plasmid DNA release from endosomes may be similar to those attributed to the IpaB protein utilized by wild-type shigellae to facilitate escape from phagosomes and into the cytosol of host cells (53). Invaplex-mediated in vitro transfection was demonstrated with both Invaplex 24 and Invaplex 50, indicating that the Invaplex components necessary for transfection were contained within both Invaplex preparations. Invaplex 50, isolated from Shigella sonnei (Mosely), and Invaplex 24, isolated from Shigella dysenteriae 1 (Ubon), also induce the uptake of heterologous antigens, whereas Invaplex deficient in IpaB and IpaC content, or purified LPS, does not mediate transfection (Kaminski and Oaks, unpublished). Collectively, these data indicate that the invasin complex has retained the biological functionality previously attributed to the bacterial effectors and may contribute to its functionality as an adjuvant.

The LPS component of the invasin complex may also contribute to adjuvanticity by binding TLR-4 on the surface of APCs, such as dendritic cells or macrophages, and is currently under investigation. Signaling through TLR-4 increases the expression of costimulatory molecules on the APC and facilitates the maturation of dendritic cells, allowing potent antigen presentation. Engagement of and signaling through TLR-4 also induces the production and secretion of cytokines, such as tumor necrosis factor alpha and CXCL8, recruiting additional APCs or immune effector cells to the site of immunization (1). Such recruitment could potentially result in higher levels of immune stimulation and contribute to the adjuvant activity afforded by Invaplex. T-cell activation also requires appropriate antigen presentation and costimulation, using interactions between B7 molecules on the APC and CD28 on the T cell. The costimulation signal provides stimulus for IL-2 production, which results in T-cell proliferation and differentiation. As TLR signaling induces upregulated expression of B7 molecules on APCs (35), several TLR agonists, such as Hsp-70 and monophosphoryl lipid A (4, 21, 41), have been reported to have DNA vaccine adjuvant activity. The LPS component of Invaplex likely also functions as a TLR agonist. Invaplex is taken up by macrophages in a nontoxic manner and induces the secretion of several key cytokines, such as CXCL-8, tumor necrosis factor alpha, IFN-γ, and granulocyte-macrophage colony-stimulating factor (unpublished observations). The transfection capabilities combined with TLR agonist activity likely contribute to the immunogenicity and adjuvanticity of Invaplex.

The implications of the described research are several-fold. A nontoxic, “universal” adjuvant that can be used for both protein- and DNA-based vaccines to induce enhanced mucosal and systemic immune responses may be useful in a variety of vaccines currently under development. Some adjuvants, which also function with protein- or DNA-based immunogens such as liposomes, require physical incorporation of the immunogen into the adjuvant during formulation, which may affect formulation or may be technically limited. Simple admixtures of Invaplex with either DNA or protein results in enhanced immunogenicity, avoiding the potential pitfalls of physical incorporation. Furthermore, the induction of Shigella-specific immunity in combination with a balanced Th1/Th2 phenotype of the immune response directed to the plasmid-encoded antigen may provide the broad-based immune response necessary for protection against a wide range of intracellular and extracellular pathogens. The ability of Invaplex to enhance mucosal and systemic immune responses elicited with DNA-based and protein-based vaccines warrants further investigation of Invaplex as a universal mucosal adjuvant.

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