Construction and Characterization of a Live Attenuated Vaccine Candidate against Shigella dysenteriae Type 1

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Vaccine candidates against Shigella dysenteriae type 1, which is associated with the most severe cases of bacillary dysentery, were constructed. The rfp and rfb gene clusters, which code for S. dysenteriae 1 O antigen biosynthesis, were randomly integrated into either the chromosome or the virulence plasmid of the rough attenuated Shigella flexneri aroD strain SFL124-27 with a minitransposon carrying an arsenite resistance selection marker. The recombinant clones efficiently expressed the recombinant O antigen, exhibited a normal growth pattern, were able to invade and survive within eukaryotic cells to the same extent as the parental strain, and expressed the recombinant antigen within invaded cells. A clone was selected as the vaccine candidate, which was demonstrated to be immunogenic and safe in animal models, leading to 47% full protection and 53% partial protection against challenge with the wild-type strain.

Bacillary dysentery caused by Shigella spp. is a major public health problem in developing countries, with at least 250 million cases per year, of which more than 650,000 are fatal, mainly in children under 5 years of age (25). Shigellosis is highly transmissible because of bacterial spread via the fecal-oral route and because it typically involves a very low infective dose (8). Different serotypes of Shigella flexneri and serotype 1 of Shigella dysenteriae are prominent causes of shigellosis in areas of developing countries in which shigellae are endemic. However, S. dysenteriae 1 has been and continues to be responsible for several epidemic outbreaks of shigellosis throughout the world (4, 44, 66). It is also the most virulent species and is associated with the most severe cases of dysentery and the highest rates of complications (e.g., hemolytic-uremic syndrome, hemorrhagic colitis, sepsis, and purpura [29]). This increased risk of complications seems to be due to the production of a powerful cytotoxin, Shiga toxin, which attacks the endothelial cells of blood vessels (10, 35). The prevalence of multiply drug-resistant strains renders treatment difficult and emphasizes the need for efficacious vaccines (37, 56, 65, 66).

Protective immunity against shigellosis is serotype specific and correlates with the stimulation of local intestinal immunity against the O-specific surface lipopolysaccharide (LPS) (42, 45, 64). Different approaches have been taken to develop vaccines against Shigella spp. (for review, see reference 34); however, no vaccine against S. dysenteriae 1 has yet proven to be efficient. A common strategy for vaccine development that has been applied for different serotypes of S. flexneri consists of the introduction of attenuating mutations into virulent strains (33, 38, 41, 49). To overcome the reactogenicity associated with live vaccines, conjugates of synthetic peptides encompassing Shiga toxin B subunit epitopes required for the elicitation of neutralizing antibodies were constructed and used for immunization of mice and rats, resulting after several immunizations in up to 70 and 93% protection, respectively, against challenge with purified Shiga toxin (1). The parenteral immunization of human volunteers with a conjugate vaccine consisting of S. dysenteriae 1 O polysaccharide coupled to tetanus toxoid resulted in the elevation of humoral antibodies specific for LPS (6, 57). However, the efficacy of this vaccine remains to be assessed. Our strategy consisted of the expression of S. dysenteriae 1 LPS on the surface of a well-characterized antigen carrier strain. S. flexneri SFL124-27 (9) is a spontaneous rough mutant of the attenuated S. flexneri auxotrophic strain SFL124, which carries a deletion of the aroD gene. The vaccine candidate SFL124 has proven to be well tolerated (only 9.5% of the vaccinees had self-limiting diarrhea lasting 1 day after the first dose), immunogenic for animals and humans (23, 30–33), and efficacious in animal models (16, 24, 36).

In S. dysenteriae 1, the genes necessary for O antigen biosynthesis lie on a 9-kb multicopy plasmid (rfp genes [62]) and on the chromosome (rfb cluster [14, 54]). The rfp genes and eight contiguous genes of the rfb cluster have been combined in an rfp-rfb cassette (55), which after introduction into Escherichia coli K-12 (55), attenuated derivatives of Salmonella spp. (39), S. flexneri SFL124, or SFL124-27 (9), directed the synthesis of S. dysenteriae 1 O antigen. The recombinant plasmids were, however, unstable when the strains were cultivated without selective pressure (39). We report here the construction and characterization of recombinant S. dysenteriae 1 and S. flexneri hybrid vaccine strains obtained by the stable random integration of the S. dysenteriae 1 rfp-rfb genes into the attenuated vaccine carrier strain S. flexneri SFL124-27. Because antibody resistance markers are undesirable in vaccines but a phenotypic marker would be particularly helpful for genetically engineered microorganisms which will be released under uncontrolled conditions, they were genetically tagged with the genes encoding arsenite resistance. The growth pattern, stability of the heterologous O antigen expression, invasiveness, intracellular survival, immunogenicity, safety, and efficacy in animals of the resulting vaccine strains have also been evaluated.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The rough auxotrophic S. flexneri (aroD) strain SFL124-27 was used as a carrier strain (9). The S. dysenteriae 1
S. flexneri carrier strain SFL124-27 and analysis of O antigen expression by recombinant clones. The expression of heterologous LPS by a smooth carrier strain may result in weak or inefficient immune responses as a result of O antigen masking by the homologous O antigen. Covalent linkage of the heterologous O antigen to the core lipid A moiety of the carrier strain, which is probably a prerequisite for immunogenicity and reactivity of the heterologous O antigen, did not occur when the smooth attenuated strains Salmonella typhi Ty21a (11, 19, 39, 52) and Vibrio cholerae CVD103-HgR (61) were used as antigen carrier strains. Therefore, LPS from Congo red-positive colonies of the remaining 21 ampicillin-sensitive transconjugants was isolated and separated by tricine-SDS-PAGE, and LPS bands were detected by silver staining (58) or by Western blotting (9) with rabbit polyclonal antiserum against O antigen of either S. dysenteriae type 1 or S. flexneri. 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S. dysenteriae wild-type strain gen (B). LPSs from the smooth strain S. flexneri S. dysenteriae or by immunoblotting with polyclonal antibodies against strains. LPS was separated by SDS-PAGE and developed by silver staining (A). The efficient expression of recombinant O antigen by the carrier strain grown in vitro does not mean that a similar behavior will be observed within host eukaryotic cells. Since efficient intracellular expression of the O antigen might be required for the efficacy of the vaccine candidate against Shigella spp. (50, 51). Therefore, the virulence plasmids of Congo red-positive and -negative colonies of the six recombinant clones were hybridized with the rfpB probe after Southern transfer. Only the virulence plasmids of Congo red-positive colonies of clones SFL124-27::Tn(rfp-rfb)-8 and -11 hybridized with the rfpB probe, suggesting an integration of the minitransposon into the virulence plasmid, whereas in clones 32, 35, 39, and 43, the chromosome was the integration site (data not shown).

Growth curves and stability of virulence plasmid and O antigen expression in recombinant strains. The integration of the minitransposon into the carrier strain might have inactivated functions that are essential for bacterial growth and viability. However, all recombinant strains exhibited a growth pattern similar to that of the wild type, excluding any effects on the growth pattern by the integration event (data not shown).

Since S. flexneri SFL124-27 is attenuated through deletion of the aroD gene and the integration of foreign genes might influence the auxotrophic properties of this strain, dependence of recombinant clones on aromatic compounds was tested by the plating of cells on minimal medium. No change in the auxotrophic phenotype in any of the recombinant clones was detected (data not shown).

The use of a suicide delivery plasmid should lead to a stable integration of the foreign genes, because the transposase gene is lost after the transposition event, as could be shown by Southern hybridization with a probe for the transposase gene. To assess the stability of the rfp-rfb expression and the virulence plasmid, the recombinant strains SFL124-27::Tn(rfp-rfb)-8, -11, -32, -35, -39, and -43 (only these strains were invasive [see below]) were sub cultured for 3 days without selective pressure (Table 1). The rfp-rfb expression was 100% stable after 21 generations in clones 32, 35, 39, and 43, which harbor the chromosomal integration of the minitransposon. The integration site was the integration site (data not shown).

Southern blot analysis of recombinant SFL124-27 strains. Total DNA from the parental and recombinant strains was digested with PvuII and used for Southern blot analysis with probes specific for the rfpB (Fig. 2A), arsA (Fig. 2B), and transposase genes. As expected for the DNA from ampicillin-sensitive clones, no hybridization signals could be detected with the transposase probe (data not shown). The sizes of the PvuII fragments that hybridized with the probes for the rfpB or arsA gene were the same in clones SFL124-27::Tn(rfp-rfb)-8 and -11 and clones -32, -35, and -43. This suggested either hot spots for integration or the presence of derivatives from the initial transposition event. In clone 39, the integration site was different from that of the other clones.

Southern analysis with total DNA could not reveal whether the minitransposon was integrated into the chromosome or into the large virulence plasmid of Shigella spp. (50, 51). Therefore, the virulence plasmids of Congo red-positive and -negative colonies of the six recombinant clones were hybridized with the rfpB probe after Southern transfer. Only the virulence plasmids of Congo red-positive colonies of clones SFL124-27::Tn(rfp-rfb)-8 and -11 hybridized with the rfpB probe, suggesting an integration of the minitransposon into the virulence plasmid, whereas in clones 32, 35, 39, and 43, the chromosome was the integration site (data not shown).

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TABLE 1. Stability of S. dysenteriae 1 O antigen production and the Congo red phenotype in S. flexneri SL124-27 hybrids carrying the rfp-rfb determinants

<table>
<thead>
<tr>
<th>No. of generations</th>
<th>% of colonies positive for the phenotype analyzed</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>SFL124-27</td>
</tr>
<tr>
<td>S. dysenteriae 1 O antigen production*</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>Congo red-positive phenotype</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>100</td>
</tr>
<tr>
<td>14</td>
<td>87</td>
</tr>
<tr>
<td>21</td>
<td>44</td>
</tr>
</tbody>
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a S. dysenteriae 1 O antigen production was determined by colony blotting.

b Congo red-positive colonies were assayed by plating of cells on TS agar supplemented with 0.01% Congo red.

immunization into the virulence plasmid (clones 8 and 11) resulted in a correlation of rfp-rfb expression with the intact virulence plasmid (Congo red-positive phenotype). Compared to the parental strain, the stability of the virulence plasmid was highly decreased in S. flexneri strains SFL124-27::Tn(rfp-rfb)-32, -35, and -43, whereas stability was unaffected in the other recombinant clones.

**Invasiveness of recombinant strains.** Considering the mechanism of action of attenuated vaccines, it seems reasonable to hypothesize that the carrier strain should retain its ability to invade and survive in eukaryotic cells to achieve an efficient immune response after vaccination. The integration of the minitransposon might result in the inactivation of functions that are essential for intracellular survival and hence for vaccine efficacy. Therefore, the capacity to interact with Henle cells of the recombinant clones SFL124-27::Tn(rfp-rfb)-8, -32, and -39, which resulted from different integration events, was compared with that of the parental strain SFL124-27 (Fig. 3). For clone SFL124-27::Tn(rfp-rfb)-8, a consistent reduction in the intracellular survival was observed 2, 5, and 24 h after infection. The number of intracellular bacteria recovered from cells infected with strains SFL124-27::Tn(rfp-rfb)-32 and -39 was lower than that of the parental strain SFL124-27 2 h after infection, whereas at the 5-h time point it was equal or higher. The invasiveness of SFL124-27::Tn(rfp-rfb)-32 was not strikingly different from that of the recombinant strain SFL124-27::Tn(rfp-rfb)-39 or the parental strain, although the stability of the virulence plasmid was highly decreased in strain 32 (Table 1). Interestingly, the short- and long-term intracellular survival of the smooth strain S. flexneri SFL124 was higher than that of the rough strain SFL124-27. This suggested a role for the O antigen in intracellular persistence, possibly by altering surface hydrophobicity, which in turn interferes with cellular clearance mechanisms (43). For all other ampicillin-sensitive transconjugants (n = 15), the number of viable counts after infection of Henle cells did not exceed that of the SFL124-27 rough strain.

It might be necessary for an efficient carrier strain to interact with cytoskeletal proteins, thereby spreading intracellularly and from cell to cell (15). We therefore examined this interaction by simultaneous labeling of cellular F actin and bacterial LPS in infected cells. Like the smooth strain S. flexneri SFL124 and its rough mutant SFL124-27, the recombinant SFL124-27::Tn(rfp-rfb) strains retained the ability to direct efficient actin polymerization in a polar fashion and thereby spread within the infected cell (data not shown). The morphological changes in the shape of intracellular bacteria described by Okada et al. (43) were not observed in this work. However, the formation of actin tails in rough Shigella mutants is in accordance with results of other investigators (43), although another rough mutant of S. flexneri with a mutation in the rfe gene was not able to form actin tails (48).

**Immunogenicity and efficacy of recombinant S. flexneri strains.** To assess whether the recombinant O antigen was immunogenic and to compare the immunogenicity with that of wild-type S. dysenteriae 1, mice were immunized with heat-killed bacteria, and titers of antibodies against S. dysenteriae 1 LPS were measured (Fig. 4A). The recombinant strain SFL124-27::Tn(rfp-rfb)-39 was selected because (i) the expression of the rfp-rfb genes was 100% stable because of the chromosomal integration of the minitransposon (Table 1), (ii) the stability of the virulence plasmid was in the same range as that in the parental strain (Table 1), and (iii) the invasiveness and intracellular survival were not reduced or were only slightly reduced compared to those of the parental strain (Fig. 3). The antibody titers of mice immunized with S. dysenteriae 1 or S. flexneri SFL124-27::Tn(rfp-rfb)-39 were significantly higher (P ≤ 0.05) than the titers in the nonimmunized group and in mice immunized with the rough strain SFL124-27 (Fig. 4A). This indicates the synthesis of enough surface-displayed LPS molecules to trigger a specific immune response, a prerequisite for a vaccine strain.

The safety and efficacy as a vaccine candidate of the strain SFL124-27::Tn(rfp-rfb)-39 was assessed in guinea pigs according to the Serény model (53). None of the four immunization doses given to the 15 guinea pigs resulted in detectable keratoconjunctivitis, thereby demonstrating the safety of this prototype vaccine candidate in this animal model. The vaccinated
FIG. 4. Immunogenicity and efficacy of the vaccine candidates. (A) Titers of serum antibodies against \textit{S. dysenteriae} 1 LPS elicited after immunization of mice with parental strain \textit{S. flexneri} SFL124-27 (a), recombinant strain SFL124-27::Tn(rfp-rfb)-39 (b), and wild-type strain \textit{S. dysenteriae} 1 W30864 (c). A nonimmunized group (d) was included as a control. The antibody titers are given as the geometric mean of the maximal reciprocal dilution which elicited an optical density equal to or above the cutoff value of 0.1 after 1 h of incubation with substrate; standard errors are represented by vertical lines. Differences between the immunization groups were calculated by the Mann-Whitney (Wilcoxon) test for two-sample comparison with Statgraphics Plus for Windows 1.11 software. The results are statistically significant compared with those for the control SFL124-27 strain at \(P \leq 0.05\) (*). (B) Efficacy of the vaccine candidate SFL124-27::Tn(rfp-rfb)-39 in the Sérény model. The numbers of animals with a strong reaction (purulent keratoconjunctivitis) were recorded for 6 days after challenge with the virulent strain \textit{S. dysenteriae} 1 W30864 and compared between vaccinated \((n = 15)\) and nonvaccinated \((n = 14)\) animals.

animals and a control group of 14 animals were then challenged with the virulent strain \textit{S. dysenteriae} 1 W30864. The animals were observed during the next 6 days, and symptoms of keratoconjunctivitis were recorded. The protection was considered full when no symptoms of the disease were observed and partial when the symptoms were milder or disease onset was later than that of the nonvaccinated animals (16). The vaccinated animals developed symptoms of keratoconjunctivitis later than animals of the control group, and the absolute number of guinea pigs showing strong reactions (defined as purulent inflammation of the whole eye) was significantly reduced (Fig. 4B). In the vaccinated group, 7 of 15 animals developed no signs of keratoconjunctivitis (47% full protection), and in the other 8 animals, later development of the disease was observed (53% partial protection), resulting in a combined protection of 100%, whereas in the nonvaccinated group, 71% of challenged animals rapidly developed severe disease.

The paucity of data available in the literature about evaluation of prototype vaccines against \textit{S. dysenteriae} 1 with animal models does not allow us to compare our results appropriately. However, these results can be compared with those obtained with \textit{S. flexneri} strains. With a similar immunization regimen, vaccination with \textit{S. flexneri} aro mutants resulted in 100% combined protection against challenge compared to 21% protection in the control group (16, 60). On the other hand, vaccination with the hybrid \textit{E. coli} and \textit{S. flexneri} 2a vaccine strain EcS2a-2 (40) led to 33% full and 67% partial protection after homologous challenge, whereas all five animals in the nonvaccinated group developed keratoconjunctivitis (17).

Current work in our laboratories is aimed at the coexpression of the Shiga toxin B subunit in the vaccine strains described here, in an attempt to further increase the efficacy of the vaccine prototype. However, the safety and efficacy data presented here demonstrate that strain SFL124-27::Tn(rfp-rfb)-39 is a promising vaccine candidate against \textit{S. dysenteriae} 1. The presence of genes encoding arsenite resistance in the recombinant strain may facilitate the differentiation between recombinant and wild-type strains and detection of any virulent revertants in countries in which \textit{Shigella} is endemic, since vaccinees may shed both the vaccine strain and endemic \textit{Shigella}.

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