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 shigelloses 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 the recombinant O 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.

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 O polysaccharide (40) was used as the vaccine carrier strain.

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W30864 (62) wild-type strain was used as a control and in challenge studies. E. coli S17-1(pAP) (7) was used to mobilize the hybrid plasmid pHBl20 into strain SFL124-27. Plasmid pHBl20 (4a) contains the rfp-rfb-cassette from plasmid pIS37 (55), which encodes the synthesis of S. dysenteriae type 1 O antigen, cloned in the XbaI site of the PLOE/ars vector (18).

Shigella strains were grown at 37°C in Triplecose soy (TS) broth (Difco Laboratories, Augsburg, Germany) or TS agar supplemented with 0.01% Congo red. Bacterial mating. Bacterial mating. Bacterial mating. Bacterial mating.


colonies of the transconjugant clones were inoculated into TS broth without antibiotics. The DNA was probed with digoxigenin-labeled oligonucleotides (DIG Oligonucleotides) and rhodamine-labeled phalloidin (Medac, Hamburg, Germany) were added. After the washing and mounting, the cells were examined by phase-contrast and immunofluorescence microscopy showed that all recombinant strains (29 clones) were resistant to ampicillin, indicating a configuration event instead of transposition.

The introduction of the genes encoding heterologous O antigens into a recipient strain does not automatically entail good expression of the heterologous O antigen. Covalent linkage of the O antigen to the core region of the carrier strain (3, 39, 52, 61). Therefore, the suicide delivery plasmid pHBl20, which contains the rfp-rfb genes and genes encoding arsenide resistance (arsA and arsB) between the inverted repeats of Tn10 (18), was transferred by filter mating from the donor strain E. coli S17-1(pAP) to the rough recipient strain S. flexneri SFL124-27, where the rfp-rfb determinants were integrated into the genome by mini-Tn10 transposon-mediated random insertion. After the mating, 50 transconjugants were selected and further characterized. Absence of the delivery plasmid was checked by streaking of colonies on TS plates supplemented with 100 μg of ampicillin per ml. More than half of the transconjugants (29 clones) were resistant to ampicillin, indicating a configuration event instead of transposition.

RESULTS AND DISCUSSION

Integration of the rfp-rfb cassette in 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 LPS (2) or may result in deficient linking of the heterologous O antigen to the core region of the carrier strain (3, 39, 52, 61). Therefore, the suicide delivery plasmid pHBl20, which contains the rfp-rfb genes and genes encoding arsenide resistance (arsA and arsB) between the inverted repeats of Tn10 (18), was transferred by filter mating from the donor strain E. coli S17-1(pAP) to the rough recipient strain S. flexneri SFL124-27, where the rfp-rfb determinants were integrated into the genome by mini-Tn10 transposon-mediated random insertion. After the mating, 50 transconjugants were selected and further characterized. Absence of the delivery plasmid was checked by streaking of colonies on TS plates supplemented with 100 μg of ampicillin per ml. More than half of the transconjugants (29 clones) were resistant to ampicillin, indicating a configuration event instead of transposition.

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signals could be detected for the parental strain SFL124-27 (data not shown).

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 S. dysenteriae 1, Henle cells were infected with the transconjugants, and the biosynthesis of LPS by intracellular bacteria was investigated. Immunofluorescence analysis showed that 5 h after infection, the heterologous O antigen was synthesized by all investigated. Since S. flexneri SFL124-27 is attenuated through deletion of the rfp-rfb 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 subcultured 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 integra-

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**FIG. 1.** Production of O antigen by recombinant S. flexneri SFL124-27 strains. LPS was separated by SDS-PAGE and developed by silver staining (A) or by immunoblotting with polyclonal antibodies against S. dysenteriae 1 O antigen (B). LPSs from the smooth strain S. flexneri SFL124 (lane 1) and from the wild-type strain S. dysenteriae 1 W30864 (lane 2) were included as controls. Lanes: 1, S. flexneri SFL124-27 (parental strain); 2, SFL124-27 containing the rfp-rfb cassette on a pUC19-derivative; 3 to 10, recombinant SFL124-27::Tn(rfp-rfb)-8, -11, -32, -35, -39, and -43, respectively.
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>
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<tr>
<th>No. of generations</th>
<th>% of colonies positive for the phenotype analyzed</th>
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<td>SL124-27::Tn(rfp-rfb)</td>
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<td>-8</td>
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<tr>
<td>S. dysenteriae 1 O antigen production*</td>
<td>SL124-27</td>
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<tr>
<td>7</td>
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<td>14</td>
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<td>15</td>
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<tr>
<td>Congo red-positive phenotype</td>
<td>SL124-27</td>
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S. *dysenteriae* 1 O antigen production was determined by colony blotting. Congo red-positive colonies were assessed by plating of cells on TS agar supplemented with 0.01% Congo red.

The 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 SL124-27::Tn(rfp-rfb)-8, -32, and -39, which resulted from different integration events, was compared with that of the parental strain SL124-27 (Fig. 3). For clone SL124-27::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 SL124-27::Tn(rfp-rfb)-32 and -39 was lower than that of the parental strain SL124-27 2 h after infection, whereas at the 5-h time point it was equal or higher. The invasiveness of SL124-27::Tn(rfp-rfb)-32 was not strikingly different from that of the recombinant strain SL124-27::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* SL124 was higher than that of the rough strain SL124-27. 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 recombinant strain. Henle cells were infected with the parental strain SFL124-27; the recombinant *S. flexneri* strains SFL124-27::Tn(rfp-rfb)-8 (A), -32 (B), and -39 (C); and a *S. flexneri* strain (D) that expressed with the intact 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.

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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 Sere ˝ny 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

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 rough 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 sre gene was not able to form actin tails (48).

**FIG. 3. Invasiveness and intracellular survival of recombinant *S. flexneri* strains.** Henle cells were infected with the parental strain SFL124-27; the recombinant *S. flexneri* strains SFL124-27::Tn(rfp-rfb)-8 (A), -32 (B), and -39 (C); a *S. flexneri* strain (D); and the smooth strain SFL124 (E). Intracellular bacteria were then harvested 2, 5, and 24 h after infection. The CFU recovered per well were compared with the number of viable bacteria harvested from cells infected with strain SFL124-27. The numbers of CFU recovered per well for strain SFL124-27 were 3.5 × 10^5 (1.2% of the initial inoculum), 6.5 × 10^5, and 5.9 × 10^5 after 2, 5, and 24 h, respectively. The results reported are mean values of three independent assays; standard errors are represented by vertical lines. The results were analyzed by Student's *t* test and are statistically significant compared with those for the control SFL124-27 strain at *P* ≤ 0.05 (*).
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} aroD 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 EcSf2a-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. 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 arsenate 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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VACCINE AGAINST S. DYSENTERIAE TYPE 1


