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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 been 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 antibiotic resistance markers are undesirable in vaccines but a phenotypic marker would be particularly helpful for genetically engineered microorganisms which will be released under uncontained 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.

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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

W30864 (62) wild-type strain was used as a control and in challenge studies. *E. coli* S17-1(λpir) (7) was used to mobilize the hybrid plasmid pHB120 into strain SFL124-27. Plasmid pHB120 (4a) contains the *rfp-rfb*-cassette from plasmid pSS37 (55), which encodes the synthesis of *S. dysenteriae* type 1 O antigen, cloned in the *Xba*I site of the pLOF/Ars vector (18).

Shigella strains were grown at 37°C in Trypticase soy (TS) broth (Difco Laboratories, Augsburg, Germany) or TS agar supplemented with 0.01% Congo red (Sigma Chemie GmbH, Deisenhofen, Germany) to detect the presence of the virulence plasmid. 121-salt minimal medium (27) supplemented with 200 μM K_2HPO_4, 0.2% glucose, 10 μg of nicotinic acid per ml, aromatic compounds (40 μg of tryptophan, tyrosine, and phenylalanine per ml, 10 μg of *p*-aminobenzoic acid and 2,3-dihydroxybenzoic acid per ml) and 1.5 mM $NaAsO_2$ was used to counterselect donor bacteria in matings. Aromatic amino acid dependence of transconjugants was checked by streaking of parental and recombinant shigellae on 121-salt minimal medium lacking aromatic compounds. *E. coli* S17-1(λpir) with pHB120 was grown on Luria-Bertani medium (47). Where required, ampicillin (100 μg/ml) was used for selection.

Bacterial mating. Plasmid pHB120 was transferred from the donor strain *E. coli* S17-1(λpir) into the recipient strain *S. flexneri* SFL124-27 by mobilization by a filter mating technique (18).

LPS isolation and immunological identification. LPS was prepared from whole-cell lysates as described by Hitchcock and Brown (20). High-degree resolution of LPS was achieved by tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (tricine-SDS-PAGE [28]), and LPS bands were detected either 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* (Behringwerke, Marburg, Germany). Colony immunoblotting was carried out by transfer of the colonies from agar plates to nylon membranes (Biodyne A; Pall, Dreieich, Germany) and then immunodetection as for the Western blots.

Southern analysis of chromosomal and virulence plasmid DNA. Chromosomal DNA or virulence plasmids of Congo red-positive and -negative colonies were obtained as previously described (21, 46), separated on 0.7% agarose gels, and transferred under vacuum conditions to Biodyne B nylon membranes (Pall). The DNA was probed with digoxigenin-labeled oligonucleotides (DIG Oligonucleotide 3′-End Labeling Kit; Boehringer, Mannheim, Germany) that were complementary to sequences of the Tn*10*-transposase (5′-CCAGGCATTACTTGA CTGTAAAACTCT-3′) (15) and the *rfpB* (5′-GGGATTTCAGTCTATGCTTT TGCTACT-3′) (12, 26) and the *arsA* (5′-GCTGGATTATCAATAACAGCCT TTCCA-3′) (5) genes. After hybridization according to the manufacturer's protocol (DIG Luminescent Detection Kit for Nucleic Acids; Boehringer), the membranes were washed stringently, and the light emission of bound probes was documented on Kodak X-ray films.

Stability of the virulence plasmid and *rfp-rfb* expression. Congo red-positive colonies of the transconjugant clones were inoculated into TS broth without arsenite, and these cultures were subcultured by dilution at 1:100 in fresh medium every day. Under these conditions, 24 h of growth corresponded to approximately 7 generations. Prior to being subcultured, aliquots of the cultures were removed after 24, 48, and 72 h, appropriately diluted, and plated on TS agar containing 0.01% Congo red to assess the Congo red-positive phenotype, whereas the synthesis of *S. dysenteriae* 1 LPS was determined by colony blotting.

Tissue culture methods and invasion assays. The human cell line Henle 407 (ATCC CCL-6) was maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine (GIBCO Laboratories; Eggenstein, Germany). Cell monolayers were infected as previously described (22, 59); at different time intervals, cells were lysed, and the number of viable intracellular bacteria (CFU) was determined by plating.

Analysis by immunofluorescence microscopy. To examine bacterial synthesis of heterologous LPS and the interaction of the bacteria with host cell actin (13) after infection of eukaryotic cells, the cells grown on coverslips were infected, and after incubation for 5 h, they were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS), permeabilized with 0.2% Triton X-100 in PBS, and washed with PBS. The coverslips were incubated with polyclonal rabbit antibodies against *S. dysenteriae* 1 O antigen, washed with PBS, and fluorescein-labeled goat anti-rabbit immunoglobulin G (IgG) (Jackson ImmunoResearch Laboratories) and rhodamine-labeled phalloidin (Medac, Hamburg, Germany) were added. After the washing and mounting, the cells were examined by phase-contrast or epifluorescence microscopy with a Zeiss axiophot microscope (Carl Zeiss, Oberkochen, Germany).

Immunization of mice and ELISA. Groups of five six-week-old female BALB/c mice were immunized on days 0, 14, and 28 by intraperitoneal injection of 0.2×10^8 to 1.0×10^8 heat-killed (60°C, 1 h) bacteria suspended in PBS. Two weeks after the last immunization, the mice were sacrificed, blood samples were collected, and antibody titers against LPS of *S. dysenteriae* 1 were determined by enzyme-linked immunosorbent assay (ELISA). Microtiter plates were coated with LPS (1 μg per well) prepared by the method of Westphal and Jann (63) and used to assay antibodies of serially diluted mice sera, with horseradish peroxidase-conjugated goat anti-mouse IgG (Fc fragment specific) and IgM (Mu chain specific) (Jackson ImmunoResearch Laboratories) used as secondary antibodies. One hour after addition of the peroxidase substrate solution (*o*-phenylenediamine dihydrochloride; Sigma Chemie GmbH), the absorbance (490 nm) was read on a microplate reader (Bio-Rad; model 3550).

Serény test with guinea pigs. The Serény test with guinea pigs was performed as follows (53). Congo red-positive colonies of the *Shigella* vaccine candidate were diluted in PBS, and 25 μl was applied to the conjunctival sacs of 15 adult Dunkin Hartley guinea pigs (body weight, 200 to 300 g) (Mollegaard, Denmark) at days 0, 7, 14, and 21 (average of four immunizing doses, 3.7×10^9 bacteria per eye). At day 35, the animals were challenged with 10^8 bacteria of the virulent strain *S. dysenteriae* 1 W30864 per eye, and the symptoms of keratoconjunctivitis were recorded for 6 days. As a control, another group of 14 nonvaccinated guinea pigs was also challenged with the virulent strain at day 35. The animal experiments were performed in accordance with the guidelines of the German animal protection law (Tierschutzgesetz) and the Swedish ethical board for research animals.

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 pHB120, which contains the *rfp-rfb* genes and genes encoding arsenite resistance (*arsA* and *arsB*) between the inverted repeats of Tn*10* (18), was transferred by filter mating from the donor strain *E. coli* S17-1(λpir) to the rough recipient strain *S. flexneri* SFL124-27, where the *rfp-rfb* determinants were integrated into the genome by mini-Tn*10* transposon-mediated random insertion. After the mating, 50 transconjugants were selected and further characterized. Absence of the delivery plasmid was checked by the 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 cointegration 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 lipid A moiety of the carrier strain, which is probably a prerequisite for immunogenicity and protectivity 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. As controls, LPS preparations from the parental rough strain *S. flexneri* SFL124-27, from SFL124-27 transformed with a pUC derivative containing the *rfp-rfb*-cassette, from the corresponding smooth strain SFL124, and from the virulent strain *S. dysenteriae* 1 W30864 were also analyzed (Fig. 1). Silver staining of the LPS bands revealed the expected rough phenotype for the parental strain *S. flexneri* SFL124-27 and a smooth phenotype for all transconjugants (Fig. 1A). Western blot analysis with antibodies against *S. dysenteriae* 1 LPS showed the synthesis of relatively large amounts of *S. dysenteriae* 1 O antigen by all recombinant strains (Fig. 1B). The yields of *S. dysenteriae* 1 O antigen were approximately the same in both the transformant with the *rfp-rfb*-cassette on a high-copy-number plasmid and in all transconjugants with the integration of one copy of the *rfp-rfb* minitransposon.

Western blot analysis could not reveal whether all or only part of the recombinant bacterial population synthesized the O antigen of *S. dysenteriae* 1. Therefore, LPS expression by individual bacteria was assessed by immunofluorescence with antibodies against *S. dysenteriae* 1 LPS. Examination under phase-contrast and immunofluorescence microscopy showed that all bacteria expressed the heterologous O antigen, whereas no

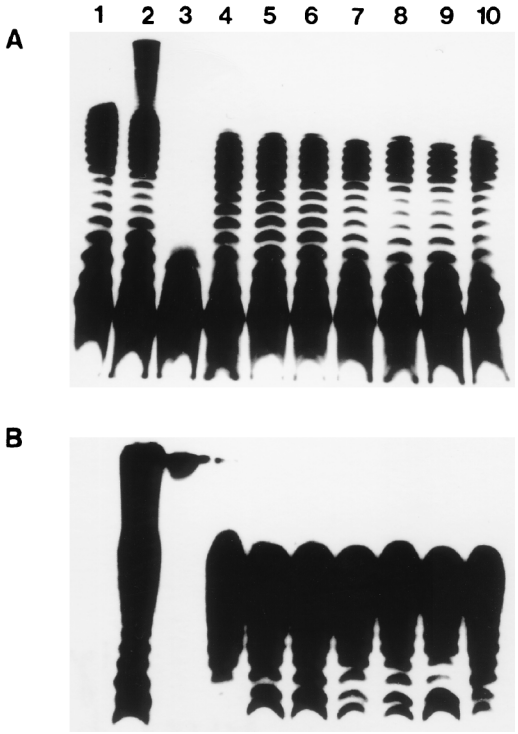

FIG. 1. Production of O antigen by recombinant *S. flexneri* SFL124-27 strains. LPS was separated by SDS-PAGE and developed by silver staining (A) or by immunoblotting with polyclonal antibodies against *S. dysenteriae* 1 O antigen (B). LPSs from the smooth strain *S. flexneri* SFL124 (lane 1) and from the wild-type strain *S. dysenteriae* 1 W30864 (lane 2) were included as controls. Lanes: 3, *S. flexneri* SFL124-27 (parental strain); 4, SFL124-27 containing the *rfp-rfb* cassette on a pUC19-derivative; 5 to 10, recombinant SFL124-27::Tn (*rfp-rfb*)-8, -11, -32, -35, -39, and -43, respectively.

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 recombinant bacteria within invaded cells under these in vivo-mimicking conditions (data not shown).

Southern blot analysis of recombinant SFL124-27 strains. Total DNA from the parental and recombinant strains was digested with *Pvu*II 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 *Pvu*II 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).

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 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-

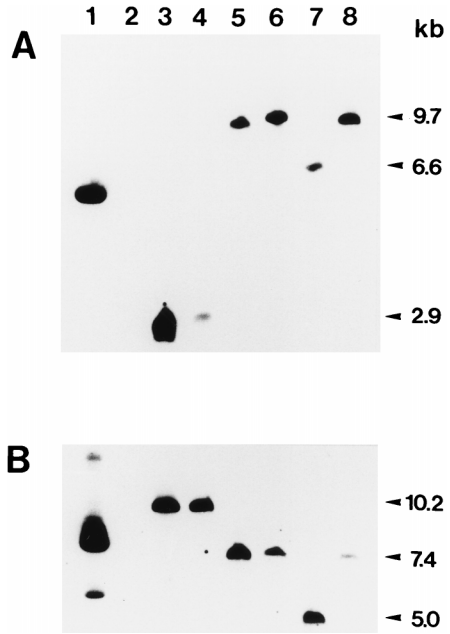

FIG. 2. Southern blot analysis of parental and recombinant *S. flexneri* SFL124-27 strains. DNAs were digested with *Pvu*II, and the fragments thereby generated were separated on agarose gels, transferred to nylon membranes, and hybridized with labeled oligonucleotides complementary to either the *rfpB* (A) or the *arsA* (B) gene. Lanes: 1, *Pvu*II-digested plasmid pHB120; 2, total DNA from SFL124-27; 3 to 8, total DNA from recombinants SFL124-27::Tn(*rfp-rfb*)-8, -11, -32, -35, -39, and -43, respectively. The sizes of the fragments are indicated in kilobases on the right.

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

No. of generations	% of colonies positive for the phenotype analyzed						
	SFL124-27	SFL124-27::Tn(*rfp-rfb*)					
		-8	-11	-32	-35	-39	-43
S. dysenteriae 1 O antigen production[a]							
7	0	100	99	100	100	100	100
14	0	93	88	100	100	100	100
21	0	15	19	100	100	100	100
Congo red-positive phenotype							
7	100	100	99	11	8	100	7
14	87	93	88	0	0	90	0
21	44	15	19	0	0	60	0

[a] *S. dysenteriae* 1 O antigen production was determined by colony blotting.
[b] Congo red-positive colonies were assessed by plating of cells on TS agar supplemented with 0.01% Congo red.

tion 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 Congo red-negative derivative used as a negative control.

It might be necessary for an efficient carrier strain to interact with cytoskeletal proteins, thereby spreading intracellularly and from cell to cell (13). 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 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 *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 ($P \leq 0.05$) 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

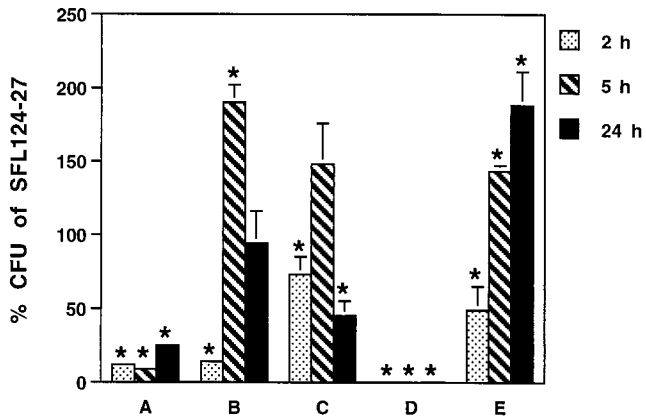

FIG. 3. Invasiveness and intracellular survival of recombinant *S. flexneri* strains. Henle cells were infected with the parental strain SFL124-27; the recombinant *S. flexneri* strains SFL124-27::Tn(*rfp-rfb*)-8 (A), -32 (B), and -39 (C); a Congo red-negative noninvasive derivative of SFL124-27 (D); 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 5.9×10^4 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 $P \leq 0.05$ (*).

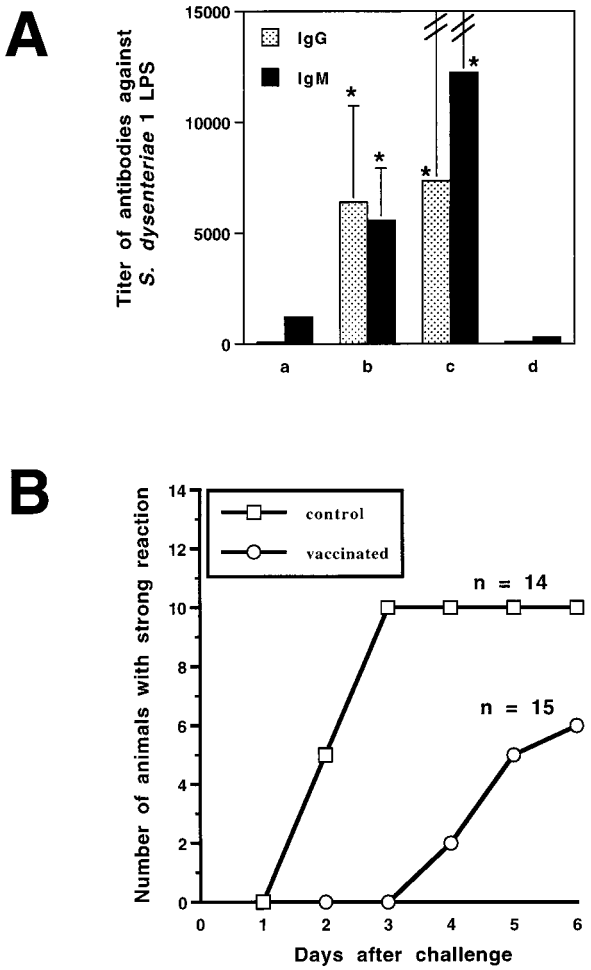

FIG. 4. Immunogenicity and efficacy of the vaccine candidates. (A) Titers of serum antibodies against *S. dysenteriae* 1 LPS elicited after immunization of mice with parental strain *S. flexneri* SFL124-27 (a), recombinant strain SFL124-27::Tn (*rfp-rfb*)-39 (b), and wild-type strain *S. dysenteriae* 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 Serény model. The numbers of animals with a strong reaction (purulent keratoconjunctivitis) were recorded for 6 days after challenge with the virulent strain *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 *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 *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 *S. flexneri* strains. With a similar immunization regimen, vaccination with *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 *E. coli* and *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. However, the safety and efficacy data presented here demonstrate that strain SFL124-27::Tn(*rfp-rfb*)-39 is a promising vaccine candidate against *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 *Shigella* is endemic, since vaccinees may shed both the vaccine strain and endemic shigellae.

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