Unusual Lipopolysaccharide Antigens of a Salmonella typhi Oral Vaccine Strain Expressing the Shigella sonnei Form I Antigen*

(Received for publication, December 5, 1983)

Robert C. Seid, Jr.,‡, Dennis J. Kopecko, Jerald C. Sadoff, Herman Schneider, Louis S. Baron, and Samuel B. Forman

From the Departments of Bacterial Diseases and Bacterial Immunology, Walter Reed Army Institute of Research, Washington, D.C. 20307

Salmonella typhi 5076-1C, a potential live, oral vaccine for protection against typhoid fever and Shigella sonnei shigellosis, expresses the S. sonnei form I antigen and normal S. typhi somatic antigens. Polysaccharide antigens of this galactose epimeraseless genetic derivative strain were hot phenol-water extracted from cells grown with (+gal) and without (−gal) galactose. Ultrafiltration of the aqueous layer from (+gal) cells resulted in a lipopolysaccharide (LPS) pellet having core-linked S. typhi O-antigen but no core-linked form I antigen; the LPS from (−gal) cells lacked O-antigen. The form I antigen, obtained from the supernatant, was purified by alcohol precipitation and ion exchange chromatography. Unlinked form I and S. typhi O-polysaccharide antigens, both present in the (+gal) supernatant, were further separated by gel filtration. Chemical analyses revealed the 5076-1C form I antigen to be a polymer (Mw = 14,000–20,000) having O-disaccharide repeating units comprised of 2-acetamido-4-amino-2,4,6-trideoxy-β-galactose and 2-acetamido-2-deoxy-1-altruronic acid. Unlike parental S. sonnei form I LPS, the 5076-1C form I antigen lacked core lipid A, had low phosphorus content, and migrated in polyacrylamide gels with lower relative mobility. In contrast to current concepts of LPS assembly, these data indicate that 5076-1C form I antigen is transported to the cell surface without covalent linkage to core lipid A, and exists as a polymerized, antigenic surface entity.

Shigella sonnei is a predominant cause of bacillary dysentery in the United States and worldwide. The major protective S. sonnei surface antigen, the form I LPS1 has been chemically defined to contain an unusual O-disaccharide repeat unit linked to core lipid A (1, 2). Presently, there are no proven effective prophylactic measures to protect against this intestinal disease. In an attempt to construct a vaccine that would stimulate local intestinal immunity to S. sonnei, the genes determining the Shigella form I antigen have been transferred to an attenuated mutant strain of Salmonella typhi, Ty2la (3); strain Ty2la had previously been shown to act as a safe, effective live oral vaccine for protection against typhoid fever (4–6). One resulting Ty2la derivative, 5076-1C, has been found to express both the Shigella form I antigen and typical S. typhi somatic antigens. Strain 5076-1C appears from other preliminary studies to be a potential bifunctional oral vaccine for protection against these two important human intestinal disease agents (3, 7).2 Due to the great practical importance of this oral vaccine strain, the present investigation was initiated to examine the physiochemical nature of the LPS antigens of this genetic derivative strain. We report here the isolation and purification of the 5076-1C LPS antigens and compare their properties to normal S. sonnei and S. typhi LPS. Previous serological studies of strain 5076-1C indicated the presence of normal form I LPS, but the chemical data presented herein demonstrate that the 5076-1C form I antigen is not covalently bound to core lipid A. Instead, this form I antigen exists as an independent, highly polymerized, immunogenic entity on the cell surface. In contrast to current concepts of LPS assembly (8–10), this unexpected finding indicates that transport of polymerized O-antigen, possibly attached to a phosphorylated carrier lipid, from the cytoplasmic membrane to the outer membrane does not require covalent attachment to core lipid A.

EXPERIMENTAL PROCEDURES3

RESULTS

Isolation and Purification of Form I Antigen and Somatic LPS Antigens from 5076-1C Cells—Ultrafiltration of the aqueous phases derived from hot phenol-water extraction of (−gal) and (+gal) 5076-1C cells afforded LPS-R and LPS-S, respectively, (1.5 and 2.3% yields based on acetone-dried cells; Fig. 1). The form I antigen did not pellet but remained in the supernatant extracts. After nuclease treatment, the form I antigen lacked core lipid A, and exists as a polymerized, antigenic surface entity.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence and reprint requests should be addressed.

1 The abbreviations used are: LPS, lipopolysaccharide; Ps, polysaccharide; galE, galactose epimeraseless; KDO, 3-deoxy-β-manno-octulosonic acid; Hpr, 1-glycerol-β-manno-heptose; 4-n-D-FucNAc, 2-acetamido-4-amino-2,4,6-trideoxy-β-galactose; L-AlNacUA, 2-acetamido-2-deoxy-1-altruronic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ACL, antigen carrier lipid; BHI, brain heart infusion; EDAC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide; LAL, Limulus amoebocyte lysate.

2 S. B. Forman, personal communications.

3 Portions of this paper (including "Experimental Procedures") are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 83M-2404, cite the authors, and include a check or money order for $2.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
column. The peak eluting at the bed volume contained free yield of a standard glucose solution, the free glucose content nucleic acids that were not retained by the DEAE-Sephacel.

addition to S.

antigens, which were retained in a dialysis membrane with a molecular mass cutoff of 14 kDa, co-precipitated during the subsequent DEAE-Sephacryl column. The form I Ps antigen (0.10% yield) derived from (+gal) 5076-1C cells was judged homogeneous due to the low amount (0.05%) of nucleic acids, protein, and neutral carbohydrates detected.

The (+gal) supernatant extract contained S. typhi O-Ps in addition to S. sonnei form I Ps antigen (Fig. 1). These two antigens, which were retained in a dialysis membrane with a molecular mass cutoff of 1 kDa, co-precipitated during ethanol addition and co-eluted during the subsequent DEAE-Sephacel chromatography, but separated on a Sephacryl gel permeation column in EDTA-containing buffer (Fig. 2). The form I Ps antigen (0.14% yield), identified by immunodiffusion assays, eluted before the S. typhi O-Ps peak (0.12% yield, fractions 47-55), which was detected colorimetrically by phenol-sulfuric acid reagent.

lesser than 0.4% of total weight (26 mg) of both the isolated S. typhi 0-Ps and the form I O-Ps antigen. The form I antigen (14 mg) and the S. typhi O-Ps (12 mg) derived from (+gal) cells (10 g dry weight) contained less than 0.1% nucleic acids and protein.

The form I Ps antigens, isolated from (-gal) and (+gal) cells, proved to be equivalent antigens by immunassays, SDS-PAGE, chemical modification studies, and NMR spectroscopy. The term form I Ps antigen is used to represent either of these two indistinguishable antigen preparations. During gel filtration in disaggregating buffer (Fig. 2), it eluted in a region behind dextran T-20 which has an average M₄ = 20,000.

Lipid A Analysis—To ascertain whether lipid A is a component of the 5076-1C somatic antigens, we employed the Limulus amoebocyte lysate assay (Table I) and also performed chemical analyses of acid-hydrolyzed products. LPS-R and LPS-S gave high LAL end points corresponding to levels observed for the control Escherichia coli LPS and LPS of parental S. sonnei and S. typhi organisms. The 5076-1C form I Ps antigen and S. typhi O-Ps were about 10-fold less effective in promoting LAL gelation, thus indicating the absence of the toxic lipid A. Moreover, treatment of both antigens with 1% acetic acid, known to cleave aciable KDO linkages in LPS (8, 9), did not result in lipid A floculation, which did occur in similar treatment of LPS-R and LPS-S.

Neither the 5076-1C form I Ps antigen nor S. typhi O-Ps contained glucosamine or fatty acids, which were readily detected in LPS-R and LPS-S (Table II). The phosphorus contents of the 5076-1C form I Ps antigen and S. typhi O-Ps were 4- to 10-fold lower than LPS-R and LPS-S. The fatty

|Antigens| Lipid A components in LPS-related antigens|
|--------|-----------------------------------|
|5076-1C form I-Ps| Relative fatty acids| Fatty acids| GlcNAc| p*| % | % | % | % |
|5076-1C S. typhi O-Ps| | | | | 12.0 | 140 | 3-OH,14 | 16.0 | 0.35 |
|5076-1C LPS-R| | | | | 1.2 | 18.1 | 69.2 | 11.5 | 14.0 | 6.0 | 2.85 |
|5076-1C LPS-S| | | | | 4.0 | 16.7 | 69.3 | 10.0 | 7.9 | 4.2 | 1.38 |
|S. typhi Ty21a LPS| | | | | 1.7 | 15.1 | 70.9 | 12.2 | 11.2 | 3.6 | 3.75 |
|S. sonnei 53G LPS| | | | | 1.8 | 17.8 | 76.4 | 4.0 | 6.3 | 2.5 | 2.20 |

* Stock solutions of each antigen were made to a concentration of 100 µg/ml.

1 Reference LPS obtained from Bureau of Biologics, Bethesda, MD.

2 Pyrogen-free water obtained from Abbott Laboratories was used as a negative control.

Fig. 1. Flow diagram of the isolation of form I Ps antigen and Salmonella somatic antigens from the S. typhi strain 5076-1C. Phenol-water extraction of the transconjugant strain.

Fig. 2. Separations of form I Ps antigen from S. typhi O-Ps by Sephacryl S-300 gel filtration. The fractions serologically reactive with form I-specific antiserum are marked by +. The S. typhi O-Ps peak, detected by phenol-sulfuric acid reagent, eluted in fractions 47-55. The arrows indicate elution positions of blue dextran (void volume, V₀); dextran T-20 (average M₄ = 20,000).
Acid profiles of LPS-R and LPS-S, which revealed a predominance of β-OH myristic acid (about 70%), exemplified the pattern seen for normal enterobacterial LPS (28), including the parental S. typhi and S. sonnei LPS (Table II).

Monosaccharide Analysis—The monosaccharide constituents which comprise the O-specific Ps and core regions of S. sonnei and S. typhi LPS are schematically depicted in Fig. 3. Except for the amino sugars and the two unusual sugars of the S. sonnei O-repeat unit, the neutral monosaccharides, as well as KDO, in these two LPS structures are readily detected by the sugar analyzer (11).

The sugar chromatograms of LPS-R, LPS-S, and S. typhi O-Ps are presented in Fig. 4. While glucose and mannose are not separated by the chromatographic system, they were distinguished by treating a portion of sample hydrolysate with glucose oxidase to remove glucose prior to analysis (29). In this manner, the glucose/mannose peak of LPS-R at 57 min in Fig. 4A contained 90% glucose and 10% mannose. In contrast, the glucose/mannose peak of LPS-S (Fig. 4B) contained 20% glucose and 80% mannose. In S. typhi O-Ps, the 57-min peak contained only mannose. The chromatograms of LPS-R and LPS-S differed markedly. In LPS-R (Fig. 4A), the core sugars heptose and glucose were predominant compared to the S. typhi O-chain components: tyvelose, rhamnose, mannose, and galactose. The reverse was true for LPS-S (Fig. 4B) where the S. typhi O-specific chain sugars were predominant. The core sugar KDO was also detected in both chromatograms even though a substantial proportion of it was presumably destroyed during hydrolysis (11). The chromatogram of the nontoxic S. typhi O-Ps (Fig. 4C) revealed only the presence of O-side chain components: tyvelose, rhamnose, mannose, and galactose. Based on their color yields, these monosaccharides were present in equimolar amounts.

The S. sonnei O-antigen sugars were not detected on the sugar analyzer, possibly due to their acid lability (1) and/or retention on the analytical column. The acid hydrolysate of S. sonnei form I LPS yielded a sugar chromatogram containing peaks only for the core components, KDO, glucose, galactose, and heptose. In contrast, acid-hydrolyzed 5076-1C form I Ps antigen yielded a blank chromatogram, suggesting the absence of the inner and outer core components (data not shown).

Chemical Modification and Analysis of 5076-1C Form I Antigen—To identify its sugar components, the 5076-1C form I Ps antigen was chemically modified, as described under "Experimental Procedures," to yield stable amino sugar derivatives detectable with the amino acid analyzer. The main reaction course for deamination of the S. sonnei O-disaccharide repeat structure (Fig. 3), should involve an SN2 attack by water at C-4 of 4-n-L-FucNAc residue to yield a quinovosa-minylic derivative (1). Reduction of the carboxyl group of L-AltNAcUA residues would lead to an altrosaminyl structure. As expected, the acid hydrolysate of deaminated form I Ps antigen gave an amino sugar peak eluting at the quinovosamine position (Table III), which was not present in the hydrolysate of unmodified form I Ps antigen. The carboxyl-reduced, deaminated 5076-1C form I Ps antigen gave a second amino sugar peak eluting in the position of 2-amino-2-deoxygalactose (Table III). These results indicated that the 5076-1C-derived form I Ps antigen contains the expected Shigella O-disaccharide repeat units.

Acid-hydrolyzed, parental S. sonnei form I LPS gave a glucosamine peak (Tables II and III), whereas its deaminated, carboxyl-reduced form yielded the expected quinovosamine and 2-amino-2-deoxygalactose peaks in addition to glucosamine. On the other hand, only glucosamine could be detected in
two LPS preparations consisted predominantly of core structures as evidenced by the staining intensity of the low molecular weight bands. Faint step-like band patterns, appearing in the upper gel region and smooth LPS structures were also produced, probably due to the cellular incorporation of trace amounts of galactose containing a varying number of O-repeat units. However, the LPS of S. sonnei form II (Lane E) and of the S. minnesota chemotype R, and R, mutants (Lanes G and H) migrated as one or more broad low M, bands, characteristic of rough incomplete LPS (30–32).

The 5076-1C LPS-R (Lane B) gave a silver-stained pattern indistinguishable from the LPS of parental galE S. typhi strain Ty21a (Lane C), also grown without galactose. These two LPS preparations consisted predominantly of core structures as evidenced by the staining intensity of the low molecular weight bands. Faint step-like band patterns, appearing in the upper gel region (Lanes B and C), suggested that some smooth LPS structures were also produced, probably due to the cellular incorporation of trace amounts of galactose constituents are not covalently bound to 5076-1C LPS-R or LPS-S.

Table III

Qualitative amino sugar composition of chemically modified antigens

| Antigens          | Components*         |
|-------------------|---------------------|
|                   | GlcN (72.9) | GalN (74.4) | AltN (75.9) | QuiN (77.1) | FucN (80.4) |
| 5076-1C form I-Ps | untreated    | -           | -           | -           | -          |
|                   | deaminated   | -           | t           | +           | -          |
|                   | carboxyl-reduced | -       | +           | -           | -          |
| S. sonnei 53G LPS| untreated    | +           | -           | -           | -          |
|                   | deaminated   | +           | -           | -           | -          |
|                   | carboxyl-reduced | +       | -           | -           | -          |
| S. typhi Ty21a LPS| untreated    | +           | -           | -           | -          |
|                   | deaminated   | +           | -           | -           | -          |
|                   | carboxyl-reduced | +       | -           | -           | -          |
| 5076-1C LPS-R    | untreated    | -           | -           | -           | -          |
|                   | deaminated   | -           | -           | -           | -          |
|                   | carboxyl-reduced | -       | -           | -           | -          |
| 5076-1C LPS-S    | untreated    | -           | -           | -           | -          |
|                   | deaminated   | -           | -           | -           | -          |
|                   | carboxyl-reduced | -       | -           | -           | -          |

* GlcN, glucosamine; GalN, galactosamine; AltN, 2-amino-2-deoxygalactose; QuiN, quinovosamine; FucN, fucosamine. Values in parentheses represent retention time (in minutes) on the amino acid analyzer. t indicates trace amount.

Fig. 5. SDS-PAGE of LPS antigens revealed by silver staining. Samples of 1 µg were analyzed except as noted. Lanes A and I, 5076-1C derived form I Ps antigen, 3 and 1 µg, respectively; Lane B, 5076-1C LPS-R; Lane C, galE S. typhi Ty21a LPS; Lanes D and J, S. sonnei form I LPS, 3 and 1 µg, respectively; Lane E, S. sonnei form II LPS; Lane F, smooth S. minnesota LPS; Lane G, rough S. minnesota chemotype R, LPS; Lane H, rough S. minnesota chemotype R, LPS. The 5076-1C form I Ps antigen (Lanes A and I) did not react with the silver-staining reagent. The asterisks in Lane I mark the position of putative 5076-1C form I Ps antigen based on its mobility (Rf) calculated from immunoblot analysis (see "Experimental Procedures" and Fig. 6). The arrows correspond to the top (T) and bottom (B) of the SDS gel.

both untreated and chemically treated LPS-R, LPS-S, and S. typhi Ty21a LPS (Table III). Thus, the S. sonnei form I O-constituents are not covalently bound to 5076-1C LPS-R or LPS-S.

SDS-PAGE and Immunoblot of 5076-1C Form I Antigen—SDS-PAGE (Fig. 5) was performed on the 5076-1C form I antigen (Lanes A and I) as well as on the LPS from smooth and rough Salmonella minnesota (Lanes F, G, and H), S. typhi Ty21a (Lane C), S. typhi 5076-1C (LPS-R, Lane B), S. sonnei form I (Lanes D and J), and S. sonnei form II (Lane E). The LPS from smooth organism of S. minnesota (Lane F) and S. sonnei form I (Lane D) strains electrophoresed as regular step-like band patterns indicative of smooth LPS structures containing a varying number of O-repeat units. However, the LPS of S. sonnei form II (Lane E) and of the S. minnesota chemotype R, and R, mutants (Lanes G and H) migrated as one or more broad low M, bands, characteristic of rough incomplete LPS (30–32).

The 5076-1C LPS-R (Lane B) gave a silver-stained pattern indistinguishable from the LPS of parental galE S. typhi strain Ty21a (Lane C), also grown without galactose. These two LPS preparations consisted predominantly of core structures as evidenced by the staining intensity of the low molecular weight bands. Faint step-like band patterns, appearing in the upper gel region (Lanes B and C), suggested that some smooth LPS structures were also produced, probably due to the cellular incorporation of trace amounts of galactose containing a varying number of O-repeat units. However, the LPS of S. sonnei form II (Lane E) and of the S. minnesota chemotype R, and R, mutants (Lanes G and H) migrated as one or more broad low M, bands, characteristic of rough incomplete LPS (30–32).

The 5076-1C LPS-R (Lane B) gave a silver-stained pattern indistinguishable from the LPS of parental galE S. typhi strain Ty21a (Lane C), also grown without galactose. These two LPS preparations consisted predominantly of core structures as evidenced by the staining intensity of the low molecular weight bands. Faint step-like band patterns, appearing in the upper gel region (Lanes B and C), suggested that some smooth LPS structures were also produced, probably due to the cellular incorporation of trace amounts of galactose con-
Stained in the growth medium.

The 5076-1C form I 0-Ps antigen (Lanes A and I) was not visualized on gels by the silver-staining reagent, possibly because of its failure to enter the SDS gel or because of the peculiarity of its O-repeat structure. Thus, we performed an immunoblot of a gel containing electrophoresed 5076-1C form I Ps antigen along with the LPS of S. sonnei form I, S. typhi 5076-1C, and S. typhi Ty21a strains. The resulting autoradiogram (Fig. 6) indicated that both the 5076-1C form I Ps antigen (Lanes A and B) and native S. sonnei form I LPS (Lane D) were serologically reactive with the form I-specific antiserum. The LPS-R from strain 5076-1C (Lane C) and S. typhi Ty21a (Lane E) were not reactive. Moreover, we have observed that the 5076-1C LPS-S, when electroblotted, failed to react with the form I-specific antiserum (data not shown). These data demonstrate that the Shigella form I 0-2s is not incorporated as part of the 5076-1C LPS structure.

The radioactive profile of the parental S. sonnei form I LPS corresponded to its silver-stained pattern (Fig. 5). The radioactivity observed in Lanes A and B of Fig. 6 indicated that the 5076-1C form I Ps antigen did indeed migrate into the SDS gel even though it was not silver stained. Its putative location on SDS-PAGE (indicated by the asterisks, Lane 1, Fig. 5) is based on its mobility (Rf value of 0.1) determined from the immunoblot autoradiogram (Fig. 6). It should be noted that Shigella O-Ps, derived from S. sonnei form I LPS by mild acid treatment, failed to electrophorese under similar SDS-PAGE conditions (data not shown). Thus, even though the 5076-1C form I Ps antigen and the S. sonnei form I O-Ps share the same antigenic determinants, they are markedly different with respect to electrophoretic behavior (see "Discussion").

\[ ^{13}C \text{ NMR Spectroscopy of Form I Antigen} \]

The presence of S. sonnei O-disaccharide repeat units in the 5076-1C form I Ps antigen was confirmed by FT-NMR spectroscopy. The \(^{13}C\) NMR spectra of 5076-1C form I Ps antigen (Fig. 7A) and of S. sonnei O-Ps (Fig. 7B), obtained from S. sonnei form I LPS by mild acid hydrolysis, are similar. Most prominent are the \(^{13}C\) signals for carbonyl carbons at 176.6 and 176.2 ppm, two anomeric carbons at 105.4 and 103.5 ppm, acetamido group(s) (CH\(_3\)CO) at 24.9 ppm, and a methyl group signal of a 6-deoxyhexose at 18.2 ppm. Signals appearing at 53.5, 54.1, and 57.3 (weak) ppm may be assigned to carbon atoms substituted with amino or acetamide groups. Secondary hydroxyl carbon atoms were also observed at 69.8, 70.2, 78.1, and 79.8 ppm. The chemical shifts observed in the two spectra (Fig. 7, A and B) are consistent with those reported by Kenne et al. (1) for Shigella O-Ps and are indicative of an O-disaccharide-repeating structure comprised of L-AltNAcUA and 4-n-DfucNAc. No other major component was revealed by the NMR spectra.

**DISCUSSION**

The LPS antigens obtained from the S. typhi vaccine derivative strain 5076-1C, which expresses the Shigella form I Ps antigen, were compared to those derived from parental S. sonnei and S. typhi strains. Chemical studies demonstrated typical core-linked O-Ps from the parental strains and confirmed that the form I O-specific disaccharide repeat unit contains L-AltNAcUA and 4-n-DfucNAc (1, 2). All LPS materials, in these studies, that sedimented during ultracentrifugation displayed typical behavior: 1) treatment with hot 1% acetic acid resulted in lipid A flocculation; 2) LPS-R and LPS-S promoted Limulus lysate gelation (Table I); and 3) chemical analyses revealed lipid A components glucosamine and \(\beta\)-OH myristic acid and inner core sugars heptose and KDO (Table II). Because galE mutants are unable to convert UDP-glucose to UDP-galactose when grown in a galactose-deficient medium, the 5076-1C strain should produce an incomplete LPS of an R, chemotype (8). In fact, LPS-R was a rough, incomplete LPS as verified by the sugar chromatogram which showed the predominance of the core sugars glucose and heptose (Fig. 4; the core sugar KDO was detected in low amounts due to its acid lability). In the presence of exogenous galactose, galE mutants should produce LPS indistinguishable from that of the wild type bacteria (8). Sugar analysis of LPS-S, derived from (+gal) 5076-1C cells, revealed a predominance of O-antigen components (tyvelose, rhamnose, mannose, and galactose; Fig. 4B) as expected.

Besides synthesizing LPS-S, 5076-1C (+gal) cells produced the form I Ps antigen in an unusual form, differing from classical LPS in these respects: 1) it did not sediment during ultracentrifugation; 2) it gave low Limulus amoebocyte lysate activity; 3) it contained no core lipid A components; and 4) it exhibited lower mobility than native S. sonnei LPS on SDS gels. The inability to silver stain the 5076-1C form I Ps antigen on SDS gels was attributed to the lack of core lipid A components, since the LPS of both S. sonnei form I and II organisms were stained (Fig. 5). As evidenced by immunoblot analysis, the 5076-1C form I antigen did electrophorese on...
LPS Antigens of \textit{S. typhi} Derivative

SDS gels, although at a much lower relative mobility compared to native \textit{S. sonnei} form I LPS (Fig. 6). Two factors may explain its low mobility. First, the 5076-1C form I antigen is a high molecular weight polymer; dialysis membrane and gel filtration results indicate $M_w = 14,000-20,000$. Second, the antigen may have a phosphorylated lipid carrier entity. Phosphorylated ACL such as undecaprenol pyrophosphate-linked moieties, are known to be involved in the biosynthesis of O-Ps chains (8–10). Since native LPS mobility in SDS gels depends on its relative lipid A content (30, 31), a P-ACL attached to 5076-1C form I Ps antigen would be the only hydrophobic region that binds with SDS, thus allowing electrophoresis into an SDS gel. A lipid requirement is based on the observation that parental \textit{S. sonnei} O-Ps, denuded of lipid A, failed to enter the SDS gel under similar conditions (data not shown). The presence of P-ACL is also supported by the detection of a low phosphorus content in the 5076-1C form I O-Ps (Table II). Unequivocal proof of a phosphorylated lipid carrier attached to the form I Ps antigen, however, must await further chemical analysis.

The (+gal) 5076-1C strain also produced some core-free \textit{S. typhi} O-Ps (6.12% yield, Fig. 4C). Like the form I Ps antigen, this O-antigen exhibited low Limulus activity (i.e. lipid A-free), remained in the supernatant during centrifugation, and exceeded 14 kDa in molecular mass as measured by dialysis. its low phosphorus content (Table II), together with the above data, suggests that this \textit{S. typhi} O-Ps antigen is linked through a P-ACL, as discussed above for the form I antigen. Evidently, transfer of P-ACL-linked \textit{S. typhi} O-Ps to the core lipid A structure is an inefficient process. This is not unexpected since, during galactose uptake in the \textit{E. coli} mutant, intracellular UDP-galactose accumulates to a toxic level (3, 4), possibly interfering with normal LPS synthesis. Note that core-free O-Ps antigens are not produced in wild type \textit{S. typhi} or \textit{S. sonnei} cells (data not shown), apparently, all of the O-Ps chains are covalently integrated into LPS molecules, signifying an efficient translocation process.

Recent studies have demonstrated that the core-linked O-specific polysaccharide chain of one bacterial species could be replaced by an O-antigen of another species via genetic manipulation, e.g. conjugal mating or transduction (9, 33–35). However, no evidence of coherent union between the form I Ps antigen and the \textit{Salmonella} core was found by chemical or by immunoblot analyses. We conclude that the 5076-1C form I Ps antigen does not form an integral part of the \textit{Salmonella} LPS molecule, but exists as a highly polymerized (i.e. an estimated 50–45 O-repeat units) autonomous cell surface entity.

Lipid A-free, O-Ps antigens are also known to be synthesized by rfa mutant bacteria (8, 36). Defective in R-core biosynthesis, these rfa mutants elaborate O-Ps antigens that are not transported out to the outer membrane, but remain attached to P-ACL at the cytoplasmic membrane, thus explaining the inability of these rfa mutant cells to agglutinate in the presence of anti-O antibodies (8, 37, 38). However, the lipid A-free, 5076-1C form I antigen is expressed on the cell surface, based on cell agglutinability in form 1-specific antiserum (3, 12). Hence, in contrast to the current theory of LPS assembly in the Enterobacteriaceae, the 5076-1C form I Ps antigen does not require translocation to core lipid A at the cytoplasmic membrane for transport to the outer membrane.

According to present knowledge, the biosynthesis of O-specific Ps in the Enterobacteriaceae is controlled by the chromosomal rfa, rfc, and rfe gene clusters (8, 9). The form I antigen genes of \textit{S. sonnei}, however, exist on a 120-MDa plasmid in natural isolates of this species. This plasmid was transferred to \textit{S. typhi} during the construction of strain 5076-1C. Although previous serological studies of the 5076-1C strain indicated that this plasmid encodes the structural genes for the polymerized form I O-antigen, the above chemical data provide further support for this conclusion. In addition, the \textit{S. sonnei} plasmids may encode other undefined traits involved in O-antigen expression. Recently, the \textit{S. sonnei} plasmid has been introduced into an \textit{E. coli} K12 strain that elaborates a complete LPS core, a prerequisite for smooth LPS synthesis. The derivative \textit{E. coli} strain is agglutinable with the \textit{S. sonnei} form I-specific antiserum. In contrast to results obtained with strain 5076-1C, SDS-PAGE analysis revealed that the form I antigen is covalently bound to \textit{E. coli} core-lipid A. These results are not altogether surprising since the \textit{E. coli} K12 core is more akin to the \textit{Shigella} core than to that of \textit{Salmonella}.

Further genetic transfer of the form I O-antigen genes to other defined LPS mutants of enteric bacteria should provide additional insights into the mechanism of LPS assembly.

Acknowledgment—We thank Jean Guidas for the typing of the manuscript.

REFERENCES

1. Kenne, L., Lindberg, B., Petesson, K., Katzenellenbogen, E., and Romanowska, E. (1980) Carbohydr. Res. 78, 119–126
2. Gen nier, A., and Romanowska, E. (1982) Eur. J. Biochem. 129, 105–109
3. Formai, S. B., Baron, L. S., Kopecko, D. J., Washington, O., Powell, C. and Life, C. A. (1981) Infect. Immun. 34, 748–750
4. Germanier, R., and Piérèr, E. (1975) J. Infect. Dis. 131, 553–558
5. Wadhan, M. H., Serie, C., Cerisier, R., Y., Guérin, N., Sallam, S., Geoffroy, P., Sadek El Tantawi, A., and Guesny, P. (1980) Bull. WHO 58, 498–499
6. Germanier, R. H., Hornick, R. B., Woodside, W. E., DuPont, H. L., Synder, M. J., Levine, M. M., and Libonati, J. B. (1977) J. Infect. Dis. 136, 717–723
7. Keren, D. F., Collins, H. H., Baron, L. S., Kopecko, D. J., and Formal, S. B. (1982) Infect. Immun. 37, 387–389
8. Nakaido, H. (1989) Adv. Enzymol 51, 77–124
9. Galanos, C., Luderitz, O., Rietschel, E. T., and Westphal, O. (1979) Int. Rev. Biochem. 14, 239–335
10. Osborn, M. J. (1971) in Structure and Function of Biological Membranes (Rothfield, L., ed.) pp. 343–400, Academic Press, New York
11. Seid, R. C., Jr., Schneider, H., Bondarew, S., and Boykins, R. A. (1982) Anal. Biochem. 124, 320–326
12. Kopecko, D. J., Washington, O., and Formal, S. B. (1980) Infect. Immun. 29, 207–214
13. Ouchterlony, O. (1958) Prog. Allergy 5, 1–78
14. Westphal, O., and Jann, K. (1965) Methods Carbohydr. Chem. 5, 95–99
15. Galanos, C., Luderitz, O., and Westphal, O. (1969) Eur. J. Biochem. 9, 245–249
16. Taylor, R. L., Shively, J. E., and Conrad, H. E. (1976) Methods Carbohydr. Chem. 7, 149–151
17. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1951) Nature (Lond.) 166, 165
18. Wong, K. H., Barrera, O., Sutton, A., May, J., Hochstein, D. H., Robbins, J. D., Robbins, J. B., Parkman, P. D., and Seligman, E. B. (1977) J. Biol. Stand. 5, 197–215
19. Lee, H. M., Bucher, D. J., and Seid, R. C., Jr., (1979) Ind. Eng. Chem. Products Res. Dev. 18, 122–126
20. Seid, R. C., Jr., and Sadow, J. C. (1981) J. Biol. Chem. 256, 7305–7310
21. Williams, J. M. (1975) Adv. Carbohydr. Chem. Biochem. 31, 9–79
22. Means, G. E., and Peoney, R. E. (1971) Chemical Modification of Proteins, Holden-Day, Inc., San Francisco
23. Schlenk, H., and Galimov, A. (1982) Anal. Chem. 34, 1412–1414

\*D. J. Kopecko, L. S. Baron, T. L. Hale, S. B. Formal, and K. Noon (1983) Annual Meeting of the American Society for Microbiology, Abstract D10, p. 60.

\*T. L. Hale, S. B. Formal, and R. C. Seid, unpublished experiments.
24. Seid, R. C., Jr., Smith, P. F., Guerra, G., Hochstein, H. D., and Barile, M. M. (1980) Infect. Immun. 33, 290-294.

25. Laemmli, U. K. (1970) Nature (Lond.) 227, 680-685

26. Tsai, C. M., and Frasch, C. E. (1982) Anal. Biochem. 122, 239-246

27. Towbin, H. T., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350-4354

28. Wilkinson, S. W. (1977) Bacteriol. Rev. 41, 572-596

29. Boykins, R. A., and Liu, T.-Y. (1980) J. Biochem. Biophys. Methods 2, 71-78

30. Jann, B., Reske, K., and Jann, K. (1975) Eur. J. Biochem. 50, 137-153

31. Pavla, E. T., and Makela, P. H. (1980) Eur. J. Biochem. 107, 299-305

32. Goldman, R. C., and Leive, L. (1980) Eur. J. Biochem. 107, 145-153

33. Kiefer, W., Gransow, K., and Westphal, O. (1976) Infect. Immun. 13, 1517-1518

34. Kiefer, W., and Westphal, O. (1976) J. Gen. Microbiol. 92, 315-324

35. Makela, P. H., and Mayer, H. (1976) Bacteriol. Rev. 40, 591-632

36. Wright, A., and Kanesaki, S. (1974) Physiol. Rev. 51, 748-784

37. Beckman, I., Subbaiah, T. V., and Stocker, B. A. D. (1964) Nature (Lond.) 201, 1299-1301

38. Subbaiah, T. V., and Stocker, B. A. D. (1964) Nature (Lond.) 201, 1298-1299

**MATERIALS AND METHODS**

**LPS Antigens of S. typhi Derivative**

**Experimental Procedures**

**Antigen Preparation and Analysis of LPS from Salmonella typhi, S. typhi var. Typhimurium, and S. typhi var. Paratyphi A**

**Materials**

- Lipopolysaccharide (LPS) from Salmonella typhi, S. typhi var. Typhimurium, and S. typhi var. Paratyphi A
- Lipopolysaccharide (LPS) from Salmonella typhi, S. typhi var. Typhimurium, and S. typhi var. Paratyphi A

**Methods**

1. **Preparation of LPS from Salmonella typhi, S. typhi var. Typhimurium, and S. typhi var. Paratyphi A**
   - **LPS from Salmonella typhi**: The method involves the isolation of LPS from the bacterial cell wall. The procedure includes the extraction of LPS from the cell wall using hot phenol-water method. The LPS is then purified by ethanol precipitation and dialysis against water. The purified LPS is then analyzed by SDS-PAGE and Western blotting.

2. **Preparation of LPS from Salmonella typhi var. Typhimurium**: The method involves the extraction of LPS from the cell wall using hot phenol-water method. The LPS is then purified by ethanol precipitation and dialysis against water. The purified LPS is then analyzed by SDS-PAGE and Western blotting.

3. **Preparation of LPS from Salmonella typhi var. Paratyphi A**: The method involves the extraction of LPS from the cell wall using hot phenol-water method. The LPS is then purified by ethanol precipitation and dialysis against water. The purified LPS is then analyzed by SDS-PAGE and Western blotting.

**Results**

- The LPS antigens from Salmonella typhi, S. typhi var. Typhimurium, and S. typhi var. Paratyphi A were analyzed by SDS-PAGE and Western blotting.
- The SDS-PAGE analysis revealed the presence of a single, prominent band corresponding to the LPS antigen. The Western blotting analysis confirmed the identity of the LPS antigen by reacting with specific antibodies.

**Discussion**

- The LPS antigens from Salmonella typhi, S. typhi var. Typhimurium, and S. typhi var. Paratyphi A were shown to be highly conserved among the different Salmonella strains, as evidenced by the presence of a single, prominent band in SDS-PAGE and Western blotting analysis.
- The high degree of conservation of the LPS antigens among different Salmonella strains suggests that they may play a crucial role in the pathogenesis of salmonellosis.

**Conclusion**

- The LPS antigens from Salmonella typhi, S. typhi var. Typhimurium, and S. typhi var. Paratyphi A were shown to be highly conserved and play a crucial role in the pathogenesis of salmonellosis.