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Introduction of *Shigella flexneri* 2a Type and Group Antigen Genes into Oral Typhoid Vaccine Strain *Salmonella typhi* Ty21a

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For protection against dysentery caused by *Shigella flexneri* 2a, an in vivo-constructed recombinant plasmid with genes specifying the *S. flexneri* type and group antigens located near the *pro* (min 6) and *his* (min 44) chromosomal markers, respectively, was made and transferred to the *galE Salmonella typhi* strain Ty21a. Strain Ty21a carrying this recombinant plasmid was shown by immunological and biochemical analyses to express the *S. flexneri* 2a type and group antigens. Mice immunized with this vaccine strain were found to be protected against challenge with virulent *S. flexneri* 2a, but not significantly against *S. typhi* challenge, presumably because synthesis of the *Shigella* antigens interfered with expression of the typhoid antigens. Elimination of the recombinant plasmid from Ty21a allowed this strain to again express typical *S. typhi* O antigens. Mouse protection against both *S. typhi* and *S. flexneri* 2a challenges was achieved with a whole-cell vaccine mixture composed of equal parts of Ty21a and the Ty21a-*S. flexneri* 2a hybrid strain.

Typhoid and dysentery remain major public health problems in many areas of the world. These diseases are endemic in developing countries due to low levels of sanitation and can periodically cause significant outbreaks of disease in developed nations. During the past 20 years, a high level of bacterial resistance to most of the useful antibiotics has developed and spread among *Shigella* species. The chemotherapeutic approach to dysentery disease control now appears to be much less effective and more unpredictable.

An important alternative medical measure is the use of vaccines. One vaccine, administered orally, which has shown great potential in protection against typhoid fever is the galactose epimeraseless (*galE*) mutant of *Salmonella typhi* Ty21a, isolated and characterized by Germanier and Furer (7). The Ty21a *galE* mutant is unable to produce the enzyme UDP galactose-4-epimerase and consequently cannot convert glucose to galactose, a component of *Salmonella* core oligosaccharide and O repeat unit structures. Unless galactose is supplied exogenously, strain Ty21a synthesizes an incomplete cell wall lipopolysaccharide (LPS) lacking the immunogenically important O antigens. When grown on galactose-containing medium, however, the organism is able to synthesize typical LPS. Administration of this strain as a live vaccine is possible because of its limited viability in vivo. In the process of synthesizing its O antigens, it also accumulates lethal amounts of galactose intracellularly in the form of galactose 1-phosphate and UDP-galactose, and bacterial osmotic lysis occurs. In a study with human volunteers, Ty21a was shown to confer statistically significant protection against typhoid fever when grown under conditions in which its 9,12 O antigens were synthesized, but not under conditions in which its O antigens were not synthesized (8). The live, attenuated oral vaccine strain Ty21a has also been tested and proven successful in preventing human typhoid fever in a field trial (11).

Current antidysentery vaccine development is based on the considerations that immunity to shigellosis is serotype specific (i.e., against the LPS) and that a safe, oral vaccine would be most effective. Kopecko et al. (9) have provided genetic and physical evidence that the *Shigella sonnei* form I surface antigen is encoded by a 180-kilobase-pair (kb) plasmid. Formal et al. (5) used this information to construct a potential vaccine strain by introduction of the form I plasmid into the *galE* oral vaccine strain Ty21a. This bifunctional vaccine strain protected mice against challenge with either *S. typhi* or *Shigella sonnei* (5) and has been shown to protect human volunteers against *S. sonnei* challenge (1).

In view of the fact that strain Ty21a can act as a carrier for the form I antigenic determinant(s), it should serve as an oral vaccine system for other enterically acquired diseases as well when appropriate protective antigen genes are introduced. Since many cases of shigellosis in the United States and elsewhere are due to *Shigella flexneri* serotype 2a, a study was undertaken to develop a similar *S. typhi* Ty21a derivative oral vaccine that specifies the *Shigella flexneri* 2a O antigens to protect against dysentery caused by this *Shigella* serotype. The construction and preliminary characterization of this Ty21a-*S. flexneri* 2a hybrid vaccine strain are reported here.

MATERIALS AND METHODS

Media, culture conditions, and conjugal transfer. As described elsewhere (5), minimal medium, PenAssay medium (Difco Laboratories), nutrient medium (Difco), MacConkey medium (Difco), and brain-heart infusion medium (BHI; Difco), with or without 1.5% agar (Difco), were used for bacterial growth. For normal supplementation and selection, streptomycin was used at 100 μg/ml, carbohydrates were used at 0.5%, and amino acids and vitamins were added at 0.1%. For immunization studies, however, growth of the attenuated *galE S. typhi* Ty21a strain and its derivatives was done in BHI broth containing 0.1% galactose. Virulent organisms for challenge studies were grown in BHI broth.

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TABLE 1. Bacterial strains

Strain	Relevant properties[a]	WRAIR[b] strain no.	Source and reference
S. typhi			
Ty2	Vi, 9,12 antigens	WR4014	WRAIR collection
Ty21a	*galE*	WR4090	R. Germanier (6)
Ty21a PHS^{r}	*pro his galE* Str^{r}	WR4085	This study
Ty21a PHS^{r}(pWR90)	Plasmid pWR90	WR4086	This study
Ty21a PHS^{r} sergeant	Plasmid-free sergeant of WR4086	WR4087	This study
O901	9,12 antigens	WR4100	W. H. Ewing (2)
E. coli K-12			
AB1133	*pro his* Str^{r}	WR3054	This study
AB1133(pWR90)	Plasmid pWR90	WR3055	This study
DK654(F'*lac*::Mu *c*ts62)	F'*lac*::Mu *c*ts62	WR2051	R. P. Silver[c]
S. flexneri (2a)			
M42-43	*nad asp*	WR1078	WRAIR collection
6023-1-1(F'*lac*::Mu *c*ts)	*nad asp* F'*lac*::Mu *c*ts62	WR1079	This study
2457T	*nad asp*	WR1080	WRAIR collection

[a] The genotypic symbols represent genes for the utilization of galactose (*galE*), genes for the synthesis of proline (*pro*), histidine (*his*), nicotinic acid (*nad*), aspartic acid (*asp*), and the somatic antigens (Vi, 9,12), and genes for resistance to streptomycin (Str^{r}) or nalidixic acid (Nal^{r}). pWR90, F'*lac*, and bacteriophage Mu *c*ts62 were present in some strains.

[b] WRAIR, Walter Reed Army Institute of Research.

[c] Strain WR2051 was kindly provided by Richard P. Silver, University of Rochester, New York.

Sterile physiological saline (0.9%) was used for cell washing and suspension of both vaccine and challenge organisms.

For conjugal transfer experiments, donor and recipient bacteria were grown to the late exponential phase in stationary PenAssay broth cultures. A 5-ml amount each of donor and recipient cultures were mixed, mating was allowed to occur for 6 to 12 h, and the resulting transconjugants were selected on the appropriate agar medium. Potential transconjugants were then purified on identical selective medium.

Bacterial strains, phage, and Mu-promoted transposition. The bacterial strains used are listed in Table 1. The *S. typhi* Ty21a spontaneous *pro his* Str^{r} mutant was obtained in sequential steps. Initially, a Str^{r} mutant was obtained by plating about 5×10^{10} nutrient-broth-grown Ty21a cells onto nutrient agar containing streptomycin (25 μg/ml). Purified Str^{r} Ty21a cells were sequentially grown in minimal broth containing penicillin (5 μg/ml) and plated on glucose minimal agar to select a *pro* mutant, which was then retreated similarly to obtain a *pro his* Str^{r} mutant, WR4085.

The procedure for Mu-promoted transposition of genes to a conjugative plasmid has been described in detail previously (4). Basically, the F'*lac*::Mu *c*ts62 plasmid was transferred conjugally at 32°C from strain WR2051 to *S. flexneri* serotype 2a strain WR1078. The resultant *S. flexneri* strain harboring F'*lac*::Mu *c*ts62 (WR1079) was grown at 37°C to induce Mu-promoted transposition of *S. flexneri* chromosomal genes to the F'*lac* plasmid and then conjugally mated at 37°C with *his pro Escherichia coli* and *S. typhi* recipient strains.

DNA isolation and agarose gel electrophoresis. The conditions and procedures for DNA isolation and electrophoresis have been described previously (2, 5, 9).

Serological studies. Antisera were prepared by immunizing rabbits with live bacterial suspensions. Agglutination tests and agglutination adsorption procedures were conducted by the methods of Edwards and Ewing (3).

Mouse protection assay. The procedures and the ICR mouse strain used have been described in detail (5). Groups of outbred ICR mice (weighing 13 to 15 g) were inoculated intraperitoneally with 0.5 ml of saline suspensions of the vaccine strain, representing a dose of 1×10^{8} Ty21a cells, 4.5×10^{6} *S. flexneri* 2a cells, or 5×10^{7} WR4086 cells, or the combination vaccine, comprising 4×10^{8} Ty21a cells and 4×10^{8} WR4086 cells. Control mice were injected similarly with 0.5 ml of saline. Immunized and control mice were challenged 1 month postimmunization with 0.5 ml of hog gastric mucin suspensions containing 1.4×10^{4} cells of virulent *S. typhi* Ty2 or 1.6×10^{6} cells of *S. flexneri* 2a strain WR1078. Deaths were recorded after 72 h.

LPS analysis. Techniques for extracting LPS and analyzing preparations by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) have been described previously (10). Basically, LPS from all bacterial strains examined was prepared by the hot phenol-water method of Westphal and Jann (12). SDS-PAGE was performed on 13.5% acrylamide slab gels with a discontinuous buffer system. Electrophoresed LPS antigens were visualized by silver staining or by immunoblot analysis. During immunoblotting, the antigens were electroblotted, as previously described (9), to nitrocellulose and sequentially reacted with anti-*S. flexneri* serotype 2a antibody and ^{125}I-labeled staphylococcal protein A. The washed immunoblot was exposed to X-Omat AR film for 24 h at −70°C in a cassette with an intensifying screen (Eastman Kodak).

RESULTS

In vivo construction of recombinant plasmids. In *Shigella flexneri* serotype 2a, the O antigen is determined by two widely separated chromosomal regions (6). The a or group antigen locus specifying the O repeat unit backbone (specifically termed the 3,4 antigen) is encoded by genes located near the *his* marker at min 44 (6). The type antigen locus, referred to as type 2, controlling the chemical modification of the O repeat unit is located near the *pro* region of the chromosome in the vicinity of min 6 (6). Transfer of the group and type antigenic determinants of *Shigella flexneri* serotype 2a to the attenuated Ty21a vaccine strain involved the construction of a recombinant plasmid specifying both of these chromosomal regions. To obtain a plasmid containing both the *pro* and *his* regions of the chromosome with adjacent type and group antigen loci, *Shigella flexneri* serotype 2a strain WR1078 was first infected via conjugal transfer with the F'*lac*::Mu *c*ts62 plasmid of *E. coli* WR2051. The resultant donor, *Shigella flexneri* serotype 2a strain WR1079 carrying F'*lac*::Mu *c*ts62, selected on minimal lactose medium containing 0.1% nicotinic acid and 0.1% aspartic acid, was grown at 37°C to induce Mu-promoted transposition of *S. flexneri* chromosomal genes to the F'*lac* plasmid and then mated at 37°C with *E. coli* K-12 strain AB1133 (*his pro*). Transconjugants of this *pro his* recipient were obtained by selection for *pro*$^+$ *his*$^+$ hybrids on minimal glucose medium containing streptomycin (500 μg/ml). *E. coli* cells receiving both the Pro$^+$ and His$^+$ traits were obtained at a frequency of 10^{-6} per donor cell. After purification, these *E. coli* hybrids were examined by slide agglutination tests with specific antisera directed against the *Shigella flexneri* group 3,4 and type 2 antigens. All of 26 *pro*$^+$ *his*$^+$ hybrids tested were found to express both the *Shigella flexneri* serotype 2a type and group antigens. The positive agglutination tests indicating the presence of the *S. flexneri* antigens on the surface of the *E. coli* hybrid cells were confirmed by adsorbing antiserum made against *S. flexneri* serotype 2a with cells of one *E. coli pro*$^+$ *his*$^+$ hybrid strain (WR3055). The adsorption resulted in removal of all of the *S. flexneri* serotype 2a-specific antibodies, verifying the presence of the *Shigella* O antigens on these *E. coli* hybrid strains. The responsible recombinant plasmid in strain WR3055, termed pWR90, putatively carrying the chromosomal regions encoding the *S. flexneri* 2a O-antigen genes, was chosen for further study. For use as a recipient of pWR90, a *pro his* spontaneous mutant (WR4085) of the *S. typhi* Ty21a vaccine strain was next isolated. In conjugation experiments, pWR90 encoding the *Shigella flexneri* serotype 2a group and type antigens was readily transferred to the WR4085 recipient, regardless of whether the selective marker was Pro or His or both Pro and His. Also, plasmid pWR90 was readily maintained in strain WR4086 by growth on a selective minimal medium lacking proline and histidine. These results indicated that these *Shigella* loci (*pro*, *his*, and O-antigen genes) were part of the same recombinant plasmid, pWR90.

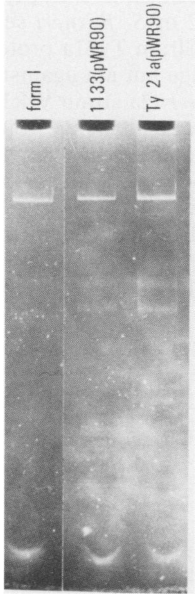

FIG. 1. Agarose gel electrophoresis of plasmid pWR90 encoding the *S. flexneri* serotype 2a O antigen. Representative plasmid DNAs were obtained from *Shigella sonnei* form I strain 53G, from *E. coli* AB1133(pWR90) (strain WR3055), and from Ty21a(pWR90) (strain WR4086) as described previously (9). Agarose gel electrophoresis of these plasmid preparations against plasmid standards of known size was conducted as described earlier (5). The direction of electrophoresis is from top to bottom.

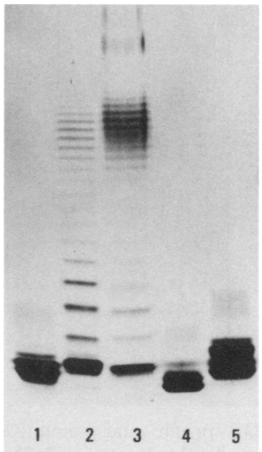

FIG. 2. SDS-PAGE profile of the LPS extracted from representative bacterial strains. LPS extraction methods and SDS-PAGE conditions have been described previously (10). The direction of electrophoresis was from top to bottom. The strains examined included (lane 1) *E. coli* AB1133 (WR3054), (lane 2) *S. flexneri* serotype 2a strain WR1078, (lane 3) *E. coli* AB1133 carrying pWR90 (WR3055), (lane 4) *S. typhi* Ty21a (WR4090), and (lane 5) Ty21a carrying pWR90 (WR4086).

Plasmid analysis. Plasmid DNA was isolated from *E. coli* K-12 strain WR3055 and *S. typhi* Ty21a strain WR4086, both carrying pWR90, and analyzed by electrophoresis on agarose gels. The form I plasmid was apparently 180 kb, while plasmid pWR90 appeared to be 195 kb in size whether obtained from *E. coli* K-12 (Fig. 1, center lane) or *S. typhi* Ty21a (right lane). These results, together with the above conjugal transfer and plasmid stability studies, suggest that the *his* and *pro* regions of the *S. flexneri* serotype 2a chromosome have become part of a recombinant plasmid approximately 195 kb in size.

LPS analysis. To characterize further the surface antigens of parental and genetic hybrid strains, the LPS extracted from several of the previously described strains was subjected to SDS-PAGE analysis as described previously (10) (Fig. 2 and 3). The LPS isolated from the phenotypically rough *E. coli* K-12 strain AB1133 was composed of lipid A and an oligosaccharide core, but lacked any core-linked O side chains (Fig. 2, lane 1). The LPS of wild-type *Shigella flexneri* serotype 2a had core lipid A and a typical ladder of O repeat bands (Fig. 2); each band represents one additional O repeat unit added to the core. The LPS of the *E. coli* AB1133 hybrid strain carrying pWR90 (Fig. 2, lane 3) showed O repeat units of the same molecular mass and chain length (10 to 18) as occurred in the wild-type *Shigella flexneri* serotype 2a strain (lane 2). LPS from strain Ty21a grown in the absence of galactose showed the pattern typical of a *galE* rough strain, with lipid A core only (Fig. 2, lane 4). However, with LPS from the Ty21a strain carrying pWR90 (WR4086), also grown in medium lacking galactose, a change relative to Ty21a (lane 4) of the lipid A and core bands, which are the predominant bands in this lane, was detected (Fig. 2, lane 5). The WR4086 LPS bands corresponding to the O side chains appeared very faintly, suggesting degradation of this LPS during hot phenol-H$_2$O extraction. Im-

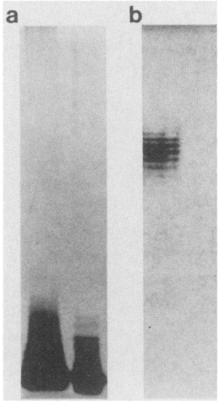

FIG. 3. SDS-PAGE profile and immunoblot analysis of LPS extracted from strain Ty21a or strain Ty21a(pWR90), which expresses the *S. flexneri* serotype 2a O antigen. These strains were grown in the absence of galactose. (a) SDS-PAGE profile of the LPS obtained from Ty21a(pWR90) (strain WR4086) (left lane) and Ty21a (strain WR4090) (right lane). The direction of electrophoresis was from top to bottom. (b) Immunoblot of the gel shown in panel a after sequential reaction with anti-*S. flexneri* 2a antibody and ^{125}I-labeled staphylococcal protein A and then examination by autoradiography as described in Materials and Methods.

munoblot studies (Fig. 3) and chemical analyses of this material (unpublished data), however, indicated that WR4086 cells were indeed making core-linked *Shigella* O antigen. The *Shigella* O antigen was apparently covalently linked to the *Shigella* core oligosaccharide or to a modified *Salmonella* core (Fig. 2, lane 5) and not to the chemically dissimilar native *Salmonella* core. Nevertheless, as described below, the *Shigella* O antigens expressed by this hybrid were highly antigenic, and this Ty21a derivative strain stimulated immunity in mice against challenge with *S. flexneri* serotype 2a cells.

Qualitative and quantitative assay for LPS expression. Initially, *S. typhi* Ty21a(pWR90) strain WR4086 was found by slide agglutination assays to agglutinate in the presence of rabbit anti-9,12 serum or rabbit anti-2a serum. Thus, both *S. typhi* and *Shigella flexneri* LPS antigens are expressed on the surface of these cells. Agglutination and serum adsorption studies were performed to detect qualitative or quantitative differences in the antigens made by strain WR4086 and by parental strains (Tables 2 and 3). Both Ty21a and the Ty21a genetic hybrid WR4086, expressing the *Shigella flexneri* serotype 2a type and group antigens, grown on BHI agar supplemented with 0.1% galactose, were agglutinated by rabbit anti-9,12 typhoid serum to a titer of 1:320. The *S. typhi* 9,12 control strain O901 had an agglutination titer of 1:640 with this antiserum. In tests with rabbit anti-*S. flexneri* serotype 2a serum, strain WR4086 was agglutinated to a titer of 1:960 (Table 2).

TABLE 2. Agglutinin titers of bacterial strains

Antigen	Unabsorbed antiserum titer[a]	
	Anti-9,12	Anti-2a
S. typhi O901	1:640	ND[b]
S. typhi Ty21a	1:320	ND
S. typhi Ty21a(pWR90)	1:320	1:960
S. flexneri (2a) 2457T	ND	1:480

[a] Reciprocal of the dilution in which agglutination was observed. Anti-9,12, Antiserum against *S. typhi* 9,12 O antigen; anti-2a, antiserum against *S. flexneri* serotype 2a O antigen.

[b] ND, No agglutination detected.

In serum adsorption tests (Table 3), all *S. flexneri* serotype 2a antibodies were removed from the antiserum by strain WR4086 or by the *S. flexneri* 2a control strain 2457T. Also, *S. typhi* strain Ty21a or O901 removed all 9,12-specific antibodies from the homologous antiserum. Strain WR4086, however, failed to remove all of the 9,12 antibodies from the antityphoid serum, leaving an agglutination titer of 1:120 when tested with the control *S. typhi* strain O901.

TABLE 3. Postadsorption agglutination titers

Serum absorbed with:	Adsorbed antiserum titer[a]	
	Anti-9,12	Anti-2a
S. typhi O901	ND[b]	1:480
S. typhi Ty21a	ND	1:480
S. typhi Ty21a(pWR90) [WR4086]	1:120	ND
S. typhi WR4086 segregant [WR4087]	ND	1:480
S. flexneri serotype 2a 2457T	1:640	ND

[a] Reciprocal of the dilution at which agglutination was observed. Adsorbed anti-9,12 serum was reacted with cells of *S. typhi* O901 to determine titer. Adsorbed anti-*S. flexneri* 2a serum was reacted with *S. flexneri* 2a 2457T cells.

[b] ND, No agglutination detected.

To determine whether strain WR4086 could regain its ability to synthesize its full complement of typhoid 9,12 antigenic components, a segregant of WR4086 was selected which had lost pWR90, the recombinant plasmid specifying the *Shigella* serotype 2a group and type antigens. In serum adsorption studies, segregant strain WR4087 was observed to have lost the ability to neutralize *S. flexneri* serotype 2a-directed antibodies and to have regained the ability to neutralize all anti-9,12 antibodies (Table 3).

Mouse protection studies. The ability of various bacterial strains to protect mice against intraperitoneal challenge with virulent *S. typhi* Ty2 or *S. flexneri* serotype 2a strains was assessed (Table 4). Strain Ty21a protected against homologous *S. typhi* challenge but not against *S. flexneri* challenge, as expected. *S. flexneri* 2a strain WR1080 protected against homologous *S. flexneri* challenge, but 14 of 15 mice died after heterologous challenge. The genetic hybrid Ty21a strain WR4086 provided significant protection against *S. flexneri* challenge but not against *S. typhi* challenge. Based on these results, a whole-cell vaccine made up of equal parts of the Ty21a parent and WR4086 was tested to determine whether such a combination vaccine would be suitably

TABLE 4. Mouse protection assay[a]

Vaccine strain	Protection (no. of mice dead/no. challenged)	
	S. typhi Ty2	*S. flexneri* serotype 2a
S. typhi Ty21a	5/16*	14/16
S. flexneri serotype 2a	14/15	3/16*
S. typhi Ty21a(pWR90) [WR4086]	11/16	4/16*
S. typhi Ty21a + WR4086	3/16*	5/16*
Saline control	7/8	7/8

[a] Mice were immunized intraperitoneally as described previously (5). Control mice received saline injections. One month postimmunization, immunized and control mice were challenged with 1.4 × 10^4 cells of virulent *S. typhi* Ty2 or 1.6 × 10^6 cells of *S. flexneri* serotype 2a strain M42-43 (WR1078) in 0.5-ml suspensions in hog gastric mucin. Deaths were recorded after 72 h. Results of protection by each vaccine preparation were compared by Fisher's exact test against the challenged, nonimmunized control population. Significant protection ($P \leq 0.05$) is indicated by asterisks.

protective against both challenge organisms. This Ty21a-WR4086 whole-cell vaccine mixture imparted equally significant protection against both the typhoid and the dysentery challenges (Table 4).

DISCUSSION

Despite numerous attempts over the last 30 years to develop effective antidysentery vaccines, this task remains undone. A recent promising approach involves use of the attenuated *S. typhi* Ty21a live, oral vaccine as a carrier of foreign antigenic determinants (5). The *S. sonnei* plasmid-borne form I O-antigen genes have been successfully transferred to Ty21a and shown to provide protection in volunteers against *S. sonnei* challenge (1, 5). The present study was undertaken to confirm the concept that the proven-effective living, attenuated Ty21a strain could be modified to provide protection against other enterically acquired disease agents.

In this report, a genetically stable recombinant plasmid, pWR90, that contains two widely separated *S. flexneri* chromosomal loci responsible for synthesizing the serotype 2a O antigen was constructed via Mu-mediated transposition (4). pWR90, found to be about 195 kb in size, was determined by agglutination studies (Tables 2 and 3) and LPS analysis (Fig. 2 and 3) to effect the synthesis of *S. flexneri* 2a O antigen in both *E. coli* K-12 and *S. typhi* Ty21a hosts. Furthermore, serum adsorption studies showed that cells of Ty21a carrying pWR90 (WR4086) did not express sufficient 9,12 *S. typhi* somatic antigens to remove the total antibody to these antigens. When strain WR4086 was tested as a vaccine in mice, this strain protected only against challenge by virulent *S. flexneri* serotype 2a and not against typhoid challenge (Table 4). Agglutinin adsorption studies (Table 3) of strain WR4086 and its plasmid-free derivative WR4087 indicated that expression of the *S. flexneri* 2a O antigen in Ty21a somehow interfered with normal synthesis or expression of the 9,12 antigens. It seems possible that the 9,12 somatic components are physically covered by the *S. flexneri* O-antigen chains in strain WR4086 or that altered 9,12 somatic components are synthesized when the *S. flexneri* serotype 2a antigens are also produced. However, the fact that the plasmid-free derivative strain WR4087 expressed typical 9,12 antigens indicates that the necessary *S. typhi* LPS genes remain intact. The inability of strain WR4086 to serve as an antityphoid vaccine is thus due to impaired expression of the typhoid antigenic components. Nevertheless, a combined, whole-cell vaccine mixture consisting of parental Ty21a and WR4086 strains was found to behave as a bifunctional antityphoid and anti-*S. flexneri* serotype 2a vaccine. Additional studies will be necessary to determine the stability of this vaccine upon lyophilization, its safety in humans, and its efficacy as a potential human vaccine.

The above results raise three important issues. First, it is now apparent that expression of certain heterologous antigens can adversely affect the antityphoid efficacy of a genetic hybrid Ty21a vaccine. Therefore, for use as a bifunctional vaccine, each Ty21a genetic hybrid strain carrying heterologous antigenic determinants, especially O antigens, should be assessed for its antityphoid efficacy as well as for protection against the second pathogen. Second, a genetic carrier vaccine strain lacking the 9,12 typhoid somatic antigens and synthesizing primarily the added heterologous determinants is envisioned as being preferable in certain situations (e.g., for use in a population already immunized orally against typhoid fever). Third, humans are the only host normally susceptible to *S. typhi* infection. Mouse protection tests that use intraperitoneal challenge to assay the efficacy of *S. typhi*-based vaccines represent a logical first step in determining the antigenic nature and, hence, the vaccine potential of a strain. These tests, however, are necessarily artificial, as they do not mimic the normal route of infection and thus cannot directly reflect the potential value of a vaccine in humans. Development of an animal system that would directly reflect the potential for human use of an *S. typhi*-based vaccine would be highly desirable.

This report and the previous study of Formal et al. (5) suggest that Ty21a or similar attenuated *S. typhi* strains can serve as carriers of major protective-antigen-producing genes of different pathogens. It is possible that a series of oral vaccines that will protect against many enterically acquired pathogens (e.g., bacterial, viral, and protozoan) can be constructed by using this type of oral vaccine system.

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