Members of the family Enterobacteriaceae cause a wide variety of human and animal diseases, including gram-negative sepsis, food poisoning, and typhoid fever. The increasing incidence of these diseases has focussed attention on the need for vaccines that would cross-protect against the numerous serotypes of enteric bacteria. Lipopolysaccharide (LPS), an amphi-pathic moiety present on the bacterial surface (Fig. 1), is both a major virulence factor and an important target for protective immune responses. The large number of studies on the role of LPS in cross-protective immunity have mainly been done in Salmonella infection models due, in part at least, to the availability of mutants expressing nearly all possible forms of truncated LPS. These studies have shown that anti-LPS responses which develop during infection or by vaccination with smooth bacteria are highly protective but are directed mainly against the structurally hypervariable O-antigenic domain (Fig. 1) (19, 20).

Unlike the O antigen, the core region of LPS is highly conserved; the >2,000 serotypes of Salmonella share only two closely related core types (16, 17, 30). Based on this fact, rough bacterial strains, which expose core epitopes, have been extensively investigated as cross-protective immunogens. The results have, however, been inconsistent with some studies demonstrating protection against challenge with virulent smooth organisms (4, 21, 22, 28, 37), while others observed no protective effects (13, 23, 29, 31). An explanation for these dichotomous findings has not been possible due to a lack of knowledge about the identities of core determinants that elicit cross-reactive responses. Despite these contradictions in experimental findings, there is evidence that core antibodies protect in clinical settings, as determined in recent studies which show that high levels of natural anti-LPS core antibodies correlate with the more weakly cross-reactive α-Gal-1→6-α-Glc terminal in Rd, LPS represent branch points along the core oligosaccharide. Therefore, branch points in endotoxin core oligosaccharides may generally be cross-reactive.

All Accessible Epitopes in the Salmonella Lipopolysaccharide Core Are Associated with Branch Residues

NDUBISI ANTHONY NNALUE*
Department of Medical Microbiology, Faculty of Medicine and Health Sciences,
United Arab Emirates University, Al Ain, United Arab Emirates

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Antisera generated against each of the nine known chemotypes of Salmonella lipopolysaccharide (LPS) core were characterized in order to delineate cross-reactive epitopes and define the bases for their accessibility. Strongly cross-reactive epitopes were associated with three chemotypes: Ra and Rb, which recognized α-GlcNAc-1→2-α-Glc, and Rd, which recognized 1-α-D-heptose-1→7-1-α-D-heptose. Both these disaccharides and the more weakly cross-reactive α-Gal-1→6-α-Glc terminal in Rd, LPS represent branch points along the core oligosaccharide. Therefore, branch points in endotoxin core oligosaccharides may generally be cross-reactive.

* Present address: Tonna Bioservices and Consulting, 8813 Allman Rd., Lenexa, KS 66219. Phone: (913) 894-9510. Fax: (913) 894-9528. E-mail: nnalue@netscape.net.
Denmark) as the conjugate and o-phenylenediamine HCl as the substrate. For immunoblot analyses, samples of sLPS (7.5 μg) or rough LPS (2.5 μg) were resolved in denaturing sodium dodecyl sulfate (SDS)–15% polyacrylamide gels and transferred electrophoretically (120 mA, 12 h) to nitrocellulose membranes. They were then tested for reactivity with sera by using the same conjugates as in ELISA but diaminobenzidine-H2O2 (Sigma, St. Louis, Mo.) as a substrate system.

All nine anticore sera showed similarly strong reactivities with their respective homologous LPSs (ELISA end point titers 524,300 to 72,900) but differed greatly in their reactivities with other core chemotypes in immunoblots. Five sera (anti-Ra, anti-Rb4, anti-Rb3, anti-Rc, and anti-Rd1) demonstrated broad reactivity with rough LPS, strongly recognizing four or more different core chemotypes (Fig. 2 and 3). Two others, anti-Rb2 and anti-Rb1, had a somewhat narrower spectrum of recognition, reacting strongly with their homologous LPSs as well as the immediately adjacent chemotypes (Fig. 3). However, anti-Rb2 also demonstrated a weaker recognition for two additional chemotypes. A third group of sera, anti-Rd2 and anti-Re, reacted with their respective homologous chemotypes only. A comparison of the reactivity profiles of sera raised against the deep-core chemotypes Rd2 and Re and those raised against more distal core determinants (Ra, Rb1, Rb2, Rb3, Rc, and Rd1) suggested a dichotomy in the recognition of LPS by these sera. None of the anti-Ra, anti-Rb1, anti-Rb2, anti-Rb3, anti-Rc, or anti-Rd1 sera reacted with Rd2 and Re LPS, showing that they lacked antibodies directed against deep-core determinants. Likewise, anti-Rd2 and anti-Re were nonreactive with all of the more distal core chemotypes, Ra and Rd1. These findings suggest that deep-core epitopes are completely masked by distal sugar residues.

Blotted against sLPS of serogroup B, anti-Ra revealed a ladder-like pattern of bands consistent with ability to bind LPS molecules with long O-chains substitutions. The recognition of long-chained LPS molecules by anti-Rb2, anti-Rc, and anti-Rd1 could be due to antibodies directed at accessible core epitopes or to O-specific antibodies generated by such other mechanisms as polyclonal activation of B cells. To further delineate the applicable mechanisms, the sera were compared for reactivity in ELISA against sLPS of two different serospecificities and their corresponding O-specific glycoconjugates. The serogroup B-specific glycoconjugate, designated AM-PAA (O:4 specific), was prepared by copolymerization of a haptenic glycoside and acrylamide (5), while that of serogroup C1, CO-BSA (O:7 specific), comprised a dodecasaccharide from Salmonella O:6,7 polysaccharide (serogroup C1) covalently coupled to BSA (8). The results (Fig. 4) showed that while anti-Rc reacted well with both serogroup B LPS and AM-PAA, it was poorly reactive with both serogroup C1 LPS and CO-BSA. This pattern of reactivity is consistent with the presence in anti-Rc of group B O-specific antibodies and the lack of both group C1 O-specific and cross-reactive core-specific antibodies. It may, therefore, be deduced that anti-Rc reacted with sLPS only because it contained O-specific antibodies of serogroup B. Unlike anti-Rc, the sera anti-Ra, anti-Rb4, and anti-Rd1 reacted with group B as well as C1 LPS and failed to react with the glycoconjugates. These results are consistent with a lack of O-specific antibodies in these sera and thus reactivity with long-chained LPS molecules via recognition of core epitopes. The cross-reactivity of these three sera with sLPSs of different serospecificities was subsequently visualized directly by electrophoretic resolution and immunoblotting (Fig. 5). Unlike these anticore sera, anti-BO (generated against a smooth Salmonella strain of serogroup B) showed differential reactivities with sLPSs of different serospecificities (Fig. 5). It showed strong reactivity with sLPS molecules of serogroup B (O:4,5,12), weak reactivity with those of serogroup D (O:9,12), and a lack of reactivity with those of serogroups C1 (O:6,7) and C4 (O:6,7,14). These findings conform to expectation, since LPSs of serogroups B and D have O factor 12 but share no determinants with LPSs of serogroups C1 and C4.

The presence of serogroup B O-specific antibodies in an-
ti-Rc serum is not surprising since strain SL805, used for its generation, is a galE mutant derived from a smooth strain of Salmonella typhimurium. While galE mutants normally make rough LPS of the Rc chemotype, they are known to switch their LPS phenotype and make some sLPS in the presence of even trace amounts of galactose (10). This unique ability to switch between the rough and smooth phenotypes is the basis for both their reduced virulence and usefulness as live vaccines (27, 32).

It seems likely, therefore, that the cross-protective effects that have been observed with Rc strains have resulted from the immune response either to shared O-specific determinants or to other cross-protective antigens, such as outer membrane proteins (18).

The data strongly suggests that the cross-reactivity of anti-Rd1 with sLPS molecules was mediated by antibodies directed against the disaccharide L-\(\alpha\)-D-heptose-1→3-L-\(\alpha\)-D-heptose (residues VII to V in Fig. 1) of the inner core domain. The lack of cross-reactive antibodies in both anti-Rc and anti-Rd2 sera supports this deduction by showing that such antibodies are not generated once this disaccharide is lost or replaced by an additional sugar moiety. Moreover, the disaccharide has been shown to be the epitope of a broadly reactive monoclonal antibody (25). It is likewise deduced that the epitope recognized by anti-Rb4 lies in the terminal core disaccharide, \(\alpha\)-GlcNAc-1→2-\(\alpha\)-Glc, based on the facts that anti-Ra recognizes this disaccharide (24) and that Rb4 differs from Ra LPS only in lacking the \(\alpha\)-Gal1→6 (VIII) branch residue. The latter residue appears to contribute, though only slightly, to cross-reactivity, as indicated by the ability of anti-Rb4 to recognize sLPS molecules containing short O chains. Therefore, taken together, the results show that only three chemotypes, Ra, Rb2, and Rd1, elicit core-specific antibodies which bind LPS molecules with long O-chain substitutions.

It is of interest that the idea of using rough mutants as broadly cross-protective vaccines gained popularity with the report (4) that equine antisera raised against a Salmonella strain of the Ra chemotype protected mice against challenge with a virulent strain of Klebsiella pneumoniae. However, E. coli J5 (Rc) and Salmonella minnesota R595 (Re) have been used in most investigations of cross-protection by rough mutants in the belief that the immunodominant epitopes in these chemotypes are the most conserved among gram-negative organisms. Passive immunization with J5 was reported to protect experimental animals against the toxic effects of LPS (2, 6) as well as against lethal gram-negative bacteremia (37, 38). In a clinical trial, human antiserum raised against J5 was found to reduce mortality in patients with gram-negative bacteremia (36). Other studies have likewise reported that active or passive immunization with S. minnesota R595 protected against challenge with endotoxins or virulent bacteria in both experimental animals (3, 21, 35) and human volunteers (7). The failure of many other studies to demonstrate similar protective
effects after immunization with J5 or R595 (14, 23, 29, 31) led to a >20-year-old controversy. The results of the present systematic analysis of immunization by core chemotypes clearly show that Rc and Re strains do not elicit cross-reactive LPS-specific antibodies. Therefore, the reported protective effects of immunization with J5 or R595 could not have resulted from an adaptive immune response to LPS core determinants and must be attributed to other mechanisms.

Two lines of evidence show that cross-reactive epitopes reside only at branch points along the core oligosaccharide.

FIG. 3. Immunoblotting of sera against Salmonella LPS chemotypes resolved by SDS-polyacrylamide gel electrophoresis.

FIG. 4. Relative reactivities in ELISA of four anticore sera with sLPS and glycoconjugates of serogroups B and C1. The bars represent LPS from the immunizing strain (□), serogroup B-specific LPS (■), serogroup B-specific glycoconjugate (□□), serogroup C1-specific LPS (□□□), and serogroup C1-specific glycoconjugate (□□□).
Firstly, all four LPS chemotypes that elicited antibodies reactive with sLPS molecules have branch points at the terminal nonreducing ends of the chains. Secondly, none of the five chemotypes (Rb1, Rb2, Rc, Rd2, and Re) which terminate other than at branch points elicited cross-reactive antibodies. However, it appears that secondary factors, such as overall conformation of the core moiety, modulate the extent to which a particular branch residue is accessible, as exemplified by the fact that the Rb3 chemotype elicited cross-reactive antibodies which recognized only low-molecular-weight chains.

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