Cross-protective potential of live *Shigella* mutants lacking immunodominant antigens

PhD thesis

Dr Valéria Szijártó

Program leader: Prof. Dr. Levente Emődy

Supervisor: Dr. Gábor Nagy

University of Pécs

Department of Medical Microbiology and Immunology

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1. Introduction

The genus *Shigella* belongs to the class of Gammaproteobacteria in the family Enterobacteriaceae and traditionally it is divided into 4 species; *S. dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei*. *Shigella* bacteria are human pathogens causing an invasive infection of the colon termed shigellosis or bacillary dysentery. As few as 10-100 bacteria can elicit the symptoms, which range from mild, watery diarrhoea to full blown dysentery with fever, tenesmus (with intestinal cramps) and mucopurulent, bloody diarrhoea. The acute complications (dehydration, hypoglycaemia, intestinal perforation, toxic megacolon, peritonitis and Gram-negative sepsis) are life-threatening, especially in children. A distinct form of *Shigella* infections is called haemolytic uremic syndrome (HUS), which (apart from those cause by enterohaemorragic *E. coli*) is nearly exclusively caused by Shiga-toxin producing *S. dysenteriae* type 1 strains.

In 1999, *Shigella* was estimated to be responsible for ~113 million cases worldwide and approximately 1 million deaths, mostly in children under the age of five. Although over 90% of shigellosis occurs in developing countries, bacillary dysentery is also common among travellers and military personnel entering endemic countries. This huge number of cases, the increasing resistance of *Shigella*e to common antibiotics, as well as the potential use of *Shigella* as a biological weapon all confirm the need for a vaccine against shigellosis. However up-to-day there is no licenced vaccine available, mainly due to the large number of different *Shigella* serotypes and the lack of cross-protection between these different serotypes. Based on the structure of the O-antigen part of the lipopolysaccharid (LPS) the four species can further divided into nearly 50 (sub)serotypes; 16 of *S. flexneri*, 18 of *S. boydii*, 13 of *S. dysenteriae* and one of *S. sonnei*. From these *S. flexneri* 2a, 3a, 6 and *S. sonnei* are responsible for 65% of all moderate-to-severe dysenteric cases in the developing countries, with *S. flexneri* being the most often isolated species. On the other hand, in industrialized areas *S. sonnei* alone accounts for close to 65% of *Shigella* infections. The geographically different and constantly changing distribution of the dominant serotypes implies the necessity of a vaccine providing protection against several – optimally against all – *Shigella* serotypes.

Lipopolysaccharide O-antigens are highly immunogenic surface antigens of shigellae. During a natural *Shigella* infection, there is a significant increase in the level of O-antigen specific antibodies. These antibodies are known to be protective, however exclusively against an infection with the identical serotype (homologous infection). Besides the O-antigen, there are further major virulence factors in *Shigella*e. Virulent strains carry a large virulence plasmid encoding several different virulence factors, among which the most important is the type 3 secretion system (T3SS). This needle like protein complex reaches through the inner and outer membrane of the bacterium to the surface. Upon contact of the T3SS tip with the target cell membrane, the so-called invasion plasmid antigens B and C (IpaB and IpaC) can pass through the „needle" and by insertion into the target cell memrane form a pore. Through this pore approximately 25 effector proteins of *Shigella* can enter the host cytoplasm and induce engulfment of the bacterium. Upon repeated contact with *Shigella* (mainly for residents of endemic countries) there is an increased antibody level against the Ipa antigens. As these proteins are conserved among different serotypes, these antibodies would suggest protection

against different (heterologous) serotype *Shigella* strains. The lack of the natural cross-protection implies, however, that these antigens are probably not exposed, most probably because they are surface located exclusively while *Shigella* entering the epithelial cells.
Current vaccine approaches against *Shigella* in clinical phase exclusively rely on smooth, invasive strains, eliciting protective antibodies generated against the O-antigen of the vaccine strains. Therefore, for broad coverage, these approaches would necessitate combination of the most prevalent serotypes. Alternatively, there are subunit vaccine approaches involving conserved *Shigella* antigens. Immune response against these conserved antigens – in case the antigens are accessible – could provide protection against all serotypes of *Shigella*. Genetically engineered outer membrane particles with conserved outer membrane proteins of *Shigella* were shown to be protective in a mouse model. Although subunit vaccines are considered as the safest vaccine types, the manufacturing costs can prevent these vaccines to be used for broad immunization in developing countries.
The most successful vaccine trial of broad-spectrum *Shigella* vaccines was carried out in the 1970's using a spontaneous avirulent *S. flexneri* 2a strain Istrati T_{32} or „Vadizen". This live strain was administered in Rumania to over 30,000 children and 4,700 adults orally and provided 78-88% protection against both homologous and heterologous (i.e. against different serotypes) infection with no major side effects recorded. The immunity lasted for at least 6 months post-vaccination and required repeated (5-times) administration of the vaccine. Genetic analysis revealed that the vaccine strain carried a deletion in the large virulence plasmid (covering the Ipa antigens) and lost its invasive phenotype. Although these early studies did not fulfil the requirements of a double-blind placebo controlled clinical trial, and the heterologous protection was against the experience of all previous and later vaccine studies, the efficacy elicited is exceptional among the anti-dysentery vaccine trials.
Based on the cross-protection observed with the Vadizen vaccine we hypothesized tha loss of the major surface antigen complex (Ipa-s) and/or the consequent non-invasive phenotype could enchance the immune response against some conserved, minor antigens of *Shigella*. To provide a cheaper, broad protective vaccination strategy, than the conserved antigen based subunit vaccines, we generated and tested live, attenuated vaccine candidate mutants.

2. Aims

1) Generation of attenuated *Shigella* mutants with site-directed mutagenesis and additional selection of non-invasive mutants lacking the whole large virulence plasmid.

2) Determination of virulence potential of these mutants using *in vitro* and *in vivo* methods.

3) Examination of vaccine potential of live vaccine candidate strains in the mouse lung model, especially with respect to potential cross-protection provided against different heterologous serotypes.

4) Identification of conserved *Shigella* (surface) antigens detected by cross-protective immune sera.

3. Materials and methods

3.1. Generation of isogenic *Shigella* mutants

Auxotrophic and rough mutants of the sequenced prototype *S. flexneri* 2a 2457T strain were generated with the Red recombinase technique. Briefly, target genes were replaced by an antibiotic cassette with the λ Red recombinase. The recombination occured between the genes and the 30-50-bp-long ends of the antibiotic cassette, which were generated to be homologous to the target gene. Both the plasmid encoding the λ Red recombinase and the antibiotic cassette were introduced into the parent strain by electroporation. Subsequently, non-invasive forms of the mutants were selected. While invasive (T3SS expressing) shigellae bind the Congo red dye on tryptic soy agar plates supplemented with Congo red and form orange to red colonies (Congo red positive, CRP), non-invasive (T3SS non-expressing) variants remain white (Congo red negative, CRN).

Similarly, phase I (invasive and smooth) and phase II (non-invasive and rough) variants of *S. sonnei* were differentiated on Congo red TSA plates.

To confirm the lack of the whole virulence plasmid in CRN isolates, presence of virulence factors encoded on the plasmid as well as the origin of replication of the plasmid was determined with PCR.

3.2. *In vitro* characterisation of the generated *Shigella* mutants

Adhesive and invasive capacity of the mutant strains was determined on HeLa and Int407 cell lines. Bacteria were added to confluent layers of these cells at a MOI (multiplicity of infection) of 10. Non adherent bacteria were removed by washing and then the cells were lysed. Bacterial concentration (Colony Forming Unit or CFU) was determined by plating different dilutions of the suspension. The result represented the cell associated bacterial number. For the detection of intracellular bacteria, following incubation with bacteria, cells were treated with gentamycin to remove all extracellular bacteria. After lysis of the cells the bacterial count determined gave the intracellular (i.e. invasive) bacterial count.

Serum sensitivity of the *Shigella* strains was tested in 50% serum from healthy human donors. Survival of the bacteria in the presence of active complement after 30-60-120 minutes and 24h was determined and compared to the survival in heat inactivated serum.

3.3. Testing of the mutants in the mouse lung model

Six to eight-week-old female specific pathogen free (SPF) BALB/c mice were used for the *in vivo* experiments. Mice were anesthetised intraperitoneally followed by a challenge infection or immunization with sub-lethal doses, intranasally.

For the detection of *in vivo* survival of our mutants, mice were subjected to sublethal doses of bacterial suspensions. On days 1, 2, 3 and 4 post-infection mice were euthanized and bacterial count from their lung homogenizate was determined.

Attenuation, therefore safety of the generated vaccine candidates was determined with the calculation of the 50% lethal dose (LD_{50}). Groups of mice were infected with different concentration of bacteria and survival of the animals was monitored for 14 days. The LD_{50} value was calculated with the statistical method of Reed and Muench.

To test the protective capacity of the 2457T-derived mutants, mice were immunized with sublethal doses of the different strains twice. Two weeks after the booster, immunized mice

were challenged with a lethal dose of the parent strain. Protection was determined based on the survival.

Similarly, cross-protection of the mutants was tested by an infection of immunized mice with a challenge strain expressing different, heterologous O-antigens. Survival and weight loss of the challenged mice was monitored daily.

Statistical analysis of the survival curves was performed with the LogRank (Mantel-Cox) test.

Immune sera were collected from immunized mice one week after the second immunization. Bronchoalveolar lavage fluid (BAL) was collected 2 weeks following the booster by the cannulation of the trachea of euthanized mice.

3.4. ELISA

Antibodies raised upon immunization of mice were detected from serum and BAL samples by ELISA. For the coating, different live bacteria were used, and specific serum IgG, IgG1, IgG2a and BAL IgA were detected with HRP conjugated secondary antibodies. The ELISA substrate was o-phenylenediamine and the optical density was measured at 492 nm wavelenght.

3.5. Identification of potential cross-protective *Shigella* antigens

To identify the target antigen of cross-protective serum antibodies, immune serum was reacted to sonicated bacterial lysates. Immune complexes were captured with protein G beads. After repeated washing of the beads, antigens and antibodies were eluted and separated on polyacrylamide gel. Protein bands detectable by the serum obtained with the rough, non-invasive but not with the control non-immune serum were excised. The identification of the proteins from these bands was performed by mass-spectrometry in collaboration with Éva Hunyadi-Gulyás in the Proteomics Research Group at the Biological Research Centre, Hungarian Academy of Sciences, Szeged.

4. Results

4.1. Construction and characterization of the vaccine candidates.

Isogenic attenuated mutants of prototype *S. flexneri* 2a 2457T were constructed by replacement of the *aroC* or the *rfbF* genes. The inactivation of the *aroC* resulted in an auxotrophic mutant unable to synthetise aromatic amino acids and multiply *in vivo*. The deletion of the *rfbF* resulted in a rough LPS phenotype. With the selection of Congo red negative clones, non-invasive forms devoid of the large virulence plasmid encoding the invasion plasmid antigens (Ipa-s) were selected. Phase II form of *S. sonnei* was isolated lacking both the invasion antigens and O-antigen encoded on the virulence plasmid. Loss of the whole virulence plasmid was verified with PCR confirming the lack of plasmid-borne virulence genes and the replication origin. Phenotypic characteristic of the mutants is summarized in Table 1.

Table 1 Phenotype of the *Shigella* mutants generated

Strain	O-antigen	Virulence plasmid
S. flexneri 2a 2457T	+	+
2457T Δ*aroC* CRP	+	+
2457T Δ*aroC* CRN	+	-
2457T Δ*rfbF* CRP	-	+
2457T Δ*rfbF* CRN	-	-
S. sonnei 598 phase I	+	+
S. sonnei 598 phase II	-	-

4.2. *In vitro* characterization of vaccine candidates

All the Congo red positive strains were confirmed to be able to invade HeLa and Int407 cells, whereas no Congo red negative strains were detected intracellularly, which confirmed the non-invasive phenotype of these mutants. Although there was a marked increase in the adherence of the rough and of the non-invasive mutants compared to the parent strain, the difference was not found to be statistically significant.

The prototype *S. flexneri* 2a 2457T as well as its isogenic mutants were found to be killed by active complement in 50% serum within 60 minutes, whereas they survived and multiplied in heat-inactivated serum. Therefore these strains were characterized to be serum sensitive.

On the other hand, phase I form of *S. sonnei* 598 was found to be serum resistant in serum originating from 5 different donors. In contrast with the phase I form, phase II *S. sonnei* mutant was rapidly killed in human serum already after 30 min confirming, that loss of the O-antigen and/or the invasion plasmid results in decrease in serum resistance.

4.3. *In vivo* survival, virulence and protective capacity of *Shigella* vaccine candidates

To assess the level of attenuation of the vaccine candidate mutants, we determined the 50% lethal dose of the strains. The virulence of the invasive Δ*aroC* and Δ*rfbF* mutant was comparable, and showed only a minimal attenuation compared to the wild-type strain, however, no mortality was detected with the non-invasive strains (CRN and phase II forms) even at the highes tested dose (10^8 or $10^{7.5}$ CFU). Therefore the non-invasive mutants of *Shigella* are considered fully avirulent in the mouse lung model.

To elucidate the protective capacity of our vaccine candidates, mice were immunized with the different *Shigella* mutants and challenged with either a strain belonging to the same serotype (homologous challenge) or to a different serotype (heterologous challenge).

As expected, both the invasive and non-invasive vaccine strain expressing O-antigen elicited partial (40%) but significant protection against the homologous challenge. Surprisingly the rough invasive Δ*rfbF* CRP strain despite of the lack of O-antigen provided even higher, 80% protection. Moreover the protection of the rough strain was found to be irrespective of the invasiveness, as the non-invasive form of the mutant (Δ*rfbF* CRN) resulted in the same level of protection.

Against a heterologous challenge by *S. flexneri* 6 no significant protection was provided by the Δ*aroC* mutants, which show the lack of cross-protection after a natural *Shigella* infection. However, immunization with the rough strains (both invasive and non-invasive form) resulted

in nearly complete protection against the heterologous challenge. The cross-protective capacity of the mutants was tested against another heterologous challenge, against a *S. sonnei* strain. Repeatedly the smooth, invasive strain did not provided protection, and in this experiment the rough invasive strain was also found to be ineffective. However the rough non-invasive double mutant provided almost full protection (14/15 mice survive) in 3 independent experiments.

The cross protection provided by rough, non-invasive strains was confirmed during immunization with phase II *S. sonnei* mutant (lacking the virulence plasmid and O-antigen). Compared to immunization with a phase I form, the phase II mutant resulted in significant protection against two heterologous strains. In this case the protection was either assessed through the higher survival of the immunized mice (against a *S. flexneri* 6 challenge) or by the significantly lower weight loss of mice (against a *S. flexneri* 2a strain).

We could also prove that the increased protective capacity of the rough strains does not result from prolonged immune stimulus, as these strains were cleared from the lung after 24 hours. In contrast the smooth invasive strain could be detected even on day 4 post-challenge.

4.4. *Shigella* specific antibodies raised upon immunization with different vaccine strains

During the detection of *Shigella*-specific antibodies in serum and in the bronchoalveolar lavage fluid we aimed to identify the immunological background of the cross-protection provided by the rough, non-invasive strains. Our results corroborated that after immunization with the rough, non-invasive strain, there is a higher immune response against „minor" antigens (non-O-antigen and non-invasion plasmid antigen specific antibodies) compared to immunization with the smooth invasive strain. Furthermore, these antibodies are significantly more likely to belong to IgG2a class, implying a Th1 dominance of the immune response upon immunization with the double mutant.

4.5. Identification of potential cross-protective antigens

During the identification of antigens detected by the immune serum from mice immunized with the double mutant, we consequently found outer membrane protein C (OmpC), bifunctional acetaldehyde-CoA/alcohol dehydrogenase, glycine dehydrogenase and dihydrolipoamide acetyltransferase. Among these proteins, only OmpC is known to be membrane localized, the remaining 3 enzymes are likely to be cytoplasmic proteins.

5. Discussion

Current vaccine approaches (in general as well as for *Shigella* in particular) usually rely on major antigens of the pathogens. However these antigen, due to the high evolutionary pressure, usually have several variants, which form the basis of classifying pathogens into serotypes. On the other hand, the various serotypes share a considerable number of conserved antigens. These antibodies could have remained conserved mainly for two reasons; either their function is so indispensable, that no modification of the antigen is allowed, or they are not accessible for antibodies, therefore non-protective. In *Shigella* infections, it was shown

earlier, that the majority of antibodies generated are against the O-antigen and the invasion plasmid antigens (Ipa-s). Given that Ipa-s are considered to be non-protective and O-antigens are highyl variable with close to 50 variants, the immunity elicited is restricted against the same O-serotype. We have proposed, that immunodominant antigens (such as Ipa-s and O-antigens) may hijack the immune response in a way that allows less antibodies to be raised against minor antigens, thereby preventing the production of cross-protective antibodies. To corroborate our hypothesis we generated and proved the cross-protective capacity of *Shigella* mutants devoid of both major antigens (rough and non-invasive, lacking the invasion plasmid antigens). These double mutants of both parental strain *S. flexneri* 2a 2457T and *S. sonnei* 598 were found to be avirulent in the mouse lung model, with no lethality and no visible signs of infection even at the highes tested dose. Furthermore, the lack of virulence plasmid in phase II *S. sonnei* resulted in sensitivity to 50% human serum compared to the serum resistant phase I variant. Therefore our results suggested, that non-invasive rough mutants of *S. flexneri* 2a and *S. sonnei* are highly arrenuated and may fulfill the requirements for vaccine candidates with respect to safety.

We showed that immunization with the double mutant 2457T Δ*rfbF* CRN strain not only provided significant protection against a homologous challenge in the mouse lung model, but elicited nearly full protection against two distinct heterologous challenge strain. Moreover immunization with a double mutant phase II *S. sonnei* confirmed the cross-protective capacity of rough non-invasive strains; it provided full protection against a 90% lethal dose of *S. flexneri* 6 and resulted in significantly lower weight loss during a sublethal *S. flexneri* 2a infection. We could prove, that the cross-protection may results from improved immune response against minor antigens as well as from an altered ratio of specific IgG subclasses; antibodies against the minor antigens were more likely to be IgG2a isotypes, whereas there was a higher ratio of IgG1 antibodies upon immunization with a smooth, invasive strain. IgG2a is known to activate complement, whereas IgG1 cannot, therefore our hypothesis was, that the difference in isotype dominance may also play an important role in protection against *Shigella*.

Attempts were made to identify these conserved minor antigens; besides OmpC we repeatedly identified three cytoplasmic enzymes. Given the intracellular lovalization, it is not likely that antibodies targeting these enzymes would contribute to protection. Nevertheless there are data in the literature proving, that metabolic enzymes can be present on the surface of bacteria, and in some cases were shown to be targets of protective antibodies.

Based on our experimental data, we feel that mutants lacking major surface antigens are safe vaccine candidates with cross-protective capacity, therefore they could serve the basis for broad protective live *Shigella* vaccines.

6. New findings

1) We succesfully generated several attenuated *Shigella* mutants with site-directed mutagenesis.

2) Our work provided evidence, that rough, non-invasive *Shigella* strains are avirulent in the mouse lung model, therefore they are considered to be safe as vaccine candidates.

3) We proved in repeated experiments and against different heterologous strains, that these rough, non-invasive mutants can elicit significant, high level cross-protection.

4) The cross-protection may result from the elevated level of antibodies raised against conserved, minor antigens upon immunization with the double mutants and from the altered ratio of IgG subclasses.

5) Identification of the potential cross-protective antigens propose the role of OmpC and implies the possibility of metabolic enzymes as targets of protective antibodies.

7. Publications

Publication related to the thesis:

Szijártó V, Hunyadi-Gulyás E, Emődy L, Pál T, Nagy G. Cross-protection provided by live *Shigella* mutants lacking major antigens. Int J Med Microbiol **2013 May**; 303(4):167-75. (IF: **4.173)**

List of additional publications:

Schneider G, Dobrindt U, Middendorf B, Hochhut B, **Szijártó V**, Emődy L, Hacker J. Mobilisation and remobilisation of a large archetypal pathogenicity island of uropathogenic *Escherichia coli in vitro* support the role of conjugation for horizontal transfer of genomic islands. BMC Microbiol **2011**; 11:210. (IF: 3.04)

Szijártó V, Pál T, Nagy G, Nagy E, Ghazawi A, al-Haj M, El Kurdi S, Sonnevend Á.. The rapidly emerging ESBL-producing *Escherichia coli* O25-ST131 clone carries LPS core synthesis genes of the K-12 type. FEMS Microbiol Lett **2012 Jul**; 332(2):131-6. (IF: 2.044)

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