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Broadly Protective *Shigella* Vaccine Based on Type III Secretion Apparatus Proteins

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***Shigella* spp. are food- and waterborne pathogens that cause severe diarrheal and dysenteric disease associated with high morbidity and mortality. Individuals most often affected are children under 5 years of age in the developing world. The existence of multiple *Shigella* serotypes and the heterogenic distribution of pathogenic strains, as well as emerging antibiotic resistance, require the development of a broadly protective vaccine. All *Shigella* spp. utilize a type III secretion system (TTSS) to initiate infection. The type III secretion apparatus (TTSA) is the molecular needle and syringe that form the energized conduit between the bacterial cytoplasm and the host cell to transport effector proteins that manipulate cellular processes to benefit the pathogen. IpaB and IpaD form a tip complex atop the TTSA needle and are required for pathogenesis. Because they are common to all virulent *Shigella* spp., they are ideal candidate antigens for a subunit-based, broad-spectrum vaccine. We examined the immunogenicity and protective efficacy of IpaB and IpaD, alone or combined, coadministered with a double mutant heat-labile toxin (dmLT) from *Escherichia coli*, used as a mucosal adjuvant, in a mouse model of intranasal immunization and pulmonary challenge. Robust systemic and mucosal antibody- and T cell-mediated immunities were induced against both proteins, particularly IpaB. Mice immunized in the presence of dmLT with IpaB alone or IpaB combined with IpaD were fully protected against lethal pulmonary infection with *Shigella flexneri* and *Shigella sonnei*. We provide the first demonstration that the *Shigella* TTSAs IpaB and IpaD are promising antigens for the development of a cross-protective *Shigella* vaccine.**

Shigellosis is a severe diarrheal disease associated with high morbidity and mortality rates, particularly in the developing world. It is also responsible for long-term effects on cognitive and physical development in children (51). The global burden has been estimated at more than 160 million cases per year, with the most affected being children under 5 years of age living in areas of *Shigella* endemicity (24). The organism can be transmitted from person to person through fecal-oral contact or through contaminated fomites; ingestion of as few as 10 organisms can cause illness in adult volunteers (11). In industrialized countries, *Shigella* is known to be responsible for cases of pediatric diarrhea and to cause occasional food-borne outbreaks (6). Other susceptible groups include travelers, military personnel, and refugees (53). Additionally, the Centers for Disease Control and Prevention lists *Shigella* as a category B bioterrorist agent (food safety threat).

The *Shigella* genus comprises four different species: *Shigella dysenteriae*, *Shigella flexneri*, *Shigella sonnei*, and *Shigella boydii*. These organisms are further divided into multiple serotypes based on the structure of the O polysaccharide portion of their outer membrane lipopolysaccharide (LPS), thereby increasing their antigenic variability. One serotype in particular, *S. dysenteriae* serotype 1, which produces Shiga toxin, is responsible for the most severe infections, including hemolytic uremic syndrome (15), and it is the cause of epidemic dysentery. Additionally, serotypes can drift during outbreaks, further limiting the efficacy of vaccines that are restricted to particular serotypes (37, 56). The emergence of strains resistant to antimicrobial drugs, including ciprofloxacin, currently the first-line antibiotic treatment against *Shigella* infections (20, 46, 55), heightens the difficulty of controlling this pathogen and makes the development of an effective vaccine even more urgent.

Despite being a longstanding priority for the World Health Organization (54) and despite the progress made in recent years (27), no licensed vaccine for *Shigella* spp. currently exists. Efforts to develop a vaccine against this pathogen have included the use of killed bacteria (31), live attenuated (1, 19, 22, 38) and recombinant carrier (21) organisms, polysaccharide conjugates (7, 36), and LPS-protein mixtures (16, 48). When tested in humans, these vaccines were either too reactogenic or poorly immunogenic. A major disadvantage of these candidates is the O antigen restriction, which limits the scope of protection they can offer and requires the development of a multiserotype vaccine to provide adequate protective coverage in areas of *Shigella* endemicity.

The type III secretion system (TTSS) is a common virulence mechanism in many Gram-negative pathogens. The TTSS apparatus (TTSA) resembles a molecular needle and syringe and is present at a density of 50 to 100 per bacterial cell (4). It provides an energized conduit for the translocation of effector proteins from the bacterial cytoplasm to the host cell cytoplasm for the benefit of the pathogen. The *Shigella* TTSA needle tip protein is IpaD, which

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resides atop the TTSA needle in a pentameric ring where it controls translocator and effector protein secretion (14). The crystal structure of IpaD (37 kDa) reveals a dumbbell-like structure, with the handle being a coiled coil that is likely required for proper TTSA assembly and with elongated domains at each end that are required for IpaD functions specific to *Shigella* spp. (18). Upon addition of bile salts, IpaD undergoes a conformational change (2, 10), allowing the mobilization of IpaB (62 kDa) to form a ring in a position distal to IpaD (35). The interaction of IpaB with IpaD forms the putative plug that controls *Shigella* type III secretion. The hydrophobic domain of IpaB then interacts with the host cell membrane to trigger the secretion of IpaC to complete the conduit between the bacterium and the host (12). The presence and proper tip localization of IpaB, IpaC, and IpaD are required for invasion of epithelial cells. Several studies have reported the presence of antibodies against Ipa proteins in serum from infected individuals (5), in subjects immunized with live attenuated organisms during clinical trials (44, 45), and following vaccination in animal models (30, 49). The conserved nature of IpaB and IpaD among the shigellae and their critical role in pathogenesis make them ideal targets for vaccine development. An Ipa-based vaccine would provide broader coverage across multiple serotypes. It would also simplify vaccine production and formulation.

We examined the immunogenicity of the *Shigella* IpaB and IpaD proteins, administered alone or together, in the presence of a double mutant heat-labile toxin (dmLT) from enterotoxigenic *Escherichia coli*, used as a mucosal adjuvant, in a mouse model of intranasal immunization. The *Shigella* proteins were purified using improved methods and were subjected to stability and conformational analysis. Protective efficacy was determined against lethal pulmonary infection with *S. flexneri* and *S. sonnei*. We provide the first demonstration that IpaB and IpaD are promising target antigens for a cross-protective *Shigella* vaccine.

MATERIALS AND METHODS

Protein purification materials. Plasmids and competent *Escherichia coli* cells were obtained from Novagen (Madison, WI). Metal affinity columns, Q Sephadex, Butyl Sepharose, and HiLoad 16/60 Superdex 200 preparative grade columns were obtained from GE Healthcare (Piscataway, NJ). *n*-Octyl-polyoxyethylene (OPOE) was obtained from Alexis Biochemicals (Lausen, Switzerland). All other chemicals were reagent grade.

Protein purification. The *E. coli* double mutant heat-labile toxin [LT(R192G/L211A) or dmLT] adjuvant was purified by galactose affinity chromatography as described previously (33). Recombinant IpaD was purified as previously described (14). Briefly, BL21(DE3) *E. coli* containing the plasmid D/pET15b was grown for ~3 h to mid-log phase and induced by isopropyl-β-D-thiogalactopyranoside (IPTG) to overexpress His-tagged IpaD. The bacteria were collected and lysed, and the His-tagged IpaD was purified from the cytoplasmic fraction via standard immobilized metal affinity column (IMAC) chromatography. Peak fractions were combined and subjected to a subsequent Q Sephadex anion-exchange chromatography step. Recombinant IpaB was purified by a modification of Birket et al. (3). Briefly, *ipaB* was subcloned into pET15b, while *ipgC* was subcloned into pACYC-Duet 1. Tuner(DE3) *E. coli* cells were cotransformed with these plasmids. This modification allowed the purification of the His-tagged IpaB/IpgC complex and the subsequent removal of the nontagged IpgC. As with IpaD, the bacteria were grown to mid-log phase and induced with IPTG to overexpress protein, and His-tagged IpaB/IpgC was purified from the clarified cytoplasmic fraction via IMAC chromatography. Peak fractions were collected and subjected to hydrophobic interaction chromatography using a Butyl Sepharose high-performance column with a linear gradient from 1 M ammonium sulfate in 50 mM sodium phosphate (pH 7.0) to 50 mM sodium phosphate. For preparation of His-tagged IpaB, the IMAC-bound protein complex was incubated in the presence of 1% OPOE. The chaperone was removed in the flowthrough and in subsequent wash steps. His-tagged IpaB was eluted in the presence of OPOE to maintain the protein in a soluble form. His-tagged IpaB was then further purified using size-exclusion chromatography with a HiLoad 16/60 Superdex 200 prep grade column. All proteins were concentrated by ultrafiltration and dialyzed into phosphate-buffered saline (PBS) (pH 7.2). Protein concentrations were determined via the absorbance at 280 nm (A_{280}) using extinction coefficients based on the amino acid composition of each protein (28).

Far-UV circular dichroism (CD) spectra were collected using a Jasco J815 spectropolarimeter equipped with a Peltier temperature controller (Jasco Inc., Easton, MD). Spectra were acquired using a cuvette with a 0.1-cm path length at 10°C. A resolution of 1.0 nm and a scanning speed of 50 nm/min with a 2-s data integration time were employed. The spectra presented are averages of three consecutive measurements. The thermal unfolding of the proteins was followed by a monitoring of the ellipticity at 222 nm over a temperature range of 10 to 90°C, with a resolution of 2.5°C and a heating rate of 15°C/h. The protein concentration was 0.5 mg/ml for IpaD and 0.3 mg/ml for IpaB and IpaB/IpgC. CD signals were converted to mean residue molar ellipticities ($[\theta]_R$), and the thermal transitions were analyzed using the Jasco spectral manager and Microcal Origin 6.0 software (25).

Challenge organisms. *S. flexneri* 2a 2457T and *S. sonnei* 53G inocula for challenge were prepared as previously described (29). Briefly, the strains were grown on tryptic soy agar (TSA) with Congo red (final concentration, 0.02%); a total of 20 to 25 colonies were picked and grown in a volume of 125 ml Luria-Bertani (LB) broth (Athena Environmental Sciences, Baltimore, MD) with agitation for 3 to 4 h at 37°C or until an A_{600} of 0.8 to 1.3 was achieved. Cultures were centrifuged, and bacterial pellets were resuspended in phosphate-buffered saline (PBS). The numbers of CFU of each challenge dose were determined by plating serial dilutions of the culture on Congo red agar plates. The 50% mouse lethal doses (MLD_{50}) for the challenge strains were calculated by the method of Reed and Muench and determined to be ~5.4×10^6 CFU for *S. flexneri* and 1.2×10^7 CFU for *S. sonnei*.

Mice, immunizations, and challenge procedures. Female BALB/c mice (8 to 10 weeks old) (Charles River Laboratories, Wilmington, MA) were immunized intranasally (i.n.) on days 0, 14, and 28 with IpaB (2.5 μg), IpaB complexed with IpgC (3.25 μg; equivalent to 0.75 μg IpgC and 2.5 μg IpaB), or IpaD (10 μg). The proteins were administered alone or together, admixed with 2.5 μg of the *E. coli* dmLT adjuvant. Control groups received 2.5 μg of dmLT or PBS. Immunization was performed under isoflurane anesthesia (Abbott Laboratories, North Chicago, IL) dispensed through a precision vaporizer. The inoculum volume was 25 μl, and it was administered by means of a pipette and tip, delivering half of the volume in each nare. Animals were allowed to completely recover before they were returned to their cages, and they were monitored daily after immunization. On day 56 after primary immunization, mice were challenged with virulent *Shigella* strains via the nasal route to induce a pulmonary infection, as previously described (29, 50). Two lethal dosage levels each were used for both *S. flexneri* (6×10^7 and 1.3×10^8 CFU, corresponding to 11 and 24 MLD_{50}, respectively) and *S. sonnei* (1.1×10^8 and 2×10^8 CFU, corresponding to 5 and 9 MLD_{50}, respectively). The challenge inoculum was given in a volume of 30 μl PBS. Mice were monitored daily for 14 days after the challenge, and their health status, daily weight, and survival were recorded. Mice were euthanized if they reached the moribund state or lost more than 20% of their initial body weight and did not recover within 48 h. Animal studies and procedures were approved by the University of Maryland Institutional Animal Care and Use Committee.

Measurement of antibodies. Serum IgG antibodies to IpaB, IpaD, and dmLT were measured by an enzyme-linked immunosorbent assay (ELISA) (39, 40). Briefly, Immulon II ELISA plates (Thermo Scientific,

Waltham, MA) were coated with IpaB (0.1 μg/ml), IpaD (1 μg/ml), or dmLT (1 μg/ml) diluted in PBS for 3 h at 37°C. Plates were washed with PBS Tween (PBST) and blocked overnight with PBS containing 10% dry milk (Nestle, Solon, OH). Serum samples were added in serial dilutions in PBST with 10% dry milk and incubated for 1 h at 37°C. Antibodies were detected with horseradish peroxidase (HRP)-labeled goat anti-mouse IgG followed by 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (KPL, Gaithersburg, MD). The reaction was stopped by adding 1 M H_3PO_4. Plates were read using an Ascent microplate reader (Thermo Scientific, Waltham, MA). Titers were calculated by the interpolation of absorbance values of experimental samples in the regression curve of a calibrated positive control; they are reported in ELISA units (EU) per ml and correspond to the inverse of the serum dilution that produces an A_{450} of 0.2 above the blank. For measurement of stool antibodies, 5 to 6 fecal pellets from individual animals were weighted and resuspended in PBS containing 0.2% sodium azide (Sigma-Aldrich, St. Louis, MO) to a final concentration of 10% (wt/vol). Samples were centrifuged, and supernatants were stored at −20°C with 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich). IgA antibodies to IpaB, IpaD, and dmLT were measured in stool supernatants by ELISA as described above, using HRP-labeled goat anti-mouse IgA as the secondary antibody (Southern Biotech, Birmingham, AL).

Antibody-secreting cells (ASC). The frequency of IgG- and IgA-secreting cells was measured by an enzyme-linked immunosorbent spot assay (ELISpot) as previously described (39, 40), with modifications. Spleen, bone marrow, and nasal mucosa-associated lymphoid tissue (NALT) were collected on days 39 and 56 after immunization, and single-cell suspensions were prepared. Cells were incubated overnight in Immulon II flat-bottom plates (Thermo Scientific) previously coated with 5 μg/ml IpaB or IpaD; PBS-coated wells were used as negative controls. HRP-labeled goat anti-mouse IgG (KPL) and IgA (Southern Biotech) were added in the agarose overlay. True Blue (KPL) was used as the substrate. For all ELISpot measurements, spots were counted using a stereomicroscope and results were expressed as the mean number of spot-forming cells (SFC) per 10^6 cells from quadruplicate wells.

IFN-γ secretion. The frequencies of gamma interferon (IFN-γ)-secreting T cells were measured as previously described (39). Spleens were collected on day 56 following primary immunization, and single-cell suspensions were prepared. Cells (in quadruplicate wells) were incubated with IpaB or IpaD (10 μg/ml) in plates previously coated with anti-IFN-γ capture antibody (IFN-γ ELISpot kit; BD Biosciences, San Diego, CA) for 48 h at 37°C and 5% CO_2. IFN-γ production was detected using a biotinylated anti-IFN-γ antibody followed by streptavidin-HRP. 3-Amino-9-ethylcarbazole was used as a peroxidase substrate (Calbiochem, San Diego, CA). Numbers of SFC were calculated and reported as described above.

Statistical methods. All graphics and statistical analyses were generated using GraphPad Prism 5. The data distribution was first examined using the D'Agostino and Pearson normality test. One-way analyses of variance (ANOVAs) and Tukey's tests were used for subsequent group comparisons. Survival curves were plotted in Kaplan-Meier curves; treatments were compared using a log rank test. A P value of <0.05 was considered significant in all analyses.

RESULTS

Purification of recombinant *Shigella* IpaB and IpaD. Purification of recombinant IpaD and IpaB was performed by modified previous methods (3, 14). In addition to the standard IMAC purification, contaminating proteins were removed from the His-tagged IpaD via anion-exchange chromatography (Fig. 1). The association of IpaB with its cognate chaperone, IpgC, is required for IpaB stability in the *Shigella* cytoplasm (32). Thus, we previously purified the overexpressed IpaB/IpgC complex via a His tag genetically fused to the N terminus of IpgC (3). To more efficiently capture IpaB, the plasmid constructs were modified to fuse the His tag to the N terminus of IpaB, leaving the IpgC untagged. After standard IMAC purification, the complex was further purified via hydrophobic interaction chromatography (Fig. 1). When His-tagged IpaB alone was required, the His-tagged IpaB/IpgC complex bound to the IMAC resin was incubated with 1% OPOE. IpgC was washed away, and the His-tagged IpaB was eluted with buffer containing OPOE. His-tagged IpaB was further purified by size-exclusion chromatography (Fig. 1).

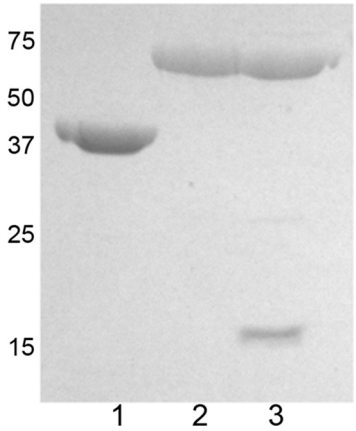

FIG 1 SDS-PAGE of purified recombinant proteins. IpaD (lane 1), IpaB (lane 2), and IpaB/IpgC (lane 3) (5 μg each) were subjected to SDS-PAGE and subsequently stained with Coomassie blue R-250. Molecular mass markers (in kDa) are on the left.

Far-UV CD spectroscopy was employed to assess the secondary structure of the purified proteins. These spectra are routinely collected to ensure that the purification process has resulted in proteins maintaining their proper conformation. Thus, CD spectroscopy was used to demonstrate that the proteins were unaffected by the additional purification steps (3, 13). Indeed, the spectra for IpaD, IpaB, and the IpaB/IpgC complex exhibit the typical α-helical minima at 208 and 222 nm (Fig. 2A). While additional purification steps have been added to remove contaminants, these spectra illustrate that the contaminants have not contributed significantly to previous measurements (3, 14). To assess the stability of the purified recombinant proteins, thermal unfolding curves were collected. As previously reported (13), IpaD exhibited an initial transition at 60°C and a second transition at 80°C in which the protein completely unfolds and precipitates (Fig. 2B). When the IpaB and IpaB/IpgC thermal unfolding curves were obtained, only one transition was seen, regardless of the presence of the chaperone (Fig. 2B). Although preliminary results have indicated that IpaB forms a multimeric complex, the IpgC still acts to stabilize IpaB, a result which is demonstrated by the increase in the thermal unfolding from 60°C for IpaB to 70°C for IpaB/IpgC.

To determine the initial stability of the proteins under standard storage conditions, IpaD, IpaB, and IpaB/IpgC in PBS were stored at 4°C or −80°C and assessed for degradation weekly over a 3-month period using SDS-PAGE. IpaD remained stable for up to 3 months regardless of temperature (data not shown). Both IpaB and IpaB/IpgC were stable for 3 months at −80°C. In contrast, IpaB and IpaB/IpgC were stable for only 3 and 4 weeks, respectively, at 4°C. In light of the thermal stability described above, it was surprising that the IpaB/IpgC complex degraded 25% faster than IpaB alone at 4°C, albeit only by 1 week. While these data

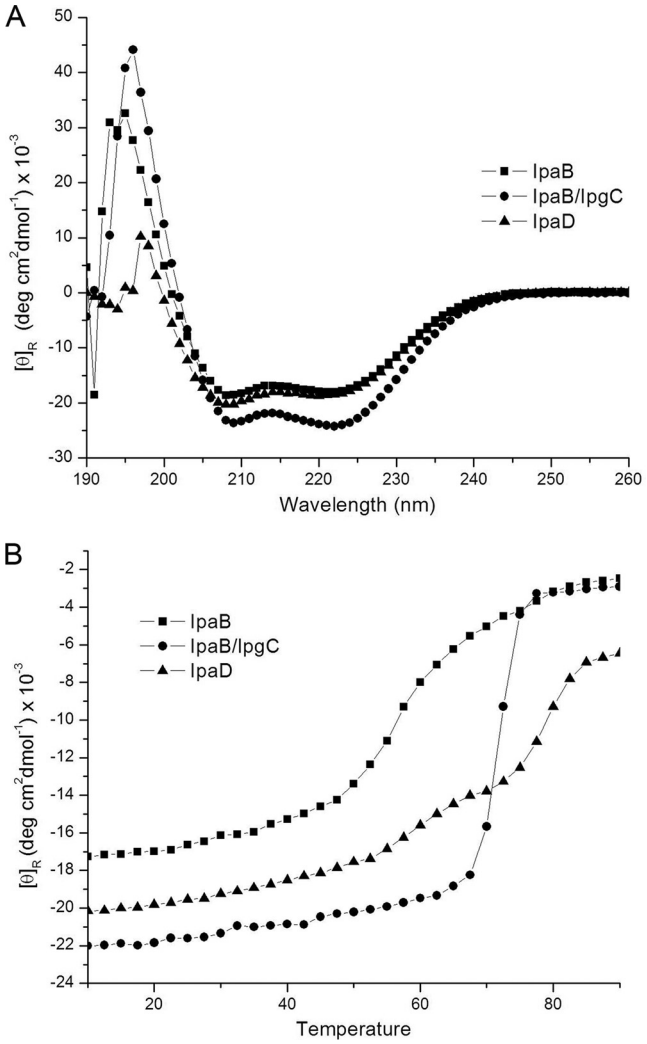

FIG 2 CD spectroscopy of recombinant proteins. (A) Far-UV spectra of recombinant proteins were recorded at 10°C in 10 mM phosphate-citrate buffer at pH 7.2. (B) Temperature dependence of the molar ellipticity at 222 nm of recombinant proteins was monitored from 10°C to 90°C at 2.5°C intervals at a rate of 15°C/h. Error bars have been omitted for clarity; uncertainty was less than 5% ($n = 3$).

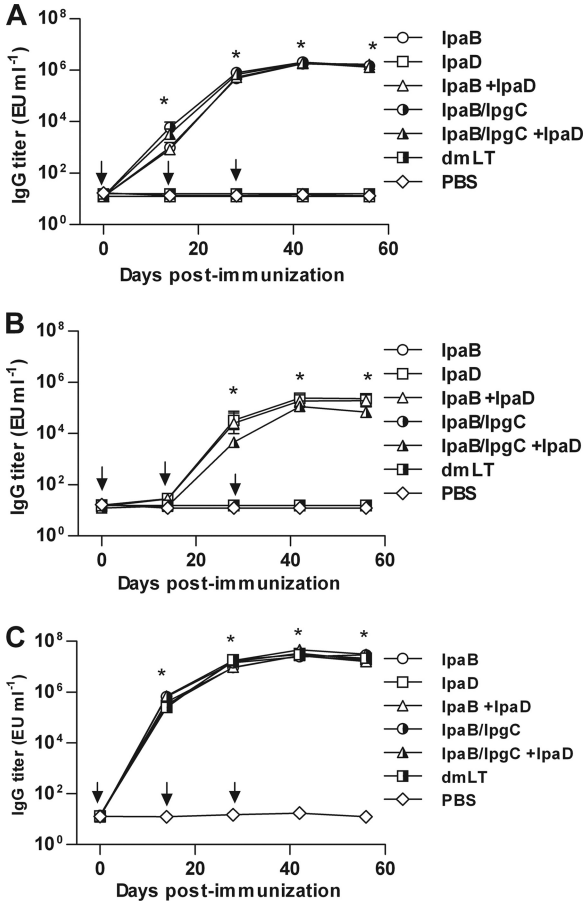

FIG 3 Serum antibody responses to IpaB, IpaD, and dmLT. Mice were immunized on days 0, 14, and 28 (arrows) with IpaB, IpaB/IpgC, and/or IpaD admixed with dmLT, as described in Materials and Methods. Serum IgG antibodies specific for IpaB (A), IpaD (B), and dmLT (C) were measured by ELISA. Data are the mean titers (EU ml^{-1}) and the standard errors (SE) from 10 mice per group. An asterisk indicates a P value of <0.05 when comparing vaccinated mice and the PBS controls. (A) No responses were seen in mice that received IpaD alone or PBS. (B) No responses were seen in mice immunized with IpaB or IpaB/IpgC or PBS. (C) No responses were detected in the PBS control.

appear contradictory to the data from the thermal unfolding, where the complex was more stable, it is likely that the binding of IpgC to IpaB results in unstructured regions that are unique to the complex and available for proteolytic cleavage. A complete biophysical characterization of IpaB and the complex is ongoing (S. P. Choudhari, R. Krammer, C. R. Middaugh, and W. L. Picking, unpublished data).

Mucosal immunization with IpaB/IpaD proteins elicits strong antibody responses. The kinetics of serum IgG responses against *Shigella* IpaB and IpaD and the *E. coli* dmLT adjuvant are shown in Fig. 3. Mice immunized i.n. with the Ipa proteins alone (i.e., IpaB, the IpaB/IpgC complex, or IpaD) or combined (i.e., IpaB and IpaD [IpaB+IpaD] or IpaB/IpgC+IpaD) in the presence of dmLT developed robust antigen-specific antibody responses. Serum IgG antibodies to IpaB were detected soon after immunization (2 weeks after the first vaccine dose), whereas antibodies against IpaD appeared later (2 weeks after the second immunization). For both IpaB and IpaD, serum IgG levels reached a plateau after the third immunization. No differences were seen in the serum IgG responses to either of the vaccine antigens among the different groups regardless of whether the Ipa proteins, admixed with dmLT, were administered alone (IpaB, IpaB/IpgC, or IpaD) or together (IpaB+IpaD or IpaB/IpgC+IpaD). Interestingly, despite the fact that the amount of IpaB used for immunization was a fraction (1:4) of the amount of IpaD (2.5 μg versus 10 μg), the antibody responses to IpaB consistently surpassed the responses induced by IpaD by at least 1 log at all time points examined ($P < 0.05$) and in all the experiments performed. The amounts of IpaB and IpaD used for immunization were established in preliminary dosing experiments in which increasing quantities of either protein (2.5 μg to 20 μg) were administered with dmLT. Increasing amounts of dmLT were also tested. We selected the 1:4 dose ratio (2.5 μg of IpaB and 10 μg of IpaD) and 2.5 μg of dmLT for subsequent experiments because, when combined in such proportions, the proteins were well tolerated and reached the highest responses within the variables tested.

The IpaB/IpgC complex was not included in dose-finding studies but was used in the same amount as IpaB.

Very strong serum IgG responses were also produced against the adjuvant *E. coli* dmLT. Elevated titers were detected 2 weeks after vaccination and reached a plateau 2 weeks after the second dose. No differences were seen in titers among the different treatment groups. The dmLT-specific IgG responses were consistently high in all the experiments performed.

Ipa-specific IgA- and IgG-secreting cells in systemic tissues. In addition to serum antibodies, we measured the frequency of vaccine-induced ASC in spleen and bone marrow. These cells represent pools of long-lived plasma cells and memory cells, respectively, which have the capacity to maintain circulating antibody levels. IgG and IgA ASC specific for IpaB and IpaD were detected in the spleens of vaccinated mice 1 month after the last immunization (Fig. 4A). Mice that received IpaB or the IpaB/IpgC complex, either alone or coadministered with IpaD, developed high frequencies of IpaB-specific IgA- and IgG-secreting cells (between 200 and 300 SFC per 10^6 cells). ASC responses against IpaD were also detected, but the frequency was lower than that of ASC responses against IpaB ($P < 0.05$). While responses to IpaB included both IgG- and IgA-secreting cells, the ASC responses to IpaD consisted primarily of IgG-secreting cells (10 to 150 SFC per 10^6 cells). Similar ASC response profiles and levels were seen in the spleen on day 39 after immunization (data not shown).

Similarly, vaccine-induced IgG- and IgA-secreting cells against IpaB were found in the bone marrow of vaccinated mice (Fig. 4B). ASC responses were also produced against IpaD, albeit at significantly lower levels ($P < 0.05$). The frequencies of ASC responses against both IpaB and IpaD in the bone marrow were lower (<30 SFC per 10^6 cells) than the frequencies measured in the spleen (40 to 300 per 10^6 cells). In bone marrow, we did not see the prevalence of IpaD-specific IgG ASC that was found in the spleen, possibly because all responses to IpaD were markedly low. When comparing ASC responses among the different groups and treatments, the ASC responses to IpaB and IpaD were similar regardless of whether IpaB was complexed with IpgC or whether IpaB and IpaD were given alone or together. No ASC responses to IpaB and IpaD were detected in mice that received dmLT alone or in mice in the PBS controls.

Stool IgA and nasal ASC responses following immunization with IpaB and IpaD. To demonstrate the induction of antibodies in the gastrointestinal mucosas, which represent the first line of adaptive immune defense against enteric pathogens, we measured IgA responses to IpaB and IpaD in stool supernatants from vaccinated and control mice (Fig. 5). The kinetics of IpaB-specific IgA production were similar among the different groups. Unlike the serum IgG responses, which appeared relatively soon after vaccination, the fecal IgA responses were somewhat delayed. IgA titers to IpaB were first seen after the second immunization; IgA responses to IpaD appeared after the third immunization, indicating that an additional vaccine dose was required to raise detectable mucosal antibodies to the levels found in the serum. As with the serological responses, the intestinal IgA responses to IpaD were significantly lower than those against IpaB. For both antigens, IgA titers reached a plateau 2 weeks after the third immunization. No differences were seen in the IpaB stool IgA titers among the groups that received IpaB or IpaB/IpgC alone or IpaB or IpaB/IpgC combined with IpaD. A trend of higher IpaD IgA responses was seen in mice immunized with IpaD alone, although it did not reach statistical

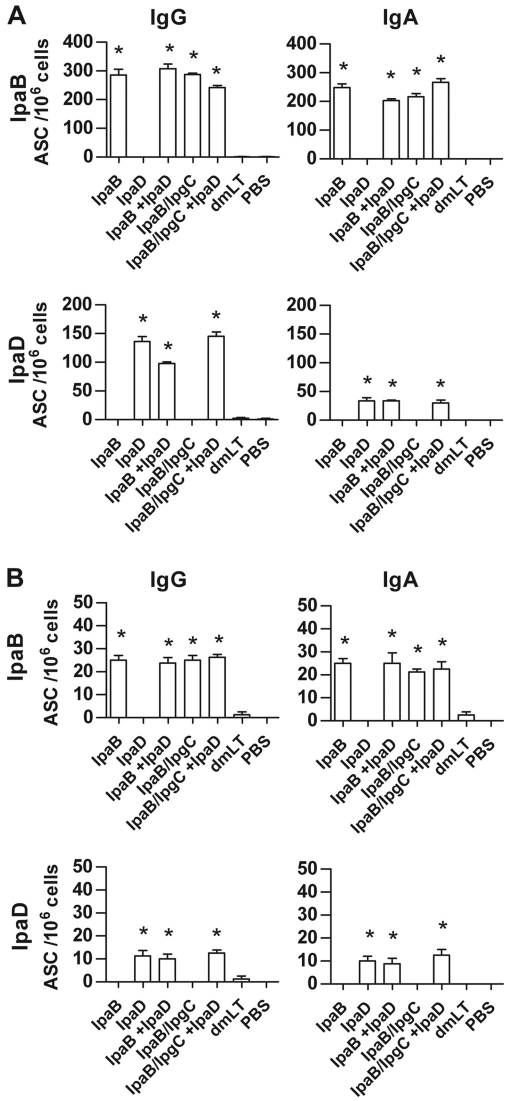

FIG 4 Antibody-secreting cells in spleen and bone marrow. Spleens (A) and bone marrows (B) were collected on day 56. Single-cell suspensions from 5 mice per group were stimulated *in vitro* with IpaB or IpaD. IgG and IgA ASC were measured by ELISpot. Bars represent the mean numbers of ASC per 10^6 cells and the standard deviations (SDs) from replicate wells. An asterisk indicates a *P* value of <0.05 when comparing that condition with PBS control mice.

significance. Very high levels of stool IgA, which were similar in all treatment groups, were produced in response to the dmLT; responses were detected after the first immunization and had already reached maximum levels after the second dose of vaccine.

IgG and IgA ASC specific for IpaB and IpaD were detected in the nasal tissue on day 56 after immunization (Fig. 6). The frequencies of IpaB-specific ASC were again higher than those measured against IpaD ($P < 0.05$). No significant differences were seen in the numbers of ASC against IpaB and IpaD among the different groups, regardless of whether IpaB or IpaB/IpgC was used or whether these proteins were given alone or coadministered with IpaD.

Intranasal immunization with IpaB and IpaD elicits robust T cell-mediated immunity. T cells, in addition to antibodies, are

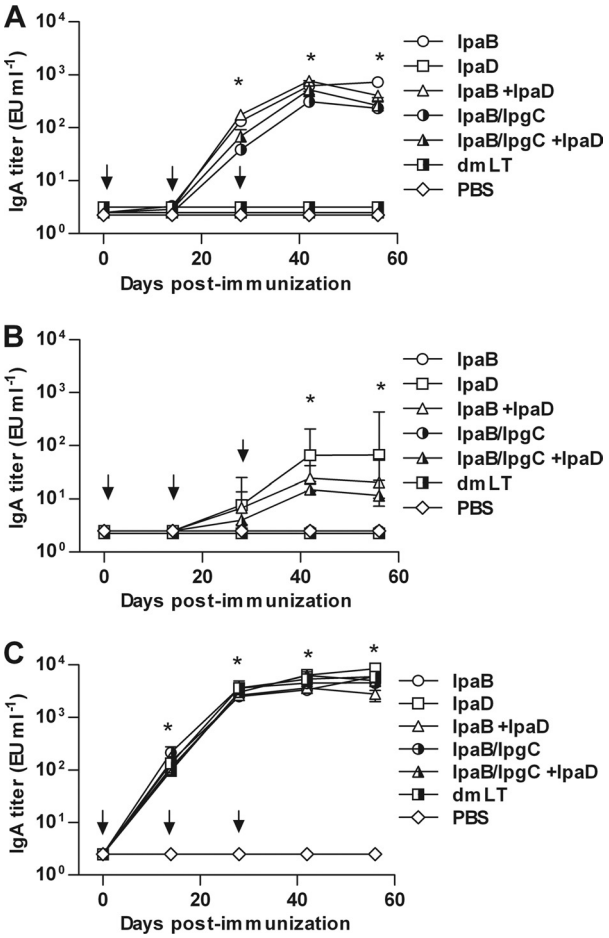

FIG 5 Fecal IgA responses to IpaB, IpaD, and dmLT. Mice were immunized on days 0, 14, and 28 (arrows). IgA specific for IpaB (A), IpaD (B), and dmLT (C) was measured by ELISA in stool supernatants. Data are the mean titers (EU ml^{-1}) and the SE from 10 mice per group, except for at day 56, when there were 5 mice in each group. An asterisk indicates a P value of <0.05 when comparing that condition with PBS control mice. (A) No responses were seen in mice that received IpaD alone or PBS. (B) No responses were seen in mice immunized with IpaB or IpaB/IpgC or PBS. (C) No responses were detected in the PBS control.

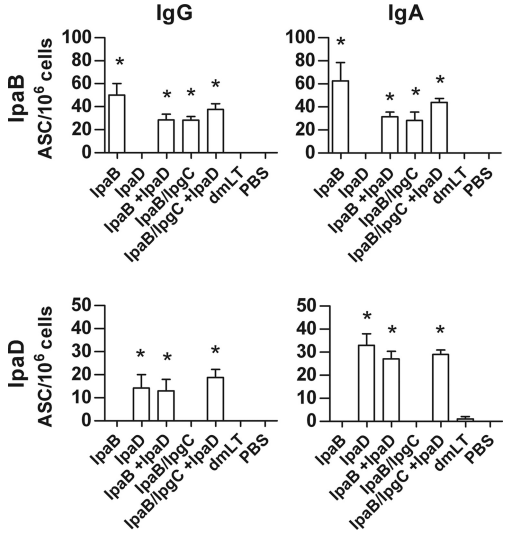

FIG 6 Antibody-secreting cells in nasal tissue. Nasal mucosa-associated lymphocyte tissue was collected on day 56, and single-cell suspensions were prepared from 5 mice per group. IgG and IgA ASC-specific IpaB and IpaD were measured by ELISpot. Bars represent the means and SDs per 10^6 cells from replicate wells. An asterisk indicates a P value of <0.05 when comparing that condition with PBS control mice.

believed to contribute to protection against *Shigella* infection through the secretion of proinflammatory mediators that will further activate innate immune cells and T helper cytokines that will support antibody production. T cells might also contribute to clearing the infection by promoting T cytotoxic cell- or NK-mediated killing of infected cells. The frequency of IFN-γ-secreting T cells specific for IpaB and IpaD was measured in the spleens of vaccinated and control mice 1 month after the last immunization (Fig. 7). Intranasal immunization with IpaB and IpaD induced antigen-specific T cells that were able to produce IFN-γ when stimulated *in vitro*. The IFN-γ responses to IpaB (50 to 150 SFC per 10^6 cells) were higher than those measured against IpaD ($<$50 SFC per 10^6 cells). An interesting finding, which was not paralleled in the serological analysis, was a trend of a higher magnitude of IFN-γ-secreting T cells in mice immunized with IpaB or IpaB/IpgC as well as IpaD ($P < 0.05$). This was true for the responses against both antigens, IpaB and IpaD. The frequencies of IFN-γ-producing cells were consistently lower in groups that received IpaB, IpaB/IpgC, or IpaD alone. No responses were detected in negative-control groups that received dmLT and PBS.

Protection against *S. flexneri* and *S. sonnei* lethal pulmonary challenge. To determine the protective efficacy of IpaB and IpaD, vaccinated and control mice were subjected to a lethal pulmonary dose of homologous *S. flexneri* 2a or heterologous *S. sonnei* (Fig. 8). Two lethal dosage levels for each species were used to perform a more stringent protection analysis: 0.6×10^8 and 1.3×10^8 CFU of *S. flexneri*, corresponding to 11 and 24 MLD_{50}, respectively, and 1.1×10^8 and 2×10^8 CFU of *S. sonnei*, corresponding to 5 and 9 MLD_{50}, respectively. Protective efficacy of the Ipa proteins when using the lower challenge dose was between 90 and 100% against *S. flexneri* (Fig. 8A) and between 60 and 80% against *S. sonnei*. The highest level of protection was seen in mice immunized with IpaB or IpaB/IpgC. When the challenge dose was doubled, the homologous and heterologous protection achieved in the vaccinated groups was somewhat lower but still significant compared with that of the unvaccinated controls (Fig. 8B). Immunization with

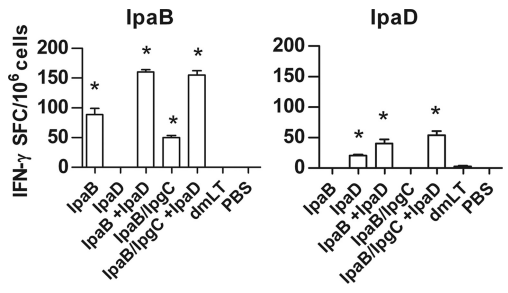

FIG 7 IFN-γ-secreting cells. Spleens were collected on day 56 from 5 mice per group. Single-cell suspensions were prepared and stimulated with 5 μg/ml IpaB or IpaD for 48 h. Results are the numbers of IFN-γ SFC per 10^6 cells and SDs from quadruple wells. An asterisk indicates a P value of <0.05 when comparing that condition with mice that received PBS.

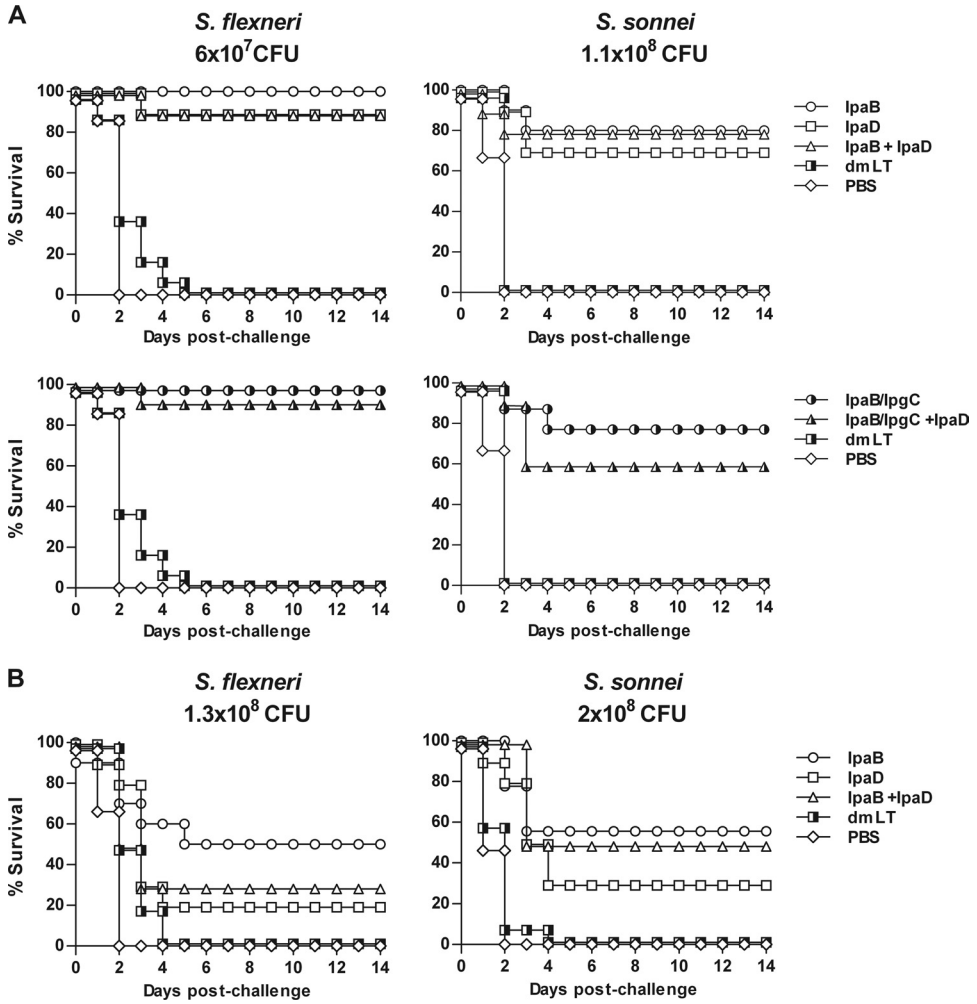

FIG 8 Protection against *Shigella* spp. in a lethal pulmonary challenge. Mice were challenged on day 56 after the first immunization. Two dosage levels were used for each strain, including 6×10^7 CFU of *S. flexneri*, corresponding to ~11 MLD_{50}, and 1.1×10^8 CFU of *S. sonnei* (~5 MLD_{50}) (A) and 1.3×10^8 CFU of *S. flexneri*, corresponding to ~24 MLD_{50}, and 2×10^8 CFU of *S. sonnei* (~9 MLD_{50}) (B). Data are the percentages of survival for 10 mice in each group.

IpaB afforded 50% protection against *S. flexneri*, whereas 30% protection was attained when IpaB was coadministered with IpaD (Fig. 8B). Protection against the higher challenge dose of *S. sonnei* was 50% in mice immunized with IpaB and 45% in mice that received IpaB coadministered with IpaD. The addition of IpaD did not increase the survival rate in any of the challenges, regardless of the challenge dose. Nevertheless, a significant proportion of mice immunized with IpaD alone (70 to 90%) were protected when challenged with the lower lethal infectious doses, although this level of protection decreased drastically (20 to 30%) when they were exposed to a higher number of organisms of either strain. A summary of the protective efficacy values and the statistical significance when comparing the vaccinated and control groups is shown in Table 1.

DISCUSSION

Despite intense efforts, the development of an effective vaccine against *Shigella* remains a daunting task. One of the main challenges in achieving an effective prophylactic tool against this pathogen is the need to protect against multiple serotype strains.

TABLE 1 Protective efficacy (percentage of survival) against *Shigella* spp. in a lethal pulmonary challenge model

Shigella organism	Dose (CFU)	Protective efficacy (%) of each vaccine[a]						
		IpaB+dmLT	IpaD+dmLT	IpaB+IpaD+dmLT	IpgC+dmLT	IpgC+IpaD+dmLT	dmLT	PBS
S. flexneri	1.2×10^8	50*	20	30*	ND	ND	0	0
S. sonnei	2.0×10^8	55.5*	30*	50*	ND	ND	0	0
S. flexneri	6.0×10^7	100*	88*	90*	100*	90*	0	0
S. sonnei	1.1×10^8	80*	70*	80*	80*	60*	0	0

[a] ND, not determined. Asterisks indicate a P value of <0.05 when comparing that condition with the group receiving dmLT (log rank test).

Antibodies to LPS have been associated with serotype-specific protection against shigellosis (8, 9, 47), yet the existence of structurally and antigenically different LPS molecules limits the applicability of an LPS-containing vaccine.

Our approach to develop a safe and effective vaccine to prevent this disease involves the use of conserved proteins that could provide broad protection against multiple serotypes. Among potential cross-protective antigens, we focused our attention on the exposed proteins of the TTSA. These proteins are encoded as part of the *mxi-spa* operon located on the large virulence plasmid that is present in all *Shigella* spp. Thus, in addition to being nearly identical among pathogenic *Shigella* strains, they mediate critical steps of bacterial pathogenesis. IpaB and IpaD were selected because of their significant roles during TTSS-mediated events that lead to bacterial invasion of epithelial cells. IpaD localizes to the needle tip, where it controls further assembly of the TTSA as well as TTSS effector secretion. IpaB is subsequently recruited to the tip of the needle, where it interacts with host membranes to initiate formation of the translocon/pore complex. Immunological effector mechanisms (i.e., antibodies) that can interfere with their function *in vivo* may potentially prevent bacterial infection, making them very attractive vaccine target antigens.

We examined the immunogenicity of IpaB and IpaD (alone or combined) in a mouse intranasal immunization model using the *E. coli* dmLT as an adjuvant and demonstrated that both proteins were able to induce robust serum and mucosal antibody levels and elevated frequencies of antibody-secreting cells in mucosal and systemic tissues. They also induced strong antigen-specific cell-mediated immunity responses, evidenced by the presence of IFN-γ-secreting T cells. Most important, very high levels of protection (80 to 100%) were achieved against a homologous strain and a heterologous strain using a lethal pulmonary challenge model. In the absence of dmLT, modest antibody responses to IpaB and IpaD were induced, but no protection was achieved (S. Heine, S. H., G. Fujii, W. L. Picking, and M. F. Pasetti, unpublished data). The dmLT molecule is a derivative of the heat-labile enterotoxin of *E. coli* with two attenuating mutations in the enzymatically active A subunit (R192G/L211A). These mutations effectively reduce enterotoxicity without impacting the adjuvant properties of the molecule (33). It was chosen as an adjuvant for our studies because it has been shown to enhance the production of vaccine-specific humoral and cell-mediated immune responses in both the systemic and mucosal compartments when it is included as part of a vaccine formulation (33).

IpaB was consistently and across measurements the more immunogenic of the two proteins. Both serum IgG and stool IgA responses to IpaB appeared faster and achieved a higher magnitude than the responses induced by IpaD. This superior immunogenicity was also seen at the T cell response level. The coadministration of proteins had no effect on their individual serological responses, although it appeared to have an impact on the T cell responses, as discussed below. The presence of high frequencies of IpaB/IpaD-specific ASC in the spleen indicates the capacity of the vaccine to induce a large pool of functional plasma cells, which are the source of systemic antibodies. The demonstration of antigen-specific ASC in the bone marrow indicates the existence of B memory cells, which can reactivate upon a subsequent antigen encounter to mount a rapid and strong anamnestic response and thus contribute to long-term protection.

The abilities to generate mucosal antigen-specific ASC and to produce intestinal secretory IgA (sIgA) are also important features of our IpaB/IpaD-based vaccine, as these immunological effectors would be necessary to prevent mucosal enteric infection in humans. Although the mechanisms of protection against *Shigella* have not been elucidated, mucosal immune defenses are sought, as they block pathogens in the intestinal lumen, preventing pathogenic attachment and infection of epithelial cells. Ipa-specific sIgA antibodies are expected to neutralize TTSS interactions at the mucosal epithelial barrier, thereby preventing bacterial invasion of host cells. IgG antibodies might also contribute to protection when infection has occurred, facilitating opsonophagocytic killing by polymorphonuclear neutrophils and macrophages (42).

In addition to producing antibodies, both IpaB and IpaD induced high frequencies of IFN-γ-secreting T cells, demonstrating the presence of robust cell-mediated immunity. Production of IFN-γ is required for the protection of mice against *Shigella* pulmonary infection (26). Likewise, T cell-mediated immunity is thought to be necessary for the protection of humans against shigellosis. IFN-γ-activated macrophages abrogate bacterial growth, whereas in the absence of IFN-γ, *Shigella* replicates intracellularly and further disseminates, a result which suggests an IFN-γ-mediated mechanism for controlling infection (52). Production of IFN-γ in response to IpaB has been demonstrated using peripheral blood mononuclear cells (PBMCs) from infected individuals (41) or from subjects that received live attenuated vaccine organisms (23).

In our studies, the production of IFN-γ appeared to be increased when IpaB/IpaD were administered together compared to that of the proteins given alone, suggesting a synergistic or more effective activation of antigen-presenting cells or Th1 lymphocytes. Interestingly, the increased responses appeared to be limited to T cells, as they were not seen in the antibody or ASC measurements. This effect was not due to cross-reactivity or contamination, as cells from mice immunized with IpaB did not respond against IpaD and vice versa.

Although IpaB degrades when expressed in the *E. coli* cytoplasm, it can be stably coexpressed and purified with its cognate chaperone, IpgC. The complex can then be separated by the addition of the detergent OPOE, which results in stable, multimeric IpaB. While IpaB would be the obvious antigen, separation from IpgC requires an additional purification step and the use of a detergent not generally recognized as safe (GRAS), OPOE, which is currently the only detergent found to efficiently separate the complex. This lengthens the purification process and increases production costs. Thus, the question emerged as to whether IpaB/IpgC is equally as immunogenic as IpaB and can serve as a vaccine antigen. We found no significant differences between IpaB and IpaB/IpgC in any of the responses measured. This finding may facilitate the production of a vaccine with a more simple production process and reduced costs.

Mice immunized with IpaB/IpaD were protected against *Shigella* lethal infection in a pulmonary challenge. Heterologous protection was also observed using *Shigella sonnei*. Protection in the lower-dose challenge was significant for all combinations of Ipa proteins tested. In agreement with the lower immune responses, immunization with IpaD afforded lower protection than immunization with IpaB. Protection conferred by IpaB/IpgC was comparable to that induced by purified IpaB.

Contrary to what we expected, the combination IpaB/IpaD did not result in increased protection in any of the challenge condi-

tions. We believe, however, that it is still advantageous to maintain both antigens in a future vaccine candidate since IFN-γ responses seemed to be improved, a result which might be important to enhance protection in humans.

Another vaccine candidate that contains *Shigella* Ipa proteins is the "Invasin-LPS complex," or Invaplex. This vaccine consists of a mixture of IpaB, IpaC, and LPS (and possibly other antigens) present in water extracts from intact *Shigella* species (49). Two chromatographic fractions, designated Invaplex 24 and 50, were immunogenic and protective against homologous virulent strains in mice and guinea pigs (34). The *S. flexneri* 2a Invaplex 50 vaccine was safe and immunogenic when administered intranasally to human volunteers in phase 1 clinical studies (43, 48). However, this vaccine failed to protect subjects from a subsequent oral challenge with virulent *S. flexneri* 2a 2457TC. Harro et al, *Shigella flexneri* 2a Invaplex 50 intranasal vaccine phase 2b challenge study, presented at the 5th Int. Conf. Vaccines Enteric Dis., 9 to 11 September 2009, Malaga, Spain. Unlike the Invaplex, our vaccine contains known quantities of highly purified IpaB, along with IpaD, and it does not contain LPS.

Although antibodies to LPS are known to mediate protection against shigellosis, our study provides the first evidence that protective immunity can be induced by the IpaB/IpaD vaccine combination and in the absence of LPS. A protein-based, LPS-free vaccine would be safer and more amenable for use in infants, young children, and immune-compromised individuals. The demonstration of cross-protective immunity using purified TTSA proteins is also novel and has broader implications, as the TTSA tip proteins from other Gram-negative enteric pathogens may prove to be protective antigens as well. Through the use of efficient antigen delivery systems, TTSA-based vaccines may potentially be administered to humans to induce mucosal immunity.

In sum, a subunit-based IpaB/IpaD vaccine may be an effective, broadly protective vaccine for prevention of shigellosis.

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