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*J Immunol* 2006; 177:4709-4717; doi: 10.4049/jimmunol.177.7.4709

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High Vaccine Efficacy against Shigellosis of Recombinant Noninvasive Shigella Mutant That Expresses Yersinia Invasin

Toshihiko Suzuki,2,3*, Yuko Yoshikawa,*, Hiroshi Ashida,*, Hiroki Iwai,4*, Takahito Toyotome,5*, Hidenori Matsui,‡ and Chihiro Sasakawa2*,†*

Live attenuated Shigella vaccines elicit protective immune responses, but involve a potential risk of inducing a strong inflammatory reaction. The bacterial invasiveness that is crucial for Ag delivery causes inflammatory destruction of infected epithelial cells and proinflammatory cell death of infected macrophages. In this study, the noninvasive Shigella mutant ΔipaB was equipped with Yersinia invasin protein, which has been shown to mediate bacterial invasion and targeting to M cells located in follicle-associated epithelium. Invasin-expressing ΔipaB (ΔipaB/inv) was internalized into epithelial cells and retained in the intraphagosomal space. ΔipaB/inv did not induce necrotic cell death of infected macrophages nor cause symptomatic damage after intranasal vaccination of mice. ΔipaB/inv was safer and more effective than the conventional live vaccine, ΔvirG. Infection by ΔipaB/inv caused polymorphonuclear neutrophil infiltration in the lung, but did not induce production of large amounts of proinflammatory cytokines. We concluded that the low experimental morbidity and high vaccine efficacy of ΔipaB/inv are primarily based on high protective immune responses, which may be enhanced by the polymorphonuclear neutrophil infiltration unaccompanied by tissue injury. The Journal of Immunology, 2006, 177: 4709–4717.

Shigellosis is a leading cause of bacillary dysentery in humans. Each year, over 164 million cases occur worldwide, with the majority occurring in children in developing countries, and 1.1 million cases result in death (1). Antibiotics are generally effective against shigellosis, but because Shigella are increasingly developing antibiotic resistance, even to the newest antibiotics (2), the World Health Organization has given priority to the development of a safe and effective vaccine against Shigella (1).

The prominent pathogenic feature of Shigella is its ability to invade a variety of host cells, including macrophages, dendritic cells, and epithelial cells, which leads to a severe inflammatory response in intestinal tissue. When Shigella reach the colon and rectum, they translocate through the epithelial barrier via M cells that overlie the solitary lymphoid nodules (3, 4). Once they reach the sites underlying M cells, Shigella encounter resident macrophages, and the phagocytosed bacteria escape from the phagosome into the cytoplasm of macrophages. Shigella multiply in the cytoplasm and induce cell death through activation of caspase-1 mediated by IpaB protein secreted via the type III secretion system, in turn leading to the maturation and release of IL-1β (5, 6). However, our recent work has shown that macrophages undergo proinflammatory necrotic cell death, and that the necrosis and caspase-1 activation are due to components common to Gram-negative bacteria (7). Another group has reported that IpaF, a member of the pathogen-associated molecular pattern-sensing Nod family proteins, is implied a role in caspase-1 activation in Shigella-infected macrophages (8). Shigella released from killed macrophages enter the surrounding enterocytes through their basolateral surface by inducing membrane ruffling and macropinocytosis (9), and the invasion eventually results in extensive inflammation and tissue destruction of the villous epithelium (10).

The use of live attenuated mutants of Shigella in vaccines is a promising approach to protection against shigellosis (11). In recent years, a number of attenuated Shigella strains have been constructed and evaluated as candidates for use in vaccines (12–14). These strains have been classified into two groups on the basis of the function of the mutated gene: 1) strains in which one or two genes in the metabolism have been inactivated, such as thyA, araA, and aroD (15–17), and 2) strains harboring mutation of virG (icsA), a gene essential for cell-to-cell spreading, in addition to one of above gene mutations (18–24). The ΔvirG strain has been widely used as an attenuated live vaccine strain or in combination with mutation in other gene(s) in several studies, including in clinical trials (14, 21, 25–27). The vaccine strain, SC602, which has mutations in the virG gene and the aerobactin ics locus, has proceeded to phase 2 trials in humans. SC502 is safe in humans at low doses, but causes symptoms such as diarrhea and fever at high doses (14). Mutations in chromosome or virulence plasmid have...
been introduced to generate noninvasive live vaccine strains, but a high bacterial dose and long period of vaccination are needed to achieve protective immunity (12).

The development of Shigella vaccines that elicit efficient protective immune response development probably requires the retention of the invasiveness to deliver protective Ags, such as LPS, to mucosal immune system, and at the same time avoid the symptom of the invasiveness to deliver protective Ags, such as LPS, to protective immune response development probably requires the retention of the bacteria to induce strong inflammatory reaction, it is very difficult to maintain a balance between invasiveness, inflammation, and immunogenicity (12, 28). After Shigella invade epithelial cells, the bacteria escape from the phagosome and are released into the cytoplasm, where they activate IL-8 production via the Nod1-NF-kB pathway (29). We recently reported that the bacteria released into the cytoplasm induce necrotic cell death accompanied by caspase-1 activation in infected macrophages (7), and the necrotic cell death of macrophages leads to a strong inflammatory reaction (30, 31). Thus, the ability of Shigella to invade cytosolic space is presumably important to inducing a strong inflammatory reaction at the site of infection.

To overcome the difficulty in maintaining the balance between bacterial invasiveness and proinflammatory responses, we focused on invasin, the outer membrane protein produced by Yersinia pseudotuberculosis (32). Invasin binds β1 integrin, and is involved in bacterial invasion of epithelial cells and in bacterial association with M cells on Peyer’s patches (33). Invasin-expressing bacteria are able to invade epithelial cells, but unable to escape from the phagosome, and they cannot induce the cell death of infected macrophages, and thus would be ideal for use in a live vaccine. In this study, we demonstrated that an invasin-expressing noninvasive Shigella mutant induced more efficacious protection against infection by wild-type Shigella than the conventional invasive vaccine strain in a mouse pulmonary infection model.

Materials and Methods

Shigella strains and plasmids

The wild-type Shigella flexneri 2a YSH6000 strain has been described previously (34). Shigella mutants, a noninvasive ipaB gene null mutant (ΔipaB) (7) and a cell-to-cell spreading deficient virG null mutant (ΔvirG) (35), were used in this study. To visualize living bacteria in infected cells, a gfp gene was cloned into pUC19-Tp (36) and transformed into Shigella strains. To construct invasin-expressing ΔipaB (ΔipaBInv)6 the invasion gene of Y. pseudotuberculosis on pRRI (37) (provided by H. Wolf-Watz, Umeå University, Umeå, Sweden) was cloned into pWKS130 (38) and transformed into ΔipaB. The Shigella strains were grown routinely in brain-heart infusion broth (BD Biosciences).

Reagents

Anti-invasin Ab was provided by R. Isberg (Tufts University, Boston, MA). The following Abs were obtained commercially: rabbit anti-mouse caspase-1 (Santa Cruz Biotechnology), rabbit anti-mouse IL-1β (R&D Systems), and goat anti-myeloperoxidase (MPO; Santa Cruz Biotechnology).

Bacterial infection of culture cells

HeLa cells were seeded at 4 × 105 cells in 6-well plates containing 10% FCS-MEM (Sigma-Aldrich). They were infected with Shigella at multiplicity of infection of ~100 per cell, and the plates were centrifuged at 600 × g for 10 min to synchronize the stage of infection. After incubation for 30 min (wild-type Shigella) or 1 h (ΔipaB and ΔipaBInv), gentamicin (100 μg/ml) and kanamycin (60 μg/ml) were added to kill the extracellular bacteria, and the cells were then incubated until the times indicated. The infected cells were fixed and immunostained, as described previously (39), and they were analyzed with a confocal laser-scanning microscope (LSM510; Zeiss). For infection of macrophages, mouse J774 macrophage-like cells were seeded at 5 × 105 cells in 24-well plates containing 10% FCS-RPMI 1640 (Invitrogen Life Technologies). The cells were infected with Shigella at multiplicity of infection of ~10 per cell. Noninvasive ΔipaB was infected with ~40 bacteria per cell. The plates were centrifuged at 600 × g for 10 min, and gentamicin (100 μg/ml) and kanamycin (60 μg/ml) were added 30 min later. At the times indicated after infection, the lactate dehydrogenase (LDH) activity of the culture supernatants of infected cells was measured by using a CytoTox 96 assay kit (Promega), according to the manufacturer’s protocol. Cytokines in the culture supernatants of the cells were measured with an ELISA kit (R&D Systems).

Mouse vaccination and challenge

Mouse handling conformed to the requirements of the Animal Care and Use Committee of the Institute of Medical Science, University of Tokyo. Six-week-old BALB/c female mice (Japan CLEA) were anesthetized and intranasally (i.n.) inoculated with the bacteria indicated in 20 μl per head, as previously described (9). The animals were vaccinated on days 0 and 21, and then challenged with wild-type Shigella (2 × 106 CFU) on day 28.

Bacterial counts in infected lung

Mice were sacrificed at different time points after infection, and the lungs were removed and homogenized in 1 ml of PBS containing 0.5% Triton X-100. Serial dilutions of the lung homogenates were plated on Luria-Bertani agar to obtain bacterial counts (CFU).

Histological analyses

Mice were sacrificed at the times indicated after infection, and their lungs were removed and fixed in 4% parafomaldehyde in PBS. To measure cytokine production, the lungs were homogenized in 1 ml of PBS containing a protease inhibitor mixture (Roche), and the supernatant was diluted and used for ELISA. The lung tissue embedded in OTC compounds (Sakura) was frozen in liquid nitrogen and sectioned with a Leica cryostat (model CM1900). TUNEL assay was performed with the DeadEnd Fluorometric TUNEL System (Promega), and the same sections were immunostained with FITC anti-Shigella LPS Ab and counterstained with hematoxylin. The sections were examined under a confocal microscope (LSM510; Zeiss), and cells containing a nucleus whose intensity was greater than an arbitrary threshold of 50 in a noninfected lung area were defined as TUNEL positive. To immunostain for MPO, the sections were incubated with anti-MPO Ab and then with donkey HRP-conjugated anti-goat Ab (Jackson ImmunoResearch Laboratories). The color reaction was developed with a Vector Nova RED substrate kit (Vector Laboratories), and the sections were counterstained with hematoxylin. Because erythrocytes also react with anti-MPO Ab, only cells that contained both a polymorphous nucleus and MPO-positive cytoplasm were counted as MPO-positive polymorphonuclear neutrophils (PMNs). Bronchoalveolar lavage (BAL) was performed by inflating the lungs with 2 ml of PBS and withdrawing the fluid through the trachea. Neutrophil elastase activity in BAL fluid was measured spectrophotometrically at A 410 nm by using the neutrophil elastase-specific peptide substrate MeO-Suc-Ala-Ala-Pro-Val-pNA (Calbiochem). The BAL fluid was incubated with 1 mM substrate in assay buffer (0.1 M Tris and 1 M NaCl (pH 7.4)) (40).

Measurement of Abs to Shigella LPS

Serum and BAL fluid samples were collected on day 28 after the first vaccination, and IgG and IgA Abs against S. flexneri 2a LPS were measured by ELISA (Bethyl Laboratories). After coating 96-well plates with 100 μl of heat-water-soluble-extracted LPS (10 μg/ml) (41), the serum and BAL fluid were serially diluted in 2-fold steps, beginning with a dilution of 1/100 for the sera and 1/10 for the BAL fluid. Incubation, washing, and development were performed, according to the manufacturer’s protocol. Linear regression curves were plotted for each sample, and titers were calculated as the inverse of the dilution that produced an OD of 0.2 above the blank.

Statistical analyses

Statistical analyses were performed by the Mann-Whitney U test (CFU assays, ELISA, changes in body weight) and log-rank test (survival assays). Differences were considered significant at p < 0.05.
Results

Generation and characterization of an invasin-expressing Shigella mutant

To generate a live vaccine that would invade epithelial cells and not induce macrophage death, we introduced the invasin gene into the S. flexneri noninvasive ΔipaB mutant, which is unable to escape from the phagosome when phagocytosed by macrophages (Fig. 1A). We then investigated whether ΔipaB/inv was capable of being internalized by HeLa cells. As shown in Fig. 1B, a gentamicin protection assay showed that the number of internalized ΔipaB/inv was 5 times higher than the number of internalized ΔipaB, indicating that ΔipaB/inv has the ability to invade epithelial cells. To confirm this, HeLa cells were infected with GFP-expressing wild-type S. flexneri YSH6000, ΔipaB, and ΔipaB/inv. The extracellular bacteria were immunostained with Cy5-labeled anti-Shigella LPS Ab under nonpermeabilized conditions and examined by confocal microscopy. As shown in Fig. 1C, some wildtype Shigella and ΔipaB/inv bacteria were visualized by GFP alone, indicating they had been internalized by the cells, whereas confocal microscopic data of ΔipaB revealed both GFP and Cy5 fluorescence; thus, all of the bacteria had remained in the extracellular space. Furthermore, after 3 h of infection, the wild-type Shigella multiplied and induced the formation of actin comets in the cytoplasm, whereas ΔipaB/inv did not multiply or induce actin assembly (Fig. 2), even though ΔipaB/inv expresses VirG protein, which induces actin assembly (data not shown). These findings indicated that ΔipaB/inv is internalized by epithelial cells, but does not escape from the surrounding phagosomal membrane.

Previous studies have shown that intracytosolic Shigella that have escaped from phagosomes induce death of the infected macrophages and dendritic cells (7, 42), and that components common to Gram-negative bacteria induce necrotic cell death and caspase-1 activation (7). Mouse J774 macrophages were infected with wild-type Shigella, ΔvirG (conventional attenuated vaccine strain), ΔipaB, and ΔipaB/inv to determine whether ΔipaB/inv was capable of inducing cell death. Wild-type Shigella and ΔvirG, but not ΔipaB or ΔipaB/inv, induced rapid LDH release, thus indicating that ΔipaB/inv, the same as ΔipaB, does not induce the necrotic cell death of infected macrophages (Fig. 3A). Caspase-1 activation and caspase-1-mediated IL-1β processing were induced by wild-type Shigella and ΔvirG, but not by ΔipaB or ΔipaB/inv (Fig. 3B). Furthermore, the levels of the proinflammatory cytokines, IL-1β and IL-18, which are also processed to the mature form by caspase-1, were elevated after infection with wild-type Shigella and ΔvirG, but to a lesser extent by ΔipaB and ΔipaB/inv (Fig. 3C). These findings suggested that ΔipaB/inv, the invasive strain that is unable to escape from phagosomes, is not cytotoxic, and that it is incapable of inducing the strong inflammatory responses associated with caspase-1 activation.

Experimental morbidity induced by the ΔipaB/inv strain in vivo

To assess the safety of the ΔipaB/inv vaccine strain in vivo, BALB/c mice were infected via the i.n. route with ~2 × 10⁸ CFU. BALB/c mice infected with 2 × 10⁶ CFU of wild-type Shigella do not survive for more than ~10 days. Changes in the body weight and survival rate of infected mice were monitored at this dose. The mice were infected with the vaccine strain ΔvirG, ΔipaB, or ΔipaB/inv twice, 21 days apart. The mice infected with ΔipaB or ΔipaB/inv survived for 28 days and exhibited no clinical manifestation and no significant body weight loss (Fig. 4A). However, all mice infected with ΔvirG had ruffled fur, a stooped posture, and

![FIGURE 1. Invasin-mediated cell invasion. A. Expression of invasin. Total bacterial lysates were analyzed by Western blotting with anti-invasin Ab. B. Quantification of the bacteria internalized in infected HeLa cells. After incubation (YSH6000, 1 h; ΔipaB and ΔipaB/inv, 2 h), the number of intracellular bacteria was counted. Representative data from two similar experiments (triplicate) are shown. Data are means ± SD, p < 0.05 (Mann-Whitney U test) for ΔipaB/inv compared with ΔipaB. C. HeLa cells were infected with GFP-expressing bacteria (green). After incubation for 30 min with YSH6000, or 2 h with ΔipaB and ΔipaB/inv, the cells were immunostained for Shigella LPS (blue) under nonpermeabilized conditions and counterstained for actin filaments (red). Scale bar, 10 μm.](http://www.jimmunol.org/)

![FIGURE 2. Intracellular ΔipaB/inv does not escape from phagosomes. HeLa cells were infected with GFP-expressing bacteria (green) for 3 h. The cells were then immunostained for Shigella LPS (blue) under nonpermeabilized conditions and counterstained for actin filaments (red) to visualize actin comets formed from intracellular Shigella. Scale bar, 10 μm.](http://www.jimmunol.org/)
dyspnea on the day after infection, indicating illness. The body weight loss of ΔvirG-vaccinated mice was significant (p < 0.05) compared with the saline-vaccinated group (Fig. 4A). Approximately 60% of the mice infected died during the course of vaccination (p = 0.004, compared with saline) (Fig. 4B). Although the ΔvirG strain has been used as an attenuated live vaccine in several studies, including in clinical trials (14, 21, 25–27), these findings suggested that bacteria with single mutation in the virG gene retain virulence in mice when inoculated with a large number of bacteria. Low dose (2 × 10^7 CFU) infection of ΔvirG also caused significant body weight loss, to a maximum of 22.3 ± 5.53% (p < 0.05; n = 10), compared with the saline-vaccinated mice.

**Superior efficacy of ΔipaB/inv vaccine against wild-type Shigella infection**

BALB/c mice were vaccinated (two i.n. doses of 2 × 10^7 CFU) with ΔvirG, ΔipaB, or ΔipaB/inv, and challenged with wild-type YSH6000 strain 28 days after the first vaccination (7 days after the second vaccination). Vaccination with ΔvirG or ΔipaB/inv considerably prevented the body weight loss in the challenged mice as compared with the saline group and ΔipaB-vaccinated group (Fig. 5A), and the ΔvirG- and ΔipaB/inv-vaccinated mice exhibited the milder pathologic features, such as ruffled fur, than the saline- or ΔipaB-vaccinated group. Although ΔvirG vaccination protected against lethal infection by wild-type Shigella (50% survival rate),
protection by ΔipaBinv was substantially more successful (90% survival rate, p = 0.043 compared with ΔvirG) (Fig. 5B). Neither saline nor ΔipaB vaccination protected against lethal infection by wild-type Shigella. Thus, these data suggested that the invasive strain ΔipaBinv is a safer and more effective vaccine strain for protection against infection by wild-type Shigella.

ΔipaBinv-induced inflammatory responses in the lungs of infected mice

To gain deeper insight into the biological mechanisms responsible for the superior vaccine efficacy of ΔipaBinv, inflammatory responses in vivo were analyzed in the early infectious stage of lung infection. First, to assess bacterial clearance in infected mice, the number of bacteria in the lungs of mice infected with \(2 \times 10^7\) CFU was counted 12 and 24 h after infection. As shown in Fig. 6A, the number of bacteria recovered from lungs infected with conventional ΔvirG was two orders of magnitude higher than the number recovered from mice infected with noninvasive ΔipaB. At 12 h after infection, however, the bacterial count in ΔipaBinv-infected mice was significantly higher (\(p < 0.05\)) than in the ΔipaB-infected mice, but the difference was not significant at 24 h, indicating that ΔipaBinv is capable of invading lung cells and transiently persists by escaping host eradication. ΔipaBinv did not induce cell death in vivo as confirmed by TUNEL staining (Fig. 6B). To determine whether the difference in inflammation in the lung was reflected in the pathology and survival of the infected mice, histological studies of the lungs of infected mice were performed at 12 and 24 h after infection. Frozen sections of lung from infected mice were stained with hematoxylin and FITC-conjugated anti-Shigella Ab. As shown in Fig. 6C, at 12 h after infection, the mice infected with ΔvirG had developed alveolitis and acute bronchopneumonia, with massive intra- and peribronchial PMN infiltration. Unexpectedly, the mice infected with ΔipaBinv were found to have moderate alveolitis with PMN infiltration. The PMN infiltration was quantified by counting MPO-positive morphonuclear cells in sections of infected lung. We observed a large number of MPO-positive cells in the ΔvirG-infected mice, and that their number was significantly higher after infection with ΔipaBinv (\(p < 0.05\)) than after infection with ΔipaB (Fig. 6D). The PMN infiltration in ΔipaBinv-infected lung was sustained at 24 h after infection. However, the same as the mice infected with ΔipaB, the ΔipaBinv-infected mice did not exhibit any signs of disease, such as dyspnea, and there was no significant body weight loss (Fig. 4). To explain these discrepancies, we measured the activity of neutrophil elastase, the enzyme released by activated PMNs that is implicated in tissue destruction (43, 44). Elastase activity was significantly higher (\(p < 0.05\)) in the BAL fluid of mice infected with ΔvirG, and its activity in ΔipaB- or ΔipaBinv-infected mice was basal, the same as in the saline-infected mice (Fig. 6E), suggesting that PMN infiltration of ΔipaBinv-infected lung is not inducible tissue injury. The levels of proinflammatory cytokines, MIP-2 (mouse ortholog of human IL-8), IL-1β, IL-6, and TNF-α, produced in the lung after infection were measured. At 12 h after infection, production of a key murine PMN attractant, MIP-2, was significantly elevated in the lungs infected with ΔvirG compared with the lung infected with ΔipaB; but also in the lungs infected with ΔipaBinv (\(p < 0.05\); Fig. 7A), suggesting that MIP-2 plays a role in PMN infiltration of lungs infected with ΔvirG and ΔipaBinv. The levels of other proinflammatory cytokines, IL-1β, IL-6, and TNF-α, were significantly elevated by infection with ΔvirG, but lesser amounts were produced when infected with ΔipaB or ΔipaBinv (Fig. 7, B–D), suggesting no symptomatic damage in mice infected with ΔipaBinv, the same as in mice infected with ΔipaB, due to the lack of a large amount of proinflammatory cytokine production. These data also suggested...
that PMN infiltration alone is not necessarily associated with lung cell damage, which may depend on both PMN infiltration and PMN activation by proinflammatory cytokines, such as IL-6 and TNF-α (see Discussion).

Vaccination with ΔipaB/inv induces a high anti-Shigella LPS IgG titer that is correlated with protective immunity against Shigella

Because LPS-specific Ab production is crucial for protection against lethal pneumonia in the mouse pulmonary infection model (45, 46), we measured the concentrations of anti-LPS IgG and IgA in serum and BAL fluid to S. flexneri serotype 2a Ags by ELISA with crude LPS prepared from YSH6000 strain as the Ag. The IgG titer in the serum and BAL fluid of both ΔvirG and ΔipaB/inv-vaccinated mice was significantly higher than in saline mock-vaccinated mice, and there was no significant titer in ΔipaB-vaccinated mice (Fig. 8, A and B). These data were correlated well with the profiles of protection of the vaccinated mice against challenge with wild-type Shigella (Fig. 5B). By contrast, there were no significant differences between the serum and BAL fluid IgA titers of

FIGURE 6. Histological profiles of the lungs of mice vaccinated with ΔipaB/inv vaccine. A, BALB/c mice were infected with 2 × 10⁷ CFU of vaccine strains. CFUs of the bacterial load in the lung were determined at the times indicated. p < 0.05 (Mann-Whitney U test) for ΔipaB/inv compared with ΔipaB at 12 h after infection. The data are mean values for four mice (±SD). B, Quantitation of the TUNEL assay in infected lung sections. The data are mean values of five digitized images (field area 0.053 mm²) from at least three sections from four mice (±SD). C, Infected lung sections were immunostained with FITC-conjugated anti-Shigella LPS (green) and hematoxylin. Scale bar, 50 μm. D, Quantitation of MPO-positive cells in sections of infected lung. p < 0.05 (Mann-Whitney U test) for ΔipaB/inv compared with ΔipaB at 12 h after infection. The data are mean values of five digitized images (field area 0.042 mm²) from at least three sections from four mice (±SD). E, Relative elastase activity in the BAL fluid of infected mice. The data are the mean of values of four mice (±SD).
\( \text{H9004} \)virG, \( \text{H9004} \)ipaB, and \( \text{H9004} \)ipaB/inv-vaccinated mice, even though a high IgA titer was observed in the mice vaccinated with these strains (Fig. 8, C and D). Thus, vaccination with \( \text{H9004} \)ipaB/inv induces a high anti-LPS IgG titer that is correlated with protection against infection by wild-type \text{Shigella}, and similar to the titer induced by conventional vaccine \( \text{H9004} \)virG. These findings also suggested a predominant role of the anti-LPS IgG rather than IgA in the vaccine-induced protective immunity in the mouse pulmonary infection model.

\textbf{Discussion}

This study describes a new invasive \text{Shigella} vaccine and its superior protective activity against shigellosis. We constructed a noninvasive \text{Shigella} \( \Delta \text{ipaB} \) mutant expressing \text{Yersinia} invasin and assessed its safety and protective activity as a vaccine in mice by comparing them with mice vaccinated with conventional \( \Delta \text{virG} \) and the parental noninvasive strain, \( \Delta \text{ipaB} \). The results showed that \( \Delta \text{ipaB} \text{inv} \) is safer and more efficacious for inducing protective immunity against \text{Shigella} infection than \( \Delta \text{virG} \) or \( \Delta \text{ipaB} \). To our knowledge, this is the first study to demonstrate an effective invasive \text{Shigella} vaccine that does not induce severe inflammatory responses, as manifested by ruffled fur, a stooped posture, dyspnea, and death of the vaccinated mice.

The ability of invasive \text{Shigella} used in vaccines to escape from the phagosome into the cytoplasm of infected cells is an important property for a safe and effective live vaccine. Bacteria in the cytoplasm of infected epithelial cells elicit NF-kB activation, and bacteria in the cytoplasm of infected macrophages cause necrotic cell death, and the host responses induce a strong inflammatory reaction at the site of infection. To prevent the escape of invading bacteria from phagosome and retain their invasiveness, we turned our attention to invasin, the outer membrane protein produced by \text{Y. pseudotuberculosis}. \text{Y. pseudotuberculosis} and \text{Yersinia enterocolitica} are enteropathogenic in humans and cause gastrointestinal disorders, such as enterocolitis and mesenteric lymphadenitis (32). Enteropathogenic \text{Yersinia} also express homologous invasin. The

\begin{figure}[h]
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\caption{Production of proinflammatory cytokines. BALB/c mice were infected i.n. with \( 2 \times 10^7 \) CFU of the vaccine strains. At the times indicated, the extract of infected lung was analyzed by ELISA for production of MIP-2 (A), IL-1\( \beta \) (B), IL-6 (C), and TNF-\( \alpha \) (D). * \( p < 0.05 \) (Mann-Whitney U test). The means (±SD) of results for four mice are shown.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig8}
\caption{Reciprocal anti-\text{Shigella} LPS IgG and IgA titer in serum and BAL fluid from vaccinated mice. BALB/c mice were vaccinated twice with \( 2 \times 10^7 \) CFU of the indicated strains for 28 days. IgG (A and B) and IgA (C and D) responses in serum (A and C) and BAL fluid (B and D) are shown. ** \( p < 0.01 \) (Mann-Whitney U test).}
\end{figure}
invasin protein binds β1 integrin and is involved in bacterial invasion of epithelial cells and association with M cells on the Peyer’s patches (33). Our findings in this study showed that invasinated-mediated cell invasion did not induce a severe inflammatory response in infected mice, because the invading bacteria remained surrounded by the phagosomal membrane. Bacterial infection of mouse lung tissue instead selectively induced production of MIP-2, which elicits PMN infiltration. Infection of human cells by invasin-expressing bacteria induces IL-8 production via the NF-κB pathway (47, 48), and in vitro evidence has shown that recombiant invasin itself induces IL-8 production when the protein binds to β1 integrin on the host-cell surface (47). Although it is still unclear whether bacterial invasion by ΔinvAΔinv in vaccine or invasin itself induces MIP-2 production in mice followed by PMN infiltration, we have shown clear evidence that this live vaccine does not elicit inflammatory responses strong enough to evoke symptomatic tissue damage despite inducing PMN infiltration.

ΔinvAΔinv vaccine elicited PMN infiltration in lung tissue without inducing high levels of the proinflammatory cytokines TNF-α, IL-6, or IL-1β. TNF-α is a potent activator of PMNs and stimulates them to release elastase, which causes tissue damage (49). In Shigella infection, TNF-α has been found to be involved in the rupture and inflammatory destruction of infected epithelium in a rabbit ligated intestinal loop model (50). IL-6 is also capable of affecting the deformability of PMNs and promoting their sequestration in the lung (51), and it augments basal elastase release in vitro (52). It is therefore tempting to speculate that TNF-α, and/or IL-6 produced in infected lung affect the activity of PMNs that damage lung tissue. In addition to eliminating invading pathogens, PMNs may play an important role in the transition from innate to adaptive immune responses by producing cytokines and chemokines (53). Indeed, such immunomodulatory functions have been reported for several intracellular parasitic bacteria, including Mycobacterium tuberculosis (54) and Legionella pneumophila (55); however, the precise mechanisms by which neutrophils function in immune responses induced by ΔinvAΔinv vaccine remain to be elucidated.

Vaccination with ΔvirG and ΔinvAΔinv in the present study showed that anti-LPS IgG, rather than anti-LPS IgA, is predominantly correlated with protection against wild-type Shigella in mouse model. However, it has been shown that there is no correlation between high anti-LPS IgG and protection (45), and a monoclonal anti-LPS IgA has been demonstrated to confer protective immunity (56), although protection was still achieved using the mice lacking IgA (57). There was no clear correlation between protective responses and high anti-LPS IgA titers in a guinea pig infection model in which two routes of inoculation were used, i.e., intravenous and intragastric (58). A significant correlation between anti-LPS IgG and resistance to shigellosis has been reported in humans (59-60). Although the above findings may suggest that production of anti-LPS IgG and IgA is associated with protection, the role of serum Abs against Shigella LPS in protective immunity is still unclear.

Several strategies have been reported to improve the efficacy of invasive Shigella vaccines and to prevent strong inflammatory reaction (12). These attenuated invasive Shigella vaccines have also served as a vector for foreign Ag delivery to the mucosal immune system (61), because the early entry of invasive Shigella occurs via M cells in rabbit ligated intestinal loop model of Shigella infection (3, 4). The most important feature of invasin is that it targets β1 integrin on M cells located in the follicle-associated epithelium, and thus introducing invasin into noninvasive Shigella strain may enable the invasin-expressing strains to target M cells by interacting with β1 integrin expressed on the surface of M cells. Although further study is needed to evaluate the efficacy of oral vaccination in inducing mucosal immune responses, invasin-mediated Ag delivery via M cells would provide a new strategy for induction of mucosal immunity.

Acknowledgments

We thank Drs. Hiroshi Kiyono and Mi-Na Kweon for helpful discussions and suggestions, Dr. Kenji Ohya for technical assistance and discussions, and the other members of the Sasakawa laboratory for critical reading of the manuscript and technical advice.

Disclosures

The authors have no financial conflict of interest.

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