Intradermal Delivery of *Shigella* IpaB and IpaD Type III Secretion Proteins: Kinetics of Cell Recruitment and Antigen Uptake, Mucosal and Systemic Immunity, and Protection across Serotypes


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Intradermal Delivery of Shigella IpaB and IpaD Type III Secretion Proteins: Kinetics of Cell Recruitment and Antigen Uptake, Mucosal and Systemic Immunity, and Protection across Serotypes

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Shigella is one of the leading pathogens contributing to the vast pediatric diarrheal disease burden in low-income countries. No licensed vaccine is available, and the existing candidates are only partially effective and serotype specific. Shigella type III secretion system proteins IpaB and IpaD, which are conserved across Shigella spp., are candidates for a broadly protective, subunit-based vaccine. In this study, we investigated the immunogenicity and protective efficacy of IpaB and IpaD administered intradermally (i.d.) with a double-mutant of the Escherichia coli heat-labile enterotoxin (dmLT) adjuvant using microneedles. Different dosage levels of IpaB and IpaD, with or without dmLT, were tested in mice. Vaccine delivery into the dermis, recruitment of neutrophils, macrophages, dendritic cells, and Langerhans cells, and colocalization of vaccine Ag within skin-activated APC were demonstrated through histology and immunofluorescence microscopy. Ag-loaded neutrophils, macrophages, dendritic cells, and Langerhans cells remained in the tissue at least 1 wk. IpaB, IpaD, and dmLT-specific serum IgG- and IgG-secreting cells were produced following i.d. immunization. The protective efficacy was 70% against Shigella flexneri and 50% against Shigella sonnei. Similar results were obtained when the vaccine was administered intranasally, with the i.d. route requiring 25–40 times lower doses. Distinctively, IgG was detected in mucosal secretions; secretory IgA, as well as mucosal and systemic IgG Ab-secreting cells, were seemingly absent. Vaccine-induced T cells produced IFN-γ, IL-2, TNF-α, IL-17, IL-4, IL-5, and IL-10. These results demonstrate the potential of i.d. vaccination with IpaB and IpaD to prevent Shigella infection and support further studies in humans. The Journal of Immunology, 2014, 192: 000–000.

Shigella spp. are among the handful of enteric pathogens that account for most of the cases of diarrhea in children <5 y of age in sub-Saharan Africa and south Asia (1). Even if mortality is averted, the disease leads to impaired health and quality of life, particularly if acquired at an early age (2, 3). Despite the long-standing interest in control measures, no commercial vaccine is available. To be used globally, a vaccine would need to prevent infection caused by Shigella dysenteriae 1 (which causes epidemic dysentery), Shigella sonnei (affecting mostly travelers and daycare centers), and all 16 Shigella flexneri serotypes (mostly responsible for endemic disease) (4). Efforts to develop an effective vaccine have produced several candidates, some of which have been tested in human clinical trials with promising results (reviewed in Refs. 5–8). However, most of these vaccines are serotype specific; therefore, their protective capacity is limited to the serotype from which they were derived. In the pursuit of a broad-spectrum prophylactic intervention, we proposed the use of Shigella type III secretion system (TTSS) proteins IpaB and IpaD, which are highly conserved among Shigella serotypes, as components of a subunit-based broad protective vaccine. These proteins have an essential role in pathogenesis, because they participate in the assembly of the TTSS needle tip complex, which creates a pore in the host cell membrane and allows the translocation of bacterial effector proteins that ultimately lead to cell death (9, 10). Individuals living in endemic areas who are constantly exposed to the organism develop Ab against Shigella O Ag (11) and Ipa (12, 13), both of which are believed to contribute to naturally acquired protective immunity (5). An association was described between the levels of IpaB-specific serum IgG and IgA B memory cells and reduced severity of disease upon experimental challenge in human adult volunteers pre-exposed to live vaccine organisms or wild-type Shigella (14). In preclinical studies, mucosally delivered IpaB and IpaD were shown to protect against lethal pulmonary Shigella infection in mice (15, 16).

Given that Shigella is an enteric pathogen, oral immunization with candidate vaccines would seem the most practical approach to induce mucosal immunity that could block and prevent the organism from breaching the intestinal barrier. Disappointingly,
the success of oral vaccination has been elusive. Routine vaccines have been less immunogenic when administered orally to children living in developing countries compared with industrialized nations. This has been attributed to multiple natural barriers that interfere with immunological priming in the gut (17, 18). In animal studies, orally delivered IpaB and IpaD failed to induce substantial protection, whereas they were vigorously immunogenic and fully protective when given intranasally (i.n.).

Intradermal immunization using improved injection devices has gained attention as a safe, practical, and effective strategy to enhance vaccine immunogenicity (19). Because of its simplicity and efficiency, this mode of vaccination is particularly attractive for use in children. The feasibility of this approach has been demonstrated by the successful administration of the Mycobacterium bovis bacillus Calmette–Guérin (BCG) to millions of newborns and infants throughout the world. Multiple human clinical studies showed successful immunization against influenza, rabies, polio, hepatitis, and other pathogens through intradermal (i.d.) delivery of commercial vaccines (19–21). Intradermal vaccination against seasonal influenza using microneedles has been approved in Europe since 2009 (22) and in the United States since 2011 (19, 23).

Vaccination via the i.d. route is simple and extremely efficient, requiring a fraction of the dosage typically given i.m. or s.c. (24). Its success has been attributed to the abundant number of specialized APC (i.e., dendritic cells [DC] and Langerhans cells [LC]) residing in the dermis and adjacent epidermal layer, which capture vaccine Ag in their proximity and process and transport them to the draining lymph nodes (DLN) for presentation and stimulation of T cells (25). Vaccine Ag that reach the DLN also activate B cells. DC activated in the skin prime CD4+ and CD8+ T cells, as well as Ab-secreting cells (ASC) producing high-affinity Ab that could mediate microbial exclusion and phagocytic killing (5). In this study, we investigated the early immunological events associated with i.d. immunization with Shigella IpaB and IpaD that lead to immunological priming. In particular, we examined the recruitment of immune cells to the injection site and adjacent tissues, as well as Ag uptake and activation of skin APC. We also fully characterized the immune responses induced in the systemic and mucosal compartments and the protective efficacy of this vaccine against different Shigella serotypes.

Materials and Methods

Vaccine components

The Escherichia coli double-mutant heat-labile toxin [dmLT; LT (R192G/L211A)] was produced at the Walter Reed Army Institute of Research (Silver Spring, MD), following previously described affinity-chromatography methods (26), and obtained through PATH. Recombinant IpaB complexed with the chaperone IpgC (IpaB/IpgC) and IpaD were also purified via affinity and size-exclusion chromatography (27) and quantified using 280-nm extinction coefficients (28). To obtain IpaB for immunological assays and for use as vaccine, IpaB/IpgC was treated with octyl-oligo-oxyethylene to release the IpgC chaperone.

Mice, vaccination, and experimental infection with virulent organisms

Female BALB/c mice (7–8 wk old; Charles River Laboratories, Wilmington, MA) were immunized i.d. by delivering a 25-μl volume of inoculum into the upper right thigh (shaved the day before) using the NanoPass MicronJet (600 needle [NanoPass Technologies, Nes Ziona, Israel]) attached to a 250-μl Hamilton 700 Series Microliter Syringe (Hamilton Company, Reno, NV). The microneedles were inserted into the bare skin at a 45° angle and locked in that position, and the inoculum was delivered slowly, monitoring the proper formation of a bleb; the needles were kept in place for 5 s before removal. Three dosage levels of IpaB/IpgC and IpaD were tested in the first experiment: 50 ng IpaB/IpgC and 100 ng IpaD (referred to as the “low” dose), 100 ng IpaB/IpgC and 250 ng IpaD (referred to as the “medium” dose), and 200 ng IpaB/IpgC and 500 ng IpaD (referred to as the “high” dose). In all instances, 100 ng dmLT was added as adjuvant. The medium and high doses, which proved the most immunogenic in the first experiment, were included in a second experiment with and without adjuvant. A group immunized i.n. with 2.5 μg IpaB/IpgC or IpaB, 10 μg IpaD, and 2.5 μg dmLT, known to generate potent responses (15, 16), was included as positive control in both experiments. Intranasal vaccination was performed by dispensing 30 μl inoculum (15 μl into each nare) with a pipette, as previously described (15, 16). Negative control groups received 100 ng dmLT or PBS i.d. All groups were vaccinated on days 0, 14, and 28. Serum samples, fecal extracts, and bronchoalveolar lavage fluid (BALF) from individual animals were prepared as previously described (15, 16). On day 56 after vaccination, mice were challenged i.n. with 5.4 × 105 to 5.8 × 106 CFU S. flexneri 2a 2457T or with 1.35 × 107 CFU S. sonnei 53G (15, 16); the doses used correspond to ∼10 mouse lethal doses for each organism, and both are human virulent strains. All animal studies and procedures were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee.

Ab, ASC, and cytokines

To confirm proper i.d. delivery and to track tissue distribution of the inoculum, mice were injected i.d. with india ink (10% in PBS) and euthanized at 4 h, 24 h, or 1 wk after injection. Skin and muscle tissue were removed, embedded in paraffin, and sliced into 5-μm-thick sections. The slides were stained with H&E, and 20× and 40× tissue images were captured using an Olympus BH-2 microscope with a SPOT 4 Mega Pixel RT color camera and imaging software to visualize overall distribution of the dye and cellular inflammatory infiltration at the injection site. For immunohistochemistry (IHC) and immunofluorescence (IF) staining, mice were injected i.d. with IpaB, IpaD, and dmLT (medium dosage level described above) and euthanized 4 h, 24 h, or 1 wk after injection. Mice injected i.d. with PBS and euthanized 30 min after injection served as negative controls. Skin tissue, including the site of injection and surrounding areas, was removed, sectioned, embedded in Tissue-Tek Optimal Cutting Temperature compound (Sakura, The Netherlands) and flash frozen in 2-methylbutane contained in liquid nitrogen. Five-micrometer frozen tissue sections were prepared and fixed with cold acetone for staining. For IHC, slides were washed in PBS and immersed in PBS/0.3% H2O2 to exhaust endogenous peroxidase activity. The tissue sections were blocked with 10% rabbit serum and stained with anti-CD4 (clone RM4-5) and anti-CD8a (clone 53-7.5) (BD Biosciences, San Jose, CA), anti-CD3 (clone 145-2C11), and anti-CD68 (Clone CCP64.1) from eBioscience, San Diego, CA), and anti-mouse macrophage F4/80 and anti-leukocytic lectin Ab (clone ER-MP23; Abcam, Cambridge, MA). Slides were washed, and biotinylated rabbit anti-rat (Molecular Probes-Invitrogen, Grand Island, NY) was added as secondary Ab. Sections stained with IHC with anti-CD11c (BD Biosciences) were blocked with 10% goat serum before incubation with primary Ab, washed, and incubated with biotinylated goat anti-rabbit Ab (Sigma-Aldrich, St. Louis, MO). All sections were rehydrated in PBS, and sections were incubated with avidin/biotinylated Enzyme Complex (Vector Laboratories, Burlingame, CA). A final wash was performed before the addition of 3, 3-diaminobenzidine and counterstaining in Mayer’s hema-toxylin. Cells staining positive for each specific Ab were counted in the 10 representative fields at 400 magnification. The IF staining was performed as previously described (29), with modifications. Briefly, slides were rehydrated in PBS, and the tissue sections were applied to neutralize endogenous biotin. Sections were washed, blocked with 3% BSA in PBS containing 0.01% Triton X-100 (primary Ab diluted), and incubated overnight at 4°C with cell-specific Ab in primary Ab diluted,
along with anti-IpaB or anti-IpaD mAb (generously provided by Dr. Edwin V. Oaks, Department of Subunit Enteric Vaccines and Immunology, Bacterial Diseases Branch, Walter Reed Army Institute of Research). The following day, sections were washed with 0.1% Triton X-100, followed by PBS. Biotin-conjugated goat anti-hamster Ab (Invitrogen) was used to detect anti-CD11c primary Ab, and goat anti-rat Ab (Innitrogen) was used to detect anti-r–ER-MP23, anti-Ly6G (Gr-1); clone RB6-8C5) (AbD Serotec, Raleigh, NC), anti-CD40 (clone: 3/23; BD Biosciences) and CD207 Ab, all prepared in the primary diluent buffer. Alexa Fluor 488 goat anti-mouse (Invitrogen) was added to detect the Ag-specific Ab. The stained slides were washed and incubated in streptavidin conjugated to Alexa Fluor 568 (Invitrogen). After another wash, DAPI was added to all of the slides to stain the nucleus, and the slides were mounted using VECTASHIELD mounting media (Vector Laboratories). IF staining was visualized using a Nikon A1 confocal laser microscope for acquisition of images, and at least six 7-μm z-stack projections were collected at 0.300-0.400-μm intervals using Nikon Elements Microscope Imaging Software (Nikon Instruments, Melville, NY). Mander’s overlap coefficient of the red channel (M red) was calculated using Velocity 3D Image Analysis Software (Improvision-PerkinElmer, Waltham, MA) to determine the extent of colocalization between cells that stained positively for a particular immune cell marker (detected with Alexa Fluor 568 in the red channel) and the Ipa (Alexa Fluor 488 under the green channel). M red represents the total sum of voxels of the overlapped red with green components divided by the total sum of red intensities. M red was reported for each treatment as an average of the four to six z-stack images where the threshold was adjusted to 5% for all images to compensate for any background noise. The confocal image acquisition and analyses were adopted from published reports (30).

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA). For IHC and IF, statistically significant differences for each cell type were determined by one-way ANOVA, with Bonferroni and Tukey multiple-comparisons tests, respectively, comparing tissue from vaccine versus PBS recipients. Two-way ANOVA with Bonferroni multiple-comparisons test was used to determine statistical significance of the differences between serum Ab titers of vaccinated versus control groups. Significant differences among ASC, cytokine, and stool IgA of vaccinated versus control mice were determined by one-way ANOVA with Bonferroni multiple-comparisons test. Unpaired t test with a 95% confidence interval was used to compare IgG1 and IgG2a subclass titers in the vaccine versus PBS groups. Mean Ab titers in mucosal fluids of recipients of vaccine versus PBS were compared by one-way ANOVA with Dunnett multiple-comparisons test.

Results

Inoculum distribution and recruitment of immune cells following i.d. vaccination

We investigated i.d. delivery of IpaB and IpD adjuvanted with dmLT as a novel and potentially more efficient approach (than oral delivery) for immunization against shigellosis. The vaccine was administered to mice on three occasions, 2 wk apart, using NanoPass MicronJet 600 microneedles. To establish the optimal conditions for vaccination, we first performed a series of experiments using india ink to better visualize the inoculum distribution. An inoculum volume of 25 μl was identified as optimal because it produced a visible and consistent skin bleb without leakage (Supplemental Fig. 1A). To confirm delivery into the dermis and to visualize changes in tissue morphology, the skin tissue was examined. During the first 4 h postinfection, the dye was seen as a thick dark blue layer confined to the dermal space (Supplemental Fig. 1B). At 24 h, the ink strip was tangential to the skin surface, and large numbers of phagocytic cells (mostly neutrophils and some macrophages) had been recruited. One week after injection, the dye had diffused throughout the tissue and had been almost completely ingested by phagocytic cells (Supplemental Fig. 1).

We next investigated the changes in cell composition following i.d. vaccination with IpaB, IpD, and dmLT. The presence of neutrophils, macrophages, DC, LC, and CD4 + and CD8 + T cells in skin tissue was evaluated by H&E and IHC staining and confirmed by morphological analysis. Tissue from mice injected with PBS was included as control. Neutrophils rapidly infiltrated the injection site; the largest numbers were detected 4 h after injection and then gradually declined, remaining elevated for at least 1 wk (Fig. 1A). Macrophages also were recruited, albeit at lower numbers and a more gradual pace, reaching their peak at the 1-wk time point (Fig. 1A). CD11c + DC and CD207 + (Langerin) LC were likewise detected; these cells increased significantly 24 h after vaccination and remained elevated for at least 1 wk. At either time point, the recruited DC outnumbered the LC. Interestingly, CD4 + and CD8 + T cells also were recruited to the vaccination site after 24 h, with the highest numbers detected 1 wk postinjection (Fig. 1C). Representative IHC images for each cell type in control and vaccinated mice at peak time points are shown in Fig. 1 (right panels).
Vaccine uptake by innate immune cells

Next, we looked at the cells that might be involved in vaccine uptake by performing IF staining and confocal laser microscopy. For this analysis, we focused the z-stacks (images of planes at various depths) on tissue sections with abundant staining to investigate the presence of IpaB and IpaD (green) within specific cells (e.g., CD11c+ cells [red]). The images displayed in Fig. 2A clearly show positive staining for IpaB and IpaD colocalized with CD11c+ DC in tissue sections from vaccinated mice, as well as the absence of relevant staining in unvaccinated controls. To determine the extent of Ag sampling, we measured colocalization of IpaB and IpaD within CD11c+ DC through calculation of $M_{\text{red}}$; a value of 1.0 indicates the maximum colocalization of vaccine Ag within CD11c+ cells, and a value $\sim 0.1$ represents baseline (mean value for IpaB and IpaD colocalization within CD11c+ cells in the PBS controls). Skin DC containing IpaB or IpaD were detected as early as 4 h after vaccination; in fact, DC containing IpaB were still observed 1 wk postvaccination (Fig. 2B). Based on these results and the superior immunogenicity observed in parallel experiments (described below), we focused on IpaB in subsequent studies to investigate cells involved in Ag sampling as a prerequisite for induction of adaptive immunity. We hypothesized that IpaB likewise would be taken up by other immune cells in the skin, particularly LC, which are extremely efficient APC. IpaB was found within neutrophils (Ly6G+) 24 h and 1 wk postvaccination. IpaB staining also colocalized with LC and skin macrophages (ER-MP23+) at all time points examined (Fig. 3). The fluorescence intensity of IpaB overlapping that of DC, LC, and macrophages was not significantly different, suggesting a similar vaccine sampling capacity for these cell types, with colocalized Ag staining seen as early as 4 h and up to 1 wk after vaccination. We also investigated the activated phenotype (CD40+) of IpaB-containing APC and found significant overlap between IpaB and CD40+ cell fluorescent staining 24 h and 1 wk after vaccination.

Serum Ab responses induced by i.d.-delivered IpaB, IpaD, and dmLT

We first conducted a dose-escalation experiment in which low-, medium-, and high-dosage levels of IpaB and IpaD, alongside a constant amount of dmLT, were administered i.d. to BALB/c mice

![FIGURE 2. IpaB and IpaD taken up by skin DC. (A) Confocal microscopy IF images of skin tissue from mice immunized with IpaB, IpaD, and dmLT 24 h following vaccination or from mice that received PBS 30 min following injection. Sections were stained with mAb specific for IpaB, IpaD (green), or CD11c+ DC (red). The cell nuclei were stained with DAPI (blue). The merged images show all three channels. Arrows on merged images indicate cells with positive staining for antigen (IpaB or IpaD) and CD11c. (B) Extent of colocalization (presented as $M_{\text{red}}$) between IpaB or IpaD (green) and CD11c+ DC (red) at different time points following vaccination. Data are mean + SD from six z-stack images. The dashed line indicates the baseline level, which corresponds to the mean $M_{\text{red}}$ of PBS tissue images. *$p < 0.05$, vaccinated mice versus PBS control mice.](http://www.jimmunol.org/Downloadedfrom/INTRADERMAL+VACCINATION+WITH+SHIGELLA+TTSS+Ag)
Groups immunized with the same Ag i.n. served as positive controls, whereas negative controls received dmLT and PBS. Intradermal immunization with IpaB and IpaD resulted in high levels of Ag-specific serum IgG Ab, regardless of the dose administered. Peak serum IgG responses to IpaB were achieved after the second vaccination, and the magnitude appeared to be similar for all groups (Fig. 4B). However, the IpaB IgG responses produced by i.d. immunization were lower than the responses induced by the protein administered i.n. For IpaD, all groups reached similar (peak) IgG levels at the time of challenge. However, in contrast to IpaB, the dosage level influenced the kinetics of IgG production. The high-dose group exhibited a faster Ab response to IpaD, attaining peak levels after the second vaccination, whereas the low-dose group required an additional immunization to reach the same level of response. It also was noticed that the IgG titers against IpaD produced by i.d. vaccination were lower than those produced by i.n. immunization. Very high IgG responses to dmLT also were elicited by the i.d.-vaccinated mice, which were very similar among groups, proving the consistency of the procedures.

Interestingly, we failed to detect Ag-specific IgA for any of the proteins (IpaB, IpaD, or dmLT) in stool supernatants following i.d. vaccination. No Ag could be detected in serum either (data not shown). However, we detected positive fecal IgA responses against all three Ag in mice immunized i.n. (Fig. 4C). No Ab responses were detected in the unvaccinated (PBS) controls.

**ASC induced by i.d. immunization with IpaB, IpaD, and dmLT**

To further investigate the induction of mucosal immune responses, we measured IpaB-, IpaD-, and dmLT-specific IgG and IgA ASC in the lungs at the time of challenge. No responses were detected in
IpaB or IpaD. All groups immunized i.d. showed increased pro-
and regulatory cytokines by spleen cells stimulated in vitro with
We next examined the production of Th1, Th2, proinflammatory,
Production of cytokines upon in vitro Ag stimulation were detected in the unvaccinated control. No responses
mice vaccinated i.d., whereas significant numbers of IgG and IgA
ASC were seen in mice immunized i.n. (Fig. 5). We also measured the frequency of IpaB-, IpaD-, and dmLT-specific ASC in spleen
and bone marrow (Fig. 5). Mice immunized i.d. exhibited IgG
ASC responses to IpaB and IpaD in both organs, which, for the
most part, increased with the vaccine dose (Fig. 5). IgG ASC
specific for dmLT were also produced following i.d. vaccination.
To our surprise, only IgG (not IgA) ASC were elicited in response
to i.d. immunization; the same was observed for all three vaccine
Ag, irrespective of the vaccine dose administered. In contrast, i.n.
vaccination induced both IgG and IgA Ag-specific ASC in all
tissues examined. The IgG ASC responses measured in the spleen
and bone marrow of the medium- and high-dose i.d. groups were
comparable to those induced by i.n. vaccination. No responses
were detected in the unvaccinated control.

Production of cytokines upon in vitro Ag stimulation
We next examined the production of Th1, Th2, proinflammatory,
and regulatory cytokines by spleen cells stimulated in vitro with
IpaB or IpaD. All groups immunized i.d. showed increased pro-
duction of IL-2, as well as IFN-γ and TNF-α, in response to IpaB
(Fig. 6). These same cytokines, and similar magnitude of re-
sponses, were detected after i.n. vaccination (Fig. 6). IL-17 also
was produced by mice immunized i.d. in response to IpaB, par-
ticularly the high- and medium-dose groups (Fig. 6). IL-2 was the
only Th1-type cytokine produced at significant levels in response
to IpaD by i.d.-vaccinated mice. Very high levels of Th2-type
cytokines (IL-4 and IL-5) were produced by spleen lymphocytes
in response to both IpaB and IpaD following i.d. vaccination (Fig.
6). The secretion of IL-4 and IL-5 by cells stimulated with IpaB
was noteworthy because it greatly exceeded that of mice immu-
nized i.n. The same was observed for IL-5 against IpaD. IL-10 was also produced by i.d.-vaccinated mice in response to IpaB and
IpaD (Fig. 6, middle panels).

Ag-specific induction of IFN-γ, IL-2, and IL-5 was increased in
the inguinal and popliteal DLN as a result of i.d. vaccination.
These responses were remarkably high in the group that received
the medium dose of vaccine and in response to IpaB (Fig. 6,
bottom panels). As expected, negligible responses were seen in
mice immunized i.n. No responses were detected in the dmLT
and PBS controls.

Protection against lethal S. flexneri pulmonary challenge
Intradermal vaccination with IpaB, IpaD, and dmLT afforded
significant protection against lethal pulmonary infection with S.
flexneri. The group that received the medium dosage level had the
highest survival rate (70%). Significant protection also was ob-
erved in the group that received the lowest dose (20%). Unex-
pectedly, all of the mice immunized with the highest dose of IpaB
and IpaD succumbed to challenge (Fig. 7). The difference in
protection between the medium- and high-dose groups prompted
us to perform a second experiment to confirm these findings.

Immune responses to IpaB and IpaD with or without dmLT
A second experiment was performed to ascertain the superior
protection of the intermediate dosage level, investigate cross-
protection against a different Shigella serotype (S. sonneti), and
assess the adjuvant contribution of dmLT in the responses induced.
Mice were immunized with the high and medium dose of IpaB and
IpaD, as described above, and the proteins were given alone or in
the presence of dmLT. A positive control group received IpaB,
IpaD, and dmLT i.n., and a negative control group received PBS
(Fig. 8A). The IpaB protein used in this experiment had the IpgC
chaperone removed. Similar to what was observed in the first
experiment, high levels of IpaB and IpaD serum IgG were pro-
duced by the proteins given i.d. admixed with dmLT. The
medium-dose group produced higher IgG responses against IpaB;
interestingly, these titers (as well as those measured in the high-
dose group) surpassed those seen in the first experiment, sug-
S. flexneri pulmonary challenge, all of the mice immunized with the highest dose of IpaB
gest – the TLR2 agonists dmLT would have been preferentially recognized by the
murine Toll-like receptors. Interestingly, mice immunized with IpaB and IpaD admixed
with dmLT showed higher IgG levels than those immunized with IpaB or IpaD alone.

In summary, our findings demonstrate that intradermal vaccination with
IpaB, IpaD, and dmLT provides superior protection against Shigella
infection compared to i.n. vaccination. The adjuvant contribution of
dmLT is significant, particularly when used in combination with IpaB and IpaD.

**References**

IgG2a also was produced, albeit at lower levels, and similarly was unaffected by the dose. The presence of dmLT allowed for increased production of IgG2a. IgG1 and IgG2a were produced in response to dmLT, and the levels were similar in all dmLT recipients.

We also investigated the presence of IgG and IgA Ab in the mucosal airways. IpaB- and IpaD-specific IgG were detected in BALF of mice immunized with the Ipa admixed with dmLT. The titers were significantly lower in the absence of dmLT, and this was

**FIGURE 5.** ASC in mucosal and systemic tissues. Mice were immunized with IpaB, IpaD, and dmLT, as described in Fig. 2A. The frequency of Ag-specific ASC was measured in lung, spleen, and bone marrow cells obtained on day 56 after vaccination (the time of challenge). The data represent mean ASC counts/10^6 cells + SEM from quadruplicate wells. *p < 0.05, vaccinated mice versus PBS controls.

**FIGURE 6.** Cytokines produced by spleen and DLN cells upon IpaD and IpaB stimulation. Mice were immunized with IpaB, IpaD, and dmLT, as described in Fig. 2A. Cytokine levels were measured in culture supernatants from spleen and DLN stimulated in vitro with IpaB and IpaD. Results show mean concentration + SEM from triplicate wells. Dashed line represents average levels produced by unstimulated cells from vaccinated animals. *p < 0.05, vaccinated mice versus PBS groups.
particularly noticeable in the responses to IpaD (Fig. 9B). IgG Ab against dmLT were also detected; titers were similar for both i.d. groups and comparable to those measured in the i.n. control group. We were unable to detect vaccine-specific IgA in the alveolar fluid of the i.d.-vaccinated mice. In contrast, IgA specific for all three vaccine Ag was present in the BALF of mice immunized i.n. The amounts of total IgG and IgA among individual samples were not significantly different, thus confirming homogenous sampling (data not shown). However, it was noticed that the fluids of mice immunized i.n. contained larger quantities of total Ab than did mice immunized i.d. (data not shown); this was deemed consistent with nasal exposure activating (local) mucosal immunity.

Protection against S. flexneri and S. sonnei lethal pulmonary challenge

In agreement with our previous results, 70% of the mice that received the medium dose of IpaB and IpaD admixed with dmLT survived the challenge with virulent S. flexneri (Fig. 10A). In the absence of dmLT, the protection dropped to 50%. In contrast, the groups immunized with the highest dose of IpaB and IpaD had 30–40% protection, with dmLT providing no apparent improvement (Fig. 10A). Immunization with the medium dose of IpaB and IpaD plus dmLT also resulted in significant (50%) protection against S. sonnei (Fig. 10B). The percentage of survival dropped to 20% in the absence of dmLT. In both challenges, 100% protection was seen in the positive control group (Fig. 10).

Discussion

The recent Global Enteric Multicenter Study identified Shigella as a top priority intervention target to reduce illness and death caused by diarrheal disease in children under the age of 5 y in low-income countries (1). We have proposed the use of Shigella TTSS proteins IpaB and IpaD as protective Ag to formulate a broadly protective Shigella vaccine. The need for practical and effective ways to deliver such a vaccine prompted us to investigate the i.d. route of immunization, which, when coupled with new technologies, has emerged as a suitable alternative to i.m. injection because it is similarly effective and yet more practical and less painful. The fact that it has been the route of immunization for eradication of smallpox and continues to be successfully used to administer BCG to millions of children around the world provides a formidable precedent for its use to prevent enteric diseases in resource-poor areas. We demonstrated in this study that i.d. delivery of Shigella IpaB and IpaD admixed with dmLT is a simple and effective approach to induce protective immunity against lethal infection caused by different Shigella serotypes. To our knowledge, this is the first report of successful i.d. immunization with a cross-protective, subunit-based Shigella vaccine.

Multiple devices have been developed to facilitate i.d. vaccine delivery that are easy to use and more reliable than the conventional needle and syringe techniques (19). The NanoPass hollow microneedles used in this study allow delivery of the inoculum (even highly viscous substances) into the dermis in a slow and controlled manner. This device has been approved by the U.S. Food and Drug Administration and was safe, effective and reliable in clinical studies of i.d. influenza vaccination (31, 32). The microneedles are barely noticeable (shorter than 1 mm) and slightly penetrate the skin, thus greatly reducing pain (and the perception of pain). Different from traditional i.d. injection with needle and syringe (i.e., Mantoux technique), which requires highly trained personnel, i.d. injection with microneedles is easier, and vaccines potentially could be self-administered at home (19, 33). To our knowledge, this is the first report of a preclinical vaccine study using the NanoPass microneedles.

FIGURE 7. Protection against S. flexneri lethal pulmonary challenge. Mice were immunized, as described in Fig. 5A, they were challenged i.n. with $5.8 \times 10^7$ CFU of S. flexneri 2a on day 56. Data represent survival curves from 10 mice/group. *p < 0.05, vaccine groups versus PBS control.

FIGURE 8. Serum IpaB-, IpaD-, and dmLT-specific IgG responses. (A) Mice were immunized i.d. with IpaB and IpaD at the high and medium doses, with and without dmLT, on days 0, 14, and 28. Controls received IpaB, IpaD, and dmLT i.n. or PBS. (B) IpaB-, IpaD-, and dmLT-specific IgG titers were measured in serum by ELISA. Data represent mean titers ± SEM from 20 mice/group measured on day 55 and 10 mice/group measured at all other time points. Arrows indicate immunization. *p < 0.05, vaccine groups versus PBS control.
Our results demonstrate consistent delivery of vaccine into the dermis and the ensuing activation of an innate immune response with immediate recruitment of large numbers of neutrophils, followed by macrophages, DC, and LC. Our finding of CD11c+ DC, LC, and macrophages containing IpaB, as well as the evidence of cell activation, imply that these cells participate in Ag sampling and shuttling to the DLN for T cell stimulation (34). In fact, the success of i.d. vaccination is predicated on the efficiency of this process. The presence of IpaB- and IpaD-specific cytokine-secreting T cells in the inguinal lymph nodes of i.d.-vaccinated mice supports this notion. In addition, the recruitment of CD4+ and CD8+ T cells and activated Ag-carrying APC in the dermis up to 1 wk after vaccination suggests the possibility of local T cell priming. The presence of CD8+ T cells is consistent with reports (34, 35) describing the involvement of skin-activated LC preferentially priming Ag-specific CD8+ T cells after i.d. vaccination. Intradermal immunization with IpaB, IpaD, and dmLT resulted in high levels of Ag-specific serum IgG and systemic (spleen and bone marrow) IgG ASC. A distinct, unanticipated observation was the lack of serum IgA, systemic IgA ASC, and mucosal (stool and BALF) IgA when the Ag were administered i.d. vaccination.

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**FIGURE 9.** Serum IgG subclasses and BALF IpaB-, IpaD-, and dmLT-specific Ab. Mice were immunized with high and medium dosages of IpaB and IpaD, with or without dmLT, as described in Fig. 8A. IgG1 and IgG2 were measured on day 56. Subclass responses were also measured in mice that received dmLT alone from Experiment 1 (Fig. 5A), because this group was not included in Experiment 2. (A) Data represent mean (+ SEM) IpaB-, IpaD-, and dmLT-specific IgG1 and IgG2a titers from 10–20 mice/group. (B) Individual IpaB-, IpaD-, and dmLT-specific IgG and IgA titers measured in BALF collected on day 35 from five mice/group; horizontal lines represent mean values. *p < 0.05, vaccine groups versus PBS groups.

**FIGURE 10.** Protection against _S. flexneri_ and _S. sonnei_ lethal pulmonary challenge. Mice were immunized, as described in Fig. 8A, and challenged i.n. on day 56 with 5.4 × 107 CFU of _S. flexneri_ 2a (A) or 1.4 × 108 CFU _S. sonnei_ (B). Data represent survival curves from 10 mice/group. *p < 0.05, vaccinated mice versus PBS controls.
process that accompanies *Shigella* infection may also lead to transudation of serum IgG into the lamina propria and, thereby, limit tissue invasion.

Precedent exists for routine parenteral vaccines that preferentially induce systemic immunity to prevent mucosal infections (e.g., pneumococcal and *Haemophilus influenzae* type b conjugates, *Salmonella* Typhi Vi and pertussis vaccines). The same principle may operate in our model of i.d. immunization with IpaB and IpaD and lung exposure to virulent *Shigella*. Meanwhile, robust mucosal and systemic IgG and IgA ASC and Ab directed to all vaccine Ag were seen in mice immunized i.n. These differences likely reflect distinct pathways of immunological priming, with i.d. immunization favoring the induction of systemic IgG, and i.n. immunization favoring both systemic and mucosal IgA and IgG.

The high (and seemingly exclusive) IgG levels produced by i.d. vaccination could be explained, in part, by the fact that Ag delivered i.d. have access to dermal DC, which induce differentiation of naive T cells into T follicular helper cells, a subset that contributes to class switch and proliferation of B cells within germinal centers (34). Strikingly high levels of IL-4 and IL-5 were produced by Ag-stimulated T cells from i.d.-vaccinated mice. Skin LC are known to induce CD4+ Th2 cell differentiation (40). Thus, the demonstration of CD207+ DC carrying vaccine Ag in our study is consistent with the unusually high levels of Th2-type cytokines observed, which largely surpassed those of the i.n. group. Vaccine-induced T cells also produced IL-2 and IFN-γ, as well as IL-10, indicating that Th1-type and T regulatory responses were likewise induced.

IpaB was the more immunogenic of the two proteins (both for Ab and T cell responses), in agreement with results from previous studies (15, 16). The dmLT adjuvant increased Ab production and particularly the level of IgG2a, promoting a Th1-type response. To our knowledge, this is the first preclinical demonstration of tolerability and robust adjuvanticity of dmLT when administered i.d. alongside a protein subunit vaccine. A phase 1 clinical study to evaluate the safety and immunogenicity of dmLT administered i.d. to humans is being planned by the Division of Microbiology and Infectious Diseases at the National Institute of Allergy and Infectious Disease of the National Institutes of Health, in collaboration with PATH.

Importantly, mice immunized i.d. with IpaB, IpaD, and dmLT were protected from *S. flexneri* and *S. sonnei* lethal infection. The medium vaccine dosage was consistently the most effective. The higher death rate in the high-dose group was unexpected, yet was reproduced in two separate experiments. Interestingly, comparable levels of serum Ab were elicited by the different dose groups, despite their distinct protective efficacy. The amount of circulating IgG against IpaB and IpaD, alone, does not correlate with protection in this model (16), which suggests that other immunological effectors contribute to protective immunity. However, Ab are believed to play an important role in this process by blocking invasion and clearing the pathogen. In fact, early studies (41) reported lack of protection in B cell–deficient mice pre-exposed to sublethal doses of *Shigella* (as opposed to wild-type) and prolonged survival of naive mice upon passive transfer of immune sera. It is possible that the different vaccine doses might have induced Ab of different quality and functional capacity that influenced the protective outcomes. A noticeable difference between the medium- and high-dose groups, which could help to explain the dissimilar protection, was the increase in Ag-specific T cell cytokine responses (IFN-γ, IL-5, IL-2) in the DLN of the former. In a previous study (16), we found a correlation between IL-2, IL-5, IL-10, and IL-17 production and protection against lung infection in mice. IFN-γ, along with TNF-α, is believed to facilitate microbial clearance through recruitment and activation of phagocytic cells (42–44). The synthesis of IL-5, which stimulates B cell proliferation and Ab secretion, is consistent with supporting the vigorous IgG responses elicited by i.d. vaccination, whereas IL-2, which induces proliferation and differentiation of T and B cells, is expected to centrally regulate the responses induced. IL-17, which was detected in culture supernatants from Ag-stimulated spleen cells of i.d.-vaccinated mice, was shown to restrict bacterial growth in infected murine lungs (45). IL-10, also produced by spleen cells, may play a role in controlling inflammation and modulating T and B cell responses. T cells primed by skin-derived APC in regional lymph nodes are presumably endowed with the capacity to migrate to distant effector sites where they can help to circumscribe the infection. It is possible that an excess of Ag in the high-dose group may have compromised the processes of Ag uptake and/or presentation to T cells. Another potential reason for the superior T cell responses and protection of the medium-dosage group is the higher adjuvant/Ag ratio that these animals received. Although the amount of IpaB and IpaD in the vaccines for the different groups increased, dmLT remained constant, and this amount might have been insufficient or masked as more Ag was added. Thus, it could be assumed that optimal adjuvanticity seemingly was achieved for the medium-dose treatment. These results emphasize the need to test different Ag–adjuvant combinations in future vaccine studies to draw valid conclusions.

Although the vaccine-sparing advantage of i.d. over parenteral vaccination has been reported, little is known about how i.d. immunization compares with mucosal immunization. Beyond the differences in the type and magnitude of immune responses induced, both the i.n. and the i.d. routes of immunization conferred significant levels of protection, with i.d. vaccination requiring 25–40 times smaller doses. The use of the same route for immunization and challenge provides an “immunological advantage” that can aid in establishing the higher protection conferred by i.d. vaccination. Although successful in mice, an IpaB/IpaD dmLT vaccine could not be administered via the nasal route to humans because of safety concerns associated with the use of an enterotoxin–derived adjuvant (46, 47). It also has been argued that i.n. vaccination of children living in poor-resource (endemic) areas might be difficult because of their frequent respiratory infections and symptomatic runny noses, which may interfere with vaccine take (48). An i.d. IpaB and IpaD *Shigella* vaccine would be preferable, because it is expected to be safer, potentially more effective (not blocked by mucosal barriers), and applicable to large-scale immunization based on the precedent of BCG.

In conclusion, to our knowledge, our results provide the first preclinical evidence of cross-protective immunity of i.d.-delivered IpaB and IpaD against *Shigella* infection. The efficiency, ease of delivery, improved safety (avoiding the use of needles), and reduced cost (due to dose sparing) of i.d. vaccination are appealing features for implementing this approach to immunize against shigellosis children who live in high-risk areas, using an IpaB and IpaD-broad spectrum vaccine. These results and considerations warrant further investigation of i.d.-delivered IpaB and IpaD in humans.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1. Intradermal injection and uptake of injected dye. 25 μl of India Ink dye was administered i.d. into the shaved thigh of Balb/c mice using the NanoPass MicronJet 600 needle. (A) Macroscopic picture of the injection site during the procedure (top panel), immediately after injection, at 4 and 24 h and 1 wk after injection (bottom panel). (B) Histological images by light microscopy of H&E stained thigh tissue sections following i.d. India ink injection obtained with higher magnification (40x) to focus on the cellular infiltrate surrounding the injected fluid (top panel) and lower magnification (2x) to display the entire tissue section (bottom panel). Arrows indicate the location of hair follicles in the epidermal layer. Tissue from untreated mice served as controls. The low magnification images of the entire section (bottom row) show: 1) the inoculum delivered into the dermis and above the muscle (4 h after injection); 2) its progressive diffusion to nearby areas and influx of phagocytic cells with dense black cytoplasm (24 h); and 3) its dispersion into the subcutaneous layer (1 wk) (Supplemental Figure 1B, bottom). These observations were consistent from histological analysis of five individual mice.
Supplemental Table I. Comparison of serum IgG antibody responses against vaccine antigens

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Route</th>
<th>Vaccine</th>
<th>IpaB&lt;sup&gt;a&lt;/sup&gt;</th>
<th>IpaD&lt;sup&gt;a&lt;/sup&gt;</th>
<th>dmLT&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>i.d.</td>
<td>IpaB/IpgC+IpaD+dmLT (High)</td>
<td>239,452</td>
<td>131,476</td>
<td>3,053,612</td>
</tr>
<tr>
<td>2</td>
<td>i.d.</td>
<td>IpaB+IpaD+dmLT (High)</td>
<td>388,638</td>
<td>130,203</td>
<td>3,278,591</td>
</tr>
<tr>
<td>1</td>
<td>i.d.</td>
<td>IpaB/IpgC+IpaD+dmLT (Medium)</td>
<td>225,112</td>
<td>89,558</td>
<td>3,392,942</td>
</tr>
<tr>
<td>2</td>
<td>i.d.</td>
<td>IpaB+IpaD+dmLT (Medium)</td>
<td>696,761&lt;sup&gt;*&lt;/sup&gt;</td>
<td>105,378</td>
<td>3,826,246</td>
</tr>
<tr>
<td>1</td>
<td>i.n.</td>
<td>IpaB/IpgC+IpaD+dmLT</td>
<td>846,368</td>
<td>718,362&lt;sup&gt;*&lt;/sup&gt;</td>
<td>12,773,227&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>i.n.</td>
<td>IpaB+IpaD+dmLT</td>
<td>1,259,924</td>
<td>394,740</td>
<td>7,636,112</td>
</tr>
<tr>
<td>1</td>
<td>i.d.</td>
<td>PBS</td>
<td>28.15</td>
<td>12.5</td>
<td>519.39</td>
</tr>
<tr>
<td>2</td>
<td>i.d.</td>
<td>PBS</td>
<td>20.33</td>
<td>17.13</td>
<td>98.87</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean EU/ml values in serum samples from day 55 (n=10 for experiment 1 and n=20 for experiment 2). Asterisks denote significant differences (p<0.05) between Ab titers against individual antigens from all samples in Experiment 1 compared to the same group in Experiment 2, determined by one-Way ANOVA with Tukey’s multiple comparisons test.