Effect of a purA Mutation on Efficacy of Salmonella Live-Vaccine Vectors

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We made ΔaroA, ΔpurA, and ΔaroA ΔpurA derivatives of a strain of Salmonella dublin and isolated a nalidixate-resistant mutant of each construct. An inoculum of each of the nearly isogenic nalidixate-resistant auxotrophs was administered to BALB/c mice by gavage. The ability of each strain to colonize, invade, persist in tissues, and evoke serum and mucosal antibody responses to the lipopolysaccharide of the parent strain was examined. Only the ΔaroA strain colonized, invaded, persisted, and (more importantly) evoked sustained significant serum and mucosal antibody responses. Neither the ΔpurA nor the ΔaroA ΔpurA strain showed any of these abilities. These observations demonstrate that the purA defect, which causes a requirement for adenine, reduces the live-vaccine efficacy of attenuated Salmonella strains and may limit the effectiveness of Salmonella strains as carriers of heterologous antigens. These findings are important in the selection of attenuated S. typhi strains for use in humans either as antityphoid live vaccines or as vectors for antigens of other pathogens.

A variety of techniques, including use of live oral vaccines, have been employed to deliver antigens to the gut-associated lymphoid tissue in an attempt to initiate production of specific secretory immunoglobulin A (IgA) antibodies. One recent approach has been to employ avirulent derivatives of Salmonella strains as carriers for plasmids which code for virulence determinants of heterologous mucosal pathogens (recently reviewed by Clements [2]). Antigens expressed by these strains would presumably be delivered directly to the antibody-forming cells in the gut-associated lymphoid tissue. This has been shown to be an effective means of stimulating significant levels of specific mucosal secretory IgA directed against the carrier strain and the heterologous antigen and has been shown to stimulate production of serum antibodies as well (4).

A number of investigators have employed a variety of Salmonella mutants for this purpose, including galE mutants, which lack the enzyme UDP-galactose-4-epimerase (3, 10–12, 16, 25, 26), and aroA mutants, which have specific nonreverting deletions in the common aromatic biosynthetic pathway leading to chorismic acid (1, 4, 8, 13, 16, 17, 20–24). Deletion mutants of Salmonella typhimurium lacking adenylyl cyclase and cyclic AMP receptor protein have also been examined as carriers for antigens of Streptococcus mutans in the development of a potential antocaries vaccine (5–7).

Strains of Salmonella typhi attenuated by auxotrophic characters have also been constructed as live-vaccine candidates (9) for immunization against typhoid fever. Two strains recently tested in volunteers (15) each had an aroA deletion, expected to cause complete loss of virulence by itself, and, as an additional safety factor, a deletion at purA. Unlike mice given an aroA (deletion) mutant of Salmonella dublin orally, volunteers who received an oral dose of even 1.0 × 10^10 CFU of either of the two ΔaroA ΔpurA live-vaccine strains, 541Ty (Vi positive) or 543Ty (Vi negative), developed no or only very low titers of humoral antibody to the O antigen of the vaccine strain. It is not clear why the S. typhi live vaccine failed to cause the expected humoral antibody response to the O antigen (or to the H or the Vi antigen); it might have resulted either from the strains used having two attenuating nutritional requirements instead of the single mutation to aromatic dependence in the S. dublin live vaccine tested in mice or just from the ΔpurA mutation, which was not tested in the mouse experiments of Clements et al. (4). To test these possibilities, we used three nonviralulent derivatives of an S. dublin strain virulent for mice, one with an aroA deletion, one with a purA deletion, and one with both deletions. We tested the ability of each strain to colonize, invade, persist in tissues, and evoke serum and mucosal (gut) antibody responses to the O antigen in mice given one or another of the three nearly isogenic strains by gavage.

Construction of bacterial strains. The organisms used for this study are listed in Table 1. All are nalidixate-resistant mutants of three nearly isogenic auxotrophic derivatives of a virulent, wild-type strain of S. dublin, SVA47 (SL5608). One derivative was attenuated by deletion ΔaroA148, causing a requirement for aromatic metabolites. A second deletion, ΔpurA155, was introduced into the ΔaroA strain by cotransduction with a silent Tn10 insertion, zbj-908::Tn10, to produce an arO purA strain; tetracycline sensitivity was restored by a mutation, presumably a transposon-generated deletion or inversion, at zbj-908. The arO and arO purA strains had been constructed (B. A. D. Stocker and A. A. Lindberg, unpublished results) for trial as live vaccines (for protection of calves against salmonellosis) by methods similar to those used to make 541Ty and 543Ty, the ΔaroA his ΔpurA strains of S. typhi described above. However, the arO deletion introduced into the S. dublin strains used in this study, ΔaroA148, is the result of spontaneous deletion mutation, unlike the transposon-generated deletion, ΔaroA407, used to make the S. typhi live-vaccine strain. The third auxotrophic derivative of S. dublin SVA47 was an arO transductant isolated from the ΔaroA ΔpurA strain to allow testing of the effect on live-vaccine efficacy of the purA defect in a strain not otherwise attenuated. Spontaneous nalidixate-resistant mutants, resistant to 100 µg of nalidixic acid per ml, were isolated from each of the three auxotrophic

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TABLE 1. Bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. dublin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SL5608</td>
<td>Wild type</td>
<td></td>
</tr>
<tr>
<td>SL5621</td>
<td>araO(scrC)1121::Tn10</td>
<td>From SL5608 by transduction</td>
</tr>
<tr>
<td>SL631</td>
<td>scrC ΔaroA148</td>
<td>From SL5621 by transduction</td>
</tr>
<tr>
<td>SL650</td>
<td>ΔaroA ΔpurA155 zbi-908::Tn10</td>
<td>From SL631 by transduction</td>
</tr>
<tr>
<td>SL659</td>
<td>ΔaroA ΔpurA CRR[zbi-908::Tn10 (Tc)]</td>
<td>From SL650 by mutation</td>
</tr>
<tr>
<td>SL7163</td>
<td>ΔaroA Nal</td>
<td>From SL653 by mutation</td>
</tr>
<tr>
<td>SL7164</td>
<td>ΔaroA ΔpurA CRR[zbi-908::Tn10 (Tc)] Nal</td>
<td>From SL659 by mutation</td>
</tr>
<tr>
<td>S. typhimu-rium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT472</td>
<td>LT2 araO(scrC)1121::Tn10</td>
<td>Hosieth and Stocker (14)</td>
</tr>
<tr>
<td>aroA148</td>
<td>LT2 ΔaroA148</td>
<td>Nishioka et al. (18)</td>
</tr>
<tr>
<td>SL5495</td>
<td>Q1 ΔpurA155 zbi-908::Tn10</td>
<td>Edwards and Stocker (9)</td>
</tr>
</tbody>
</table>

*Allele symbols are abbreviated after first mention. araO(scrC)::Tn10, Tn10 insertion in proximal gene, scrC, or promoter region of scrCarO operon; zbi::Tn10, Tn10 insertion in 92 min segment of the linkage map; CRR, Tn10-generated complex rearrangement mutation causing phenotypic characters indicated in parentheses (in this case, sensitivity to tetracycline). Strain SL5608 and its descendents have the nitocinate requirement typically found in S. dublin.

strains to be tested in mice to facilitate isolation from tissue: SL7163 (ΔaroA), SL7165 (ΔpurA), and SL7164 (ΔaroA ΔpurA). As only the three nalidixate-resistant mutants were used in the animal experiments described below, we refer to them below as the araO, purA, and araO purA strains rather than by their strain numbers and genotypes, which are given in Table 1.

Colonization, invasion, and persistence in mouse tissues. Strains were tested in mice to determine the abilities of the auxotrophic mutants to colonize the small intestine and to invade and persist in mouse tissues were conducted with groups of 20 female BALB/c mice essentially as previously described (4). On days 1, 3, 7, 8, 14, and 21 postinoculation, three to five animals from each group were sacrificed, and tissues (liver, spleen, blood, Peyer’s patches, and small intestine) from each animal were removed aseptically and cultured for the presence of the infecting organism. There was evidence for colonization of the small intestines and invasion of and persistence in mouse tissues only with the araO strain, which was isolated from the small intestines, Peyer’s patches, livers, and spleens of animals up through day 3 postinoculation (Table 2). Thereafter, only the livers and, beginning at day 8 postinoculation, the small intestines were infected. The araO strain could be isolated from the blood of only one animal on one day (day 3 postinoculation). Of the 20 animals in this group, the araO strain was isolated from the small intestines of 9, from the Peyer’s patches of 5, from the livers of 7, from the spleens of 2, and from the blood of 1 (Table 2).

The purA strain was isolated from the small intestine, liver, and blood of only a single animal and only on day 1 postinoculation. Thereafter, the purA strain was not detected in any tissue throughout the 21 days of the study. Similarly, the araO purA strain was isolated from the Peyer’s patches of a single animal at day 1 postinoculation and was not subsequently detected in any tissue throughout the 21 days of the study.

Humoral response following immunization with the attenuated mutants. A major consideration in the selection of an appropriate live vaccine or carrier organism is the ability of that organism to evoke an appropriate immunologic response. As an indicator of that response, we examined the serum IgG and mucosal IgA responses against the lipopolysaccharide (LPS) of the parent S. dublin strain, SL5608. Anti-LPS was determined by enzyme-linked immunosorbent assay as previously described (4). For this study, groups of female BALB/c mice were immunized by gavage as described above with two doses, each containing 10^{10} CFU of one or another of the three auxotrophic strains by gavage. Each group contained 20 mice.

Mice immunized orally with the araO, purA, or araO purA strain developed serum anti-LPS antibodies and maintained them throughout the course of the experiment, 5 weeks post-primary inoculation (Table 3). There was, however, great variability between individual animals in all groups; statistical differences between values from immunized groups and control values from unimmunized animals were not consistent at 1, 2, or 3 weeks following the primary inoculation. By the end of week 5, serum anti-LPS IgG had increased from 0 to 14.1 μg/ml in animals immunized with the araO strain, a value significantly greater than that obtained after immunization with either the purA strain (2.89 μg/ml) or the araO purA strain (2.37 μg/ml).

Less variable variability was observed between animals examined for the presence of mucosal anti-LPS IgA. Mucosal anti-LPS IgA was consistently significantly higher in animals immunized with the araO strain than in animals from the other two groups (Table 3). By the end of week 5,
mucosal anti-LPS IgA had increased to 27.3 ng/ml in animals immunized with the \( aroA \) strain, to 1.36 ng/ml in animals immunized with the \( purA \) strain, and to 0.97 ng/ml in animals immunized with the \( araO\ purA \) strain.

These studies demonstrate that of the three strains tested, only SL7163, with the single \( araO \) mutation, was able to colonize significantly, invade, and persist in tissues. More importantly, this strain was shown to consistently evoke appropriate serum and mucosal antibody responses. Neither the \( purA \) nor the \( araO\ purA \) mutant demonstrated these characteristics. We do not know if the presence or lack of observed statistical significance (as determined by Student \( t \) test) during the 5 weeks of the experiment correlates with biological significance. Clearly, immunization with SL7163, with the single \( araO \) mutation, was more effective at eliciting appropriate antibody responses, especially at the mucosal surface.

These observations suggest that the \( purA \) defect, which causes an adenine requirement, reduces the live-vaccine efficacy of attenuated Salmonella strains and may limit the effectiveness of Salmonella strains as carriers of heterologous antigens. This was also suggested by the findings of O'Callaghan et al. (19) in a study characterizing aromatic- and purine-dependent S. typhimurium for virulence, persistence, and the ability to induce protective immunity following intravenous and oral immunization of BALB/c mice. Those authors did not measure serum and mucosal antibody responses but did demonstrate that organisms containing the single \( araO \) deletion, given orally or intravenously, were more effective at protecting against intravenous challenge than were mutants containing either a single \( purA \) deletion or both deletions together. Our findings differed from theirs in the degree of persistence of the various mutants. In their study all three mutants persisted in livers and spleens for up to 10 weeks after intravenous inoculation. We immunized orally and found that only the \( araO \) mutants were able to colonize the small intestine and to invade and persist in mouse tissues. These differences can probably be ascribed to the different routes of inoculation.

We conclude from these results that even though the \( purA \) mutation decreases the virulence of these organisms, it so attenuates the organisms as to make them unsuitable for use in live vaccines. It is important to note, however, that the \( purA \) deletion in these strains was generated by P22-mediated transduction in which strain SL5631 (\( araO \)) was used as the recipient, with strain SL5495, which is \( S. typhimurium \) LT2,\( purA155 \) \( zbj-908::Tn10 \), as the donor. \( zbj-908::Tn10 \) is a silent \( Tn10 \) insertion at a point such that cotransduction of \( purA \) with it occurs at about 30% (M. F. Edwards and B. A. D. Stocker, unpublished observation). A tetracycline-sensitive mutant not detectably altered in other properties, inferred to have arisen by a \( Tn10 \)-generated deletion-inversion mutation at \( zbj-908::Tn10 \), was subsequently isolated for use as the \( araO \ purA \) strain. We cannot completely rule out the potential influence of \( zbj-908::Tn10 \) on the observed results or the possibility that cryptic stretches of genomic DNA may have been replaced during the P22-mediated transduction. As noted previously, the methods used were similar to those used to make 541Ty and 543Ty, the \( araO\ his\ D\ purA \) strains of \( S. typhi \) used in the human vaccine studies, and the comparison with those strains is valid. These findings may be important, then, in the selection of an attenuated \( S. typhi \) strain for use in humans, either as an antityphoid live vaccine or as a vector for antigens of other pathogens.
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LITERATURE CITED


