O-antigenic determinants in *Salmonella* species of serogroup C, are expressed in distinct immunochemical populations of chains

Ndubisi A. Nnalue¹ and Alf A. Lindberg²

Abstract

The O-antigenic specificities found among salmonellae of serogroup C, are O:6,7, O:6,7, O:6,6,7 and O:6,7,14, as defined by classical serology. Factor O:7 is the group-wide determinant while factors O:6, O:6, and O:14 are found in some strains but not others. Strains of the O:6,7 specificity are subject to lysogenic conversion by phages 6, and 14 to the O:6,7 and O:6,7,14 specificities, respectively. To further delineate antigenic complexity and serological relationships among strains of this serogroup monoclonal antibodies (mAbs) were generated against the O:6,6,7 polysaccharide of *Salmonella* thompson. Five mAbs of either the O:6, or the O:6, specificities did not bind O:6,7,14 strains or LPS, showing that the O:6 determinant in these strains is neither O:6, nor O:6,. Thus antigenic conversion of O:6,7 strains by phage 14 is accompanied by addition of O:14 as well as loss of O:6,. Three mAbs which demonstrated group-wide reactivity, and were thus specific for O:7, recognized clearly separable epitopes hereby defined as sub-specificities, O:7, O:7, and O:7,. Immunoblotting of mAbs against electrophoretically resolved LPS showed that factors O:6, and O:6, are expressed only in LPS molecules of high molecular mass whereas O:7, and O:7, are expressed only in relatively low-molecular-mass chains. These results are consistent with the expression of different antigenic determinants in structurally distinct subpopulations of O chains. The implication of the existence of distinct subpopulation of chains is that the published structure of the O:6,7 repeat unit is not fully representative of the O-antigenic structure of this group.

Keywords: *Salmonella* LPS, LPS epitopes, O-antigenic complexity, O-antigen populations, antigenic conversion

INTRODUCTION

Lipopolysaccharides (LPSs) are important virulence determinants and major constituents of the outer membrane of Gram-negative bacteria. They play a major role in the interaction of the bacterial cell with its environment: during infection, for example, they protect the organism from destruction by host defence systems and mediate various pathophysiological processes. LPSs from enteric bacteria share a common architecture comprised of three distinct domains: lipid A, the core oligosaccharide (core-OS) and the O-antigenic polysaccharide (O-PS). Lipid A is anchored in the outer membrane and is invested with biological properties such as pyrogenicity and endotoxicity while the outer polysaccharide domains, core-OS and O-PS, modulate surface properties and much of the interaction of the organism with the external milieu. The binding of LPS to macrophages causes the release of mediators which trigger the generalized inflammatory response associated with Gram-negative sepsis (Beutler & Cerami, 1988). The O-PS domain activates the alternative complement pathway leading to bacterial phagocytosis and subsequent intracellular digestion (Liang-Takasaki et al., 1983). Furthermore, several reports suggest that the outer polysaccharide domains of the LPS participate in the invasion of cell lines and in the penetration of the gastrointestinal mucosa by *Salmonella* species (Finlay et
The chemical and structural heterogeneity of LPSs from *Salmonella* species and other enteric organisms resides mainly in the O-PS domain, which comprises repeat units of one to seven sugars (Lindberg & LeMinor, 1984; Orskov et al., 1977). Antigenic determinants present in the O-PS chains have been usefully exploited for seroclassification and serodiagnosis (Kauffman, 1966). These determinants were originally characterized by classical serology using cross-absorbed antisera. Using monoclonal antibodies (mAbs), Elkins & Metcalf (1984) demonstrated seven epitopes in the LPS from *S. typhimurium* strain TML (serogroup B) whereas only three determinants (factors 1, 4 and 12) were originally identified in this strain by classical serology. Although carbohydrate epitopes can be as small as a monosaccharide (Kalisiak et al., 1991; Nnalue et al., 1994), previous studies of rabbit antibodies (Jörbeck et al., 1979) or murine mAbs (Lind, 1992) which recognize determinants in *Salmonella* serogroup B O-PS show that their epitopes comprise at least a trisaccharide. Therefore the presence of these many epitopes in a supposedly homogeneous polysaccharide comprised of penta-saccharide repeat units is unexpected and it remains to be examined whether this degree of antigenic complexity is a universal feature of bacterial LPS.

We therefore considered it of interest to investigate the complexity and expression of antigenic determinants in another *Salmonella* serogroup. We chose serogroup C1 O-antigen 6,7 PS because the structural basis for its serological properties is the least well understood among O-antigens from the major pathogenic serogroups. According to the Kauffman–White table four antigenic determinants, O:6, O:6, O:7 and O:14, are found in the O-PSs of serogroup C1 strains (now comprised of the former serogroups C1 and C2), of which the O:6 determinant undergoes form variation (Edwards, 1945; Escobar & Edwards, 1968; LeMinor & Rhode, 1989; LeMinor & Popoff, 1988). Members with the O:6 determinant are subject to lysogenic conversion with changes in serospecificity (Fuller & Staub, 1968; LeMinor, 1965, 1968) as well as in virulence (Nnalue et al., 1990; Smith & Parsell, 1974). Because of the complexity of the O:6,7 antigen, only partial structures could be derived from earlier structural studies of O-PSs from serogroup C1 strains (Fuller & Staub, 1968; Lindberg & LeMinor, 1984). Following the availability of powerful modern methods Lindberg et al. (1988) re-investigated the O:6,7 PS of *Salmonella thompson* and established a more-complete structure for the biological repeat unit (Fig. 1). We report here the generation of mAbs against epitopes of O antigen 6,7 of *S. thompson* and their use for characterization of antigenic determinants in serogroup C1 salmonellae and their LPS.

**METHODS**

**Bacterial strains.** Bacterial strains were from the collection of the Division of Clinical Bacteriology, Huddinge Hospital. *S. choleraesuis* var. Kunzendorff strains SL2824 (O:6,7), SL2839 (O:6,7) and SL4388 (O:6,7,14) have been described previously (Nnalue & Lindberg, 1990; Nnalue et al., 1990). Other strains, of well-established serospecificities, were originally obtained from Professor L. LeMinor of the World Health Organization Collaborating Centre for *Salmonella*, Institut Pasteur, Paris, France. These were *S. paratyphi* C, strain IS33 (O:6,6,7); *S. choleraesuis* var. Kunzendorff, IS36 (O:6,7); *S. choleraesuis* var. Kunzendorff, IS37 (O:6,7); *S. bareilly*, IS44 (O:6,6,7); *S. miiawasima*, IS45 (O:6,6,7); *S. montevedeo*, IS46 (O:6,7); *S. newport*, IS50 (O:6,8); *S. bouismorbidicans*, IS53 (O:6,8); *S. catrau*, IS93K (O:6,14,24); *S. onderstepoort*, IS94 (O:6,14,25); *S. kentucky*, IS98 (O:8,20); and *S. muenchen*, IS222 (O:6,8). *S. choleraesuis* var. Kunzendorff strains SN58 (O:6,7) and SN61 (O:6,7,14) were derived from strain IS37 (O:6,7,7) by lysogenization with converting phages 6,7 and 14, respectively, as previously described (Nnalue et al., 1990).

**Antisera.** Agglutinating antisera specific for factors O:6 and O:6 were kindly provided by Professor L. Le Minor of the World Health Organization Collaborating Centre for *Salmonella* Research. Other antisera were purchased from Difco.

**Media.** The solid medium used for bacterial cultivation was Oxoid blood agar base (code CM35). L-broth (10 g tryptone, 5 g yeast extract, 10 g NaCl·1·) was used for batch cultivation...
Whole-cell lysates. Bacterial cells in growth were pelleted in microcentrifuge tubes and washed once in 1 ml PBS. The pellets were resuspended in 80 μl lysis buffer [1 M Tris pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 4% (v/v) 2-mercaptoethanol, 0001% (w/v) bromophenol blue] and heated at 100 °C for 20 min. This was followed by the addition of 20 μl proteinase K solution (2.5 mg ml⁻¹ in lysis buffer) to each boiled lysate and further incubation at 60 °C for 2 h. Aliquots (5–10 μl) were then subjected to SDS-PAGE.

Cell culture methods. The rat myeloma Y3.AG.1.2.3 (Y3M) (Galfre et al., 1979) used as fusion partner was maintained in RPMI 1640 (Gibco) supplemented with 10% (v/v) heat-inactivated foetal bovine serum (Flow Laboratories), 1 mM sodium pyruvate, 4 mM glutamine, and 100 U of a penicillin-streptomycin mixture (Gibco). The same medium supplemented with hypoxanthine/aminopterin/thymidine (HAT) was used for hybridoma selection after fusion.

Antigen, rats and immunization. LOU/C rats were purchased from ALAB (Stockholm, Sweden) and immunized as previously described (Nnalue et al., 1991) using heat-killed, homologous-LPS-coated S. thompson bacteria as antigen (Luk et al., 1990).

Production of hybridomas. The procedure for cell fusion between spleen cells from immunized rats and Y3M cells has been described (Nnalue et al., 1991). Putative hybrids were tested by enzyme immunoassay (ELISA) for reactivity with S. thompson LPS. Positive clones were subjected to two cycles of subcloning by limiting dilution. Culture supernatants from these clones containing mAbs were collected and used for isotyping by a commercial kit, RMT 01K (Serotec); they were then further characterized and used for immunochromatographic analysis of LPS.

Table 1. Reactivity of rat mAbs with LPSs and glycoconjugates

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Serogroup</th>
<th>Isotype...</th>
<th>ELISA reactivity (A405) of 1:100 dilution of culture supernatants from mAb clones</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MR9 IgM</td>
<td>MR11 IgG2b</td>
</tr>
<tr>
<td>S. thompson, IS40 (O:6,7)</td>
<td>C₁</td>
<td>0.4</td>
<td>1.4</td>
</tr>
<tr>
<td>S. choleraesuis, SL2824 (O:6,7)</td>
<td>C₁</td>
<td>0.9</td>
<td>1.6</td>
</tr>
<tr>
<td>S. choleraesuis, SL2839 (O:6,7)</td>
<td>C₁</td>
<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
<td>S. choleraesuis, SL4388 (O:6,7,14)</td>
<td>C₁</td>
<td>0.4</td>
<td>0</td>
</tr>
<tr>
<td>S. choleraesuis, IS37 (O:6,7)</td>
<td>C₁</td>
<td>0.6</td>
<td>2.1</td>
</tr>
<tr>
<td>S. choleraesuis, SN58 (O:6,7)</td>
<td>C₁</td>
<td>0.3</td>
<td>0.6</td>
</tr>
<tr>
<td>S. choleraesuis, SN61 (O:6,7,14)</td>
<td>C₁</td>
<td>0.8</td>
<td>0</td>
</tr>
<tr>
<td>S. newport, IS50 (O:6,8)</td>
<td>O:8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>S. kentucky, IS98 (O:8,20)</td>
<td>O:8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IS40 decasaccharide-BSA (CO-BSA) (O:7)</td>
<td>–</td>
<td>0.8</td>
<td>0</td>
</tr>
<tr>
<td>S. newington (O:3,15)</td>
<td>E</td>
<td>0.7</td>
<td>0</td>
</tr>
<tr>
<td>S. typhimurium, SL3201 (O:4,5,12)*</td>
<td>B</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Candida species mannan</td>
<td>–</td>
<td>1.6</td>
<td>0</td>
</tr>
</tbody>
</table>

* LPSs from Salmonella strains of serogroups A and D₁ were similarly non-reactive.
† Formerly classified under serogroup C₄, which is no longer recorded in the Kauffman–White table (LeMinor & Popoff, 1988; LeMinor & Rhode, 1989).
‡ Strains of the O:6,8 and O:8,20 specificities formerly belonged to serogroups C₂ and C₃, respectively. These groups are now lumped together under serogroup O:8 (LeMinor & Popoff, 1988; LeMinor & Rhode, 1989).

LPSs and glycoconjugate. Bacterial strains were streaked out on CM55 plates from cultures kept at −80 °C and incubated overnight at 37 °C. Individual colonies were tested for expression of the appropriate O-antigenic determinants by agglutination in O-factor-specific antisera. Several colonies which showed satisfactory agglutination were pooled and grown with shaking to mid-exponential phase in 100 ml L-broth at 37 °C; this culture was then used to seed a 10 l fermenter in which growth was continued to late exponential phase under aeration at a constant pH of 7. Formaldehyde was added to a final concentration of 1% (w/v) and the culture stored overnight at 4 °C to kill the bacteria. The bacteria were harvested by centrifugation (9000 g, 30 min) at 4 °C and washed once with PBS; LPS was then extracted from the pellet by the hot phenol/water method (Westphal & Jann, 1965). The resulting crude LPS preparations were extensively treated with DNase, RNase and proteinase K to eliminate proteins and reduce nucleic acid contamination to less than 5%; this was followed by lyophilization (Nnalue et al., 1992). The glycoconjugate CO-BSA was prepared by hydrolysis of the O:6,7 PS of S. thompson with phage 14 and then chemically coupling the resulting decasaccharide to BSA (Ekwall et al., 1982).
**ELISA.** LPS ELISA was done as previously described in detail (Carlin & Lindberg, 1983). Briefly, a 96-well microtitre plate (Nunc) was coated overnight at 20 °C with LPS (10 μg ml⁻¹) in 0.05 M sodium carbonate buffer (pH 9.6), overcoated with 1% (w/v) BSA for 1 h at 37 °C and washed three times with washing buffer [0.15 M NaCl, 0.05% (v/v) Tween 20]. Hybridoma culture supernatant containing mAb was diluted in PBS containing 0.05% Tween 20; 100 μl aliquots were added to wells followed by a 4 h incubation at room temperature and then washing as described above. Alkaline-phosphatase-labelled rabbit antibody to rat immunoglobulin was diluted as recommended by the manufacturer (Sigma) and 100 μl was added to each well. The plate was incubated overnight at room temperature and washed again. Substrate solution (100 μl p-nitrophenyl phosphate in 1 M diethanolamine/0.5 mM MgCl₂ buffer, pH 9.8) was added and the plate incubated for 100 min at 37 °C, then the A₄₀₅ was read in a Titertek Multiscan photometer (Flow Laboratories).

**SDS-PAGE and Immunoblotting.** Samples of LPS (7.5 μg) were resolved in 18% SDS-polyacrylamide-urea gels and visualized by silver staining (Tsai & Frasch, 1982). For immunoblot analysis the resolved LPSs were electrophoretically transferred (60 mA for 1 h, 30 mA for 14 h, then 60 mA for 1 h) to nitrocellulose membranes and tested for reactivity with mAbs as described before (Nnalue et al., 1992), using peroxidase-labelled rabbit antibodies to rat immunoglobulin as conjugate (Dakopatts) and 3,3’-diaminobenzidine tetrahydrochloride (Sigma) as substrate. Immunoblotting of mAbs against whole cells (dot blotting) was tested by a similar procedure after deposition of about 10⁷ heat-killed (70 °C, 1 h) bacteria per dot on the nitrocellulose membrane using a dot-filtration manifold (Bio-Rad). The bacterial cultures used for the preparation of heat-killed suspensions were each derived from a pool of three single colonies verified by slide agglutination to express the appropriate O antigenic factors.

**RESULTS**

**Generation of mAbs and characterization of their antigenic specificities by ELISA**

Hybridomas secreting mAbs were generated by fusion of spleen cells from two immunized rats with myeloma Y3M cells. Seven mAbs reactive with *S. thompson* LPS were cloned, isoytyped and tested for reactivity with a set of antigens comprised of LPS from the five major *Salmonella* serogroups (A–E) and mannan from *Candida* species (Table 1). One of the mAbs, MR9, which cross-reacted with mannan as well as with *Salmonella* of serogroup E LPS (O:3,10 as well as O:3,15) has been characterized in detail (Nnalue et al., 1991, 1994). The other mAbs did not react with mannan or with LPS from other serogroups. They were titrated against another set of antigens comprising LPS from *Salmonella* strains of well characterized O-antigenic specificities and a glycoconjugate specific for O factor 7 (Ekwall et al., 1982); this glycoconjugate is hereinafter referred to as CO-BSA. The results (Table 1, Fig. 2) enabled a classification of the set of seven mAbs into four distinct specificities. One mAb, MR11, is O:6₂⁻ specific because it reacted with all strains known to have this determinant and failed to react with all others. For example, culture supernatants containing this mAb reacted with *S. choleraesuis* strain IS37 LPS (O:6,7) over a wide range of dilutions but not LPS from SN58 (O:6,17) and SN61 (O:6,7,14), even at high concentrations (Fig. 2). The latter strains were both derived from IS37, by lysogenization with the converting phages 6₁ and 14, respectively. Similarly four mAbs (MR13,
MR14, MR15 and MR16) were determined to be O:6, specific on the basis of their patterns of reactivity with these LPSs; they bound LPS from SN58 (O:6,7) but not those of other specificities. All of these mAbs were further tested against a second set of LPSs from strain SL2824 (O:6,7), its phage 14 lysogenic derivative SL4388 (O:6,7,14) and an unrelated wild-type strain, SL2839 (O:6,7) with results identical to that above. The two other specificities found were those of mAbs MR9 and MR17, which reacted with all LPSs from serogroup C, strains tested and therefore recognize group-specific determinants. These two mAbs recognize clearly separable epitopes because MR9 reacted with three antigens (mannan, serogroup E LPS and CO-BSA) not recognized by MR17 (Table 1, Fig. 2).

Binding of mAbs to whole bacterial cells

The binding specificities of the mAbs were further investigated by dot immunoblotting against a collection of heat-killed bacterial strains belonging to serogroups C, O:8 and H. The results (Fig. 3) were fully consistent with the ELISA data and showed that MR11 bound all strains known to have O:6 either alone (IS37 and SL2824) or in addition to O:6, (IS33, IS49, IS44, IS45). It did not bind any of the strains which have O:6, alone.
Fig. 4. Characterization of LPS: (a) SDS-PAGE and silver staining of pwLPS; (b) comparison of pwLPS and wcLPS by SDS-PAGE and silver staining; (c, d) SDS-PAGE and immunoblotting against mAbs.

(IS36, SN58, SL2839 and SL2840) or were lysogenic for phage 14 (SN61 and SL4388). The four mAbs MR13, MR14, MR15 and MR16 (data for MR14 and MR15 not shown) similarly bound only strains with O:61 but not those which lacked this factor. The non-reactive strains included those with O:62 only (IS37 and SL2824) or which were lysogenic for phage 14 and thus serologically designated O:6,7,14 (SN61 and SL4388). As was observed in ELISA, MR9 and MR17 also bound all serogroup C1 strains in dot blots. However, none of the mAbs reacted with serogroup O:8 strains of either the O:6,8 (IS50, IS53, IS222) or the O:8,20 (IS98) specificities. The strains of serogroup H tested, *S. carrau*

strain IS93k (O:6,14,24) and *S. onderstepoort* strain IS94 (O:6,14,25) also failed to bind these mAbs with the exception of MR17, with which they reacted very weakly.

**Binding of mAbs to electrophoretically resolved LPS**

LPS samples from *S. choleraesuis* strains SL2839 (O:6,7), SL4388 (O:6,7,14) and SL2824 (O:6,y,7) as well as from *S. thompson* strain IS40 (O:6,y,7) were resolved by electrophoresis and stained with silver (Fig. 4a). As expected of smooth LPS, the samples showed ladder-like patterns composed of low- as well as high-
molecular-mass bands except that of SL2824, which was composed mainly of low-molecular-mass chains. This unusual pattern of bands in SL2824 LPS raised the question of whether high-molecular-mass LPS components from this strain were selectively lost during extraction by the phenol/water procedure. To examine whether LPSs prepared from bacterial strains by phenol/water extraction (pwLPS) were representative of whole-cell LPS, we compared the electrophoretic profile of each pwLPS with that of the proteinase-K-treated whole-cell lysate (wcLPS) of the same strain. The results (Fig. 4b) showed that wcLPS and pwLPS from strains SL2839, SL4388 and IS40 had identical patterns. With SL2824, the pattern of bands in pwLPS and wcLPS were almost but not quite identical. Their profiles were composed mainly of low-molecular-mass chains but differed somewhat in that wcLPS contained traces of high-molecular-mass chains absent from pwLPS. We have previously demonstrated that LPS prepared from SL2824 by a method involving selective degradation of other macromolecules and subsequent recovery of LPS by ultracentrifugation also displayed a pattern of bands characterized by short chains (Nalue et al., 1990). One further difference of note is the presence of a band of similar molecular mass (Fig. 4b, arrowheads) in wcLPS but not pwLPS from both SL2824 and IS40. The band was also seen in wcLPS but not pwLPS from SL2839 and SL4388 when larger amounts than presented in the figure were analysed (data not shown). This band is considered to be a macromolecule other than LPS. Therefore our data overall are consistent with the conclusion that pwLPS from all of these strains was quite similar to wcLPS and therefore suitable for use in analysis of antigenic determinants by immunoblotting.

In initial immunoblot analyses we examined the binding of our mAbs to three of the above LPSs (O:6,7; O:6,7, and O:6,7,14). The mAbs bound only to LPS of the appropriate specificity as previously established by ELISA and dot blotting. However, the patterns of binding were peculiar. The two type-specific mAbs, MR11 and MR16, recognized only bands of high molecular mass while the group-specific mAbs, MR9 and MR17, recognized only bands of relatively low molecular mass (Fig. 4c). Electrophoretic and immunoblot analyses of LPS samples from a different set of strains comprised of S. cholerasuis strain IS37 (O:6,7) and its phage-lysogenized derivatives SN38 (O:6,7) and SN61 (O:6,7,14) yielded identical results (data not shown). LPS from S. thompson IS40 (O:6,6,7,7), the strain used for generation of the mAbs, was subsequently analysed. The results (Fig. 4d) were consistent with those obtained with other group C LPSs, showing the dichotomous size distribution of bands recognized by MR11 and MR16 on the one hand and MR9 and MR17 on the other. One slight difference was the fact that in two batches of IS40 LPS the epitope of MR16 was poorly expressed and that of MR11 barely detectable while those of MR9 and MR17 were characterized by heavy expression. This peculiarity notwithstanding, our data overall would lead to the conclusion that in LPS from serogroup C strains, epitopes associated with factors O:6, and O:6, are expressed only in high-molecular-mass LPS while those associated with factor O:7 reside only in relatively low-molecular-mass chains.

One more observation from our analyses of LPS with mAbs is that while high-molecular-mass chains were not demonstrated by silver staining of SL2824 (O:6,7) LPS (Fig. 4a), they were clearly revealed by subsequent immunoblotting with mAb MR11 (Fig. 4c). A similar observation was also made with LPS (O:6,7) from IS37 (data not shown). These data would agree with previous findings that immunoblotting with a mAb was more sensitive than silver staining for detection of LPS (Di-Padova et al., 1994). On the other hand, it is quite possible that certain LPS moieties may not be readily amenable to silver staining.

DISCUSSION

The results of this study show that O antigen polysaccharides from salmonellae of serogroup C, are antigenically more complex than previously realized. Our mAb analyses would agree with classical serology that the O-antigenic determinants O:6, O:6, and O:7 are distinct and unrelated. However, while the O:6, and O:6, determinants appear to represent single epitopes, the O:7 determinant comprises at least three distinct specificities. There are two pieces of evidence for the presence of more than one epitope in O:7. One is the fact that two mAbs, MR9 and MR17, which showed group-wide reactivity, and were thus O:7 specific, had non-identical specificities. The other derives from the properties of a previously described murine mAb, MACO-3, which showed O:7 specificity. MACO-3, unlike MR17, reacted with CO-BSA and, unlike MR9, did not react with mannan or serogroup E LPS (Luk & Lindberg, 1991): it thus defines a third O:7 specificity. It is therefore reasonable to expect that O:7-specific sera generated by immunization and cross-absorption will comprise a complex of the specificities of MR9, MACO-3 and MR17 in varying proportions depending on the strains used. These three epitopes are hereby designated subspecificities of factor 7:O:7 (MACO-3), O:7 (MR9) and O:7 (MR17).

The failure of our O:6,- and O:6,-specific mAbs to react with the two group H strains tested would agree with previous observations that the O:6 antigen of this group is distinct from O:6, and O:6, of serogroup C (Lindberg & LeMinor, 1984). However, the fact that the mAbs also failed to react with the three O:6,8 strains tested (S. newport, S. muenchen and S. bovismorbificans) was surprising because it is well accepted that the O:6 antigen of group O:8 is the same as O:6 (LeMinor & Rhode, 1989; Lindberg & LeMinor, 1984). Our mAb analysis therefore shows that the determinant designated O:6, in serogroup C, is distinct from that given the same name in serogroup O:8. The implication is that the cross-agglutination which occurs between the two groups must be due to as yet undefined determinants. We also found that after
lysogenization of O:6,7 Salmonella strains by phage 14, the resulting strains of the O:6,7,14 specificity failed to react with O:6-specific mAbs. The loss of the O:6, determinant following lysogenic conversion by phage 14 was not previously recognized. Since, at the structural level, lysogenic conversion by phage 14 involves a change of the glycosylation site from α-D-mannopyranosyl III to α-D-mannopyranosyl IV (Fig. 1), it seems reasonable to expect that the serological consequences of conversion would involve more than the simple addition of a new factor, O:14. Furthermore, the fact that none of four O:6,-specific mAbs reacted with whole cells from phage 14 lysogens or their O:6,14 LPS means that their 'O:6' determinant is neither O:6, nor O:6,. It is possible that the antigenic relationship between O:6,7,14 strains and other members of serogroup C, is limited to O:7. On the other hand there may be other, as yet unidentified, determinants in LPSs from serogroup C, strains.

Much confusion currently surrounds the structural basis for the O:6, and O:6,- determinants. Some earlier studies (Fuller et al., 1968; Lindberg & LeMinor, 1984) presented evidence that glycosylation of either D-mannose II or III (Fig. 1) was the likely structural background for the O:6,- determinant. More recently Lindberg et al. (1988) reported that the O:6,6,7 PS of S. typhimurium strain IS40 comprises two populations of chains which differ only because one population lacks the α-D-glucopyranosyl group 3-linked to the α-D-mannopyranosyl III residue of each repeat unit. As the non-glycosylated, phage-14-endomannosidase-susceptible population has been shown to display only the O:7 specificity (Ekwall et al., 1982), it follows that chains substituted with α-glucose at the α-D-mannopyranosyl III residue have both the O:6, and O:6,- determinants. Taken together, these previous studies would tend to suggest that the same structural element is responsible for both the O:6, and O:6,- determinants. Our immunochromal analysis shows that the O:6,- epitope recognized by mAb MR11 was poorly expressed in two batches of LPS extracted from S. typhimurium strain IS40 even though the strain agglutinated well in cross-absorbed O:6,-specific rabbit antiserum. This further highlights the complexity of O antigenic determination in this serogroup.

The current concept of antigenic determinants in O-PS chains from Enterobacteriaceae strains is that they comprise distinct structural motifs which are present in each and every O repeat unit. It is, however, clear from our data that there is a size dichotomy between chains which express the group-wide determinants, O:7, and O:7, and those which express the strain-specific determinants, O:6, and O:6,-. Our data further indicate that chains which express O:7, are distinct from those which express O:7, because O:7, but not O:7, is present in the O:7-specific dodecasaccharide (part of CO-BSA) derived from S. typhimurium. Although MACO-3 was not tested in this study it is clear that its epitope (O:7,) could not lie in the same chain as O:7, again for the reason that MACO-3 reacts with CO-BSA. The evidence for the expression of O:6, and O:6,- on different chains is the fact that most serogroup C, strains possess only one or the other of these determinants. Our results are therefore consistent with the expression of each of the antigenic determinants O:6, O:6,- O:7, and O:7, in a structurally distinct population of O chains. This deduction is supported by the similarity between our immunochromal observations and those of other workers who have reported the expression of more than one structurally distinct O-antigenic chain in other pathogenic species (Lam et al., 1989; Lightfoot & Lam, 1991; Rivera et al., 1988; Whitfield et al., 1991). Antigenic heterogeneity associated with the so-called non-essential modifications involving acetylation or glucosylation of specific residues in the O repeat units have been previously observed in Salmonella (Make A & Stocker, 1984). The glucosylation of D-galactose residues which determines factor O:12, in S. typhimurium is known to be incomplete and random (Weintraub et al., 1988) and to involve only chains longer than six repeat units (Helander et al., 1992). Slauch et al. (1995) have detected the O:5 determinant of S. typhimurium LPS only in chains of relatively high molecular mass, but suggested that this might have resulted from selective cleavage of O-acetyl groups (the O:5 determinant) from chains of low molecular mass during electrophoresis. In the above examples (even if the selective expression of O:5 in high-molecular-mass LPS is real rather than artifactual) there is an underlying assumption that all O repeat units and therefore all O chains are otherwise serologically identical: that is, that they share other antigenic determinants of the group unaffected by modification. The kind of heterogeneity we have observed in LPS from serogroup C, strains is different because each of at least four O determinants is found in a serologically distinct population of chains which apparently does not share known antigenic determinants with other populations of chains in the same strain. To our knowledge, therefore, this is the first description of the expression of Salmonella O determinants in independent populations.

The observed differential expression of epitopes in LPSs from serogroup C, strains has implications for the recently proposed structure for the biological repeat unit of serogroup C, O-PS. Since mAb MR9 (O:7,-specific) recognizes only terminal non-reducing β-mannosyl residues in LPS (Nnalue et al., 1994) it is readily deduced that high-molecular-mass LPS chains from serogroup C, strains do not have β-mannosyl residues at their terminal non-reducing ends as required by the published repeat unit structure (Fig. 1; Lindberg et al., 1988). In fact, the structure of the repeat unit was derived from analysis of oligosaccharides generated by hydrolysis with phage 14, whose endomannosidase cleaves only non-glycosylated (O:7,-specific) chains. It is therefore concluded on immunochromal grounds that the published structure is applicable only to chains expressing O:7, and not to those that express O:6, O:6,- and O:7,.

Carbohydrate antigens are particularly complex. The
nature of their epitopes and the factors that govern their recognition by antibody are still poorly understood. The proposal by Elkins & Metcalf (1984) for the presence of conformational determinants in LPS remains a formidable idea to explain their somewhat confounding finding that O-PS chains (serogroup B) comprised of repeat units of only a few sugar residues contained as many as seven epitopes. The mAbs we have described in this study, however, all seem to recognize structural elements. The evidence shows that antigenic complexity in serogroup C, O-PS seems to be contrived instead by the expression of different epitopes in structurally distinct LPS molecules. It is tempting to speculate that a facility to produce distinct antigenic populations of O chains would provide an organism with an effective mechanism to regulate its exact surface antigen composition and that this might serve a useful purpose during the course of infection. It would be of interest to examine the generality of these findings among other Salmonella serogroups as well as other enteric organisms.

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