Meningococcal Lipopolysaccharides: Virulence Factor and Potential Vaccine Component

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INTRODUCTION

Neisseria meningitidis is a gram-negative bacterium that cause diseases such as meningitis and bacteremia, sometimes with septic shock. This human pathogen was first identified as the causative agent of bacterial meningitis by Weichselbaum in 1887 (19, 147). Today, Neisseria, Streptococcus, Staphylococcus, Haemophilus, and Pseudomonas species are recognized as an important cause of morbidity in developing areas of the world (60). It has been estimated that in the third-world countries more than 310,000 persons per year suffer from infections caused by N. meningitidis, resulting in 35,000 deaths (110). Larger numbers of cases and deaths per year are estimated for the encapsulated pathogens Haemophilus influenzae and Streptococcus pneumoniae (800,000 cases with 145,000 deaths and 100,000,000 cases with 10,000,000 deaths, respectively) (110). At the beginning of the 1980s, the World Health Organization initiated a program for the development of vaccines against encapsulated bacteria (Programme for Transdisease Vaccinology) (60). The World Health Organization program started with the development of vaccines for the prevention of endemic and epidemic meningococcal disease in infancy since vaccine development against S. pneumoniae, H. influenzae, Pseudomonas species, and group A and B streptococci was already receiving high priority by either U.S. governmental agencies (National Institute of Allergy and Infectious Diseases, U.S. Agency for International Development) or the pharmaceutical industry (110). At that time, the development of a Staphylococcus vaccine was not considered feasible within a reasonable period and therefore received a low priority (110).

The meningococcus has been the subject of numerous studies, and so there is a large body of knowledge about its epidemiology, pathogenesis, surface components, virulence factors, genome, and host defense mechanisms; the human immune response against this pathogen; host factors predisposing to meningococcal disease; and the identification of (potential) components for use as a vaccine (2, 34, 38, 42, 50, 58, 89, 103, 104, 121, 128). This review, however, will focus mainly on one class of surface components of N. meningitidis, i.e., the lipopolysaccharides (LPS), in relation to most of the above-mentioned subjects.

CLASSIFICATION SYSTEM AND EPIDEMIOLOGY

N. meningitidis is a diplococcus with a typical gram-negative cell envelope. Four different groups of (antigenic) surface structures can be distinguished on the meningococci: capsular polysaccharides (CPS), outer membrane proteins (OMP), LPS (located in the outer membrane), and surface appendages known as pili (30, 39). An extensive phenotypic classification system has been developed on the basis of the first three groups of surface components (Table 1) (52, 64, 78, 99, 105, 132, 136, 150): (i) serogroups, based on differences in the structure of the CPS; (ii) serotypes and subserotypes, based on differences in class 2/3 and class 1 OMP, respectively; and (iii) immunotypes, based on differences in the oligosaccharide structure of the meningococcal LPS (Fig. 1). For phenotyping of meningococcal strains, techniques such as whole-cell enzyme-linked immunosorbent assay (ELISA) in combination with specific monoclonal antibodies or sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) are being used (1, 78, 132).

Bacteria belonging to groups A, B, and C cause by far the largest number of cases (19, 99). Group A dominates in Africa during both epidemic and endemic periods, whereas groups B and C are the prevalent serogroups isolated during endemic periods and localized outbreaks of meningococcal disease in the western world (50, 99, 121). The (sub)sero-
TABLE 1. Phenotypic classification system of *N. meningitidis*

| System         | Basis          | No. of groups or types | Names               |
|----------------|----------------|------------------------|---------------------|
| Serogroups     | CPS            | 12                     | A, B, C, X, Y, Z, 29E, W-135, H, I, K, L<sup>a</sup> |
| Serotypes      | Class 2/3 OMP  | 20                     | 1, 2a, 2b, 2c, ..., 21<sup>c</sup> |
| Subserotypes   | Class 1 OMP    | 10                     | P1.1, P1.2, ..., P1.16<sup>c</sup> |
| Immunotypes    | LPS            | 12                     | L1-L12              |

<sup>a</sup> The complete classification of a meningococcal strain is, for example: B:15:P1.16:L3 (serogroup B, serotype 15, subserotype P1.16, immunotype L3). A strain can express more than one serotype or immunotype-specific epitope, e.g., P1.7,16 or L3,7,9.

<sup>b</sup> The structures of the repeating units of the most prevalent serogroups are as follows: A, [n(1→6)ManNac-P]; B, [n(2→8)NeuNAc]; C, [n(2→9)NeuNAc].

<sup>c</sup> Group A and C CPS are O acetylated at the C-3 of ManNac and the C-7 and C-8 of NeuNAc, respectively. See reference 42 for the structures of most of the other serotypes.

<sup>d</sup> Not all the numbers between 1 and 21 or P1.1 and P1.16 are used.

Types of meningococcal strains associated with disease vary from one geographical region to another. Also, (sub)serotypes may change over a period of years (50, 125). The number of studies concerning the distribution of the meningococcal immunotypes is limited (78, 105, 115, 150, 151). Immunotypes L8, L9, L10, and L11 are found within group A meningococci; of these, L10 and L11 are prevalent and are uniquely associated with this serogroup (77, 115, 150).

\[
R_i(1\rightarrow4){A\alpha-D-Glc(1\rightarrow4)-L\alpha-D-Hepp(1\rightarrow5)-KDO}
\]

L1: \(R_1 = \alpha-D-Galp(1\rightarrow4)-\beta-D-Galp, A = \cdot, R_2 = PEA \text{ and } R_3 = H, \) partially O-acetylated.

L2: Three oligosaccharides were isolated, which differ in the amount or location of the PEA groups. \(R_1 = \beta-D-Galp(1\rightarrow4)-\beta-D-GlcNac(1\rightarrow3)-\beta-D-Galp, A = \cdot, R_2 = \alpha-D-Glc \text{ and } R_3 = PEA(1\rightarrow6) \) or \(R_3 = PEA(1\rightarrow7) \) (major oligosaccharides) or \(R_3 = H \) (minor oligosaccharide). GlcNacP(1→2) is partially O-acetylated.

L3: \(R_1 = \beta-D-Galp(1\rightarrow4)-\beta-D-GlcNacP(1\rightarrow3)-\beta-D-Galp, A = \cdot, R_3 = PEA \text{ and } R_3 = H.\)

L5: \(R_1 = \beta-D-Galp(1\rightarrow4)-\beta-D-GlcNacP(1\rightarrow3)-\beta-D-Galp, A = \beta-D-Glc(1\rightarrow4), R_3 = \alpha-D-Glc, R_3 = H, \alpha-D-GlcNac \text{ is for } 40\% \text{ O-acetylated.} \) Two smaller oligosaccharides were also isolated which differed with respect to the \(R_1 \) structure and O-acetylation of \(\alpha-D-GlcNacP: 1. R_1 = \beta-D-Galp(1\rightarrow4), A = \beta-D-Glc(1\rightarrow4), 50\% \text{ O-acetylated, } 2. R_3 = \cdot, A = \beta-D-Glc(1\rightarrow4), \) and 30\% O-acetylated.

L6: \(R_1 = \beta-D-GlcNacP(1\rightarrow3)-\beta-D-Galp(1\rightarrow4), A = \cdot, R_3 = H, R_3 = PEA(1\rightarrow7), \) partially O-acetylated.

L7: The same as L3. Immunochimical L3 = L7.

L8: Structure not elucidated, immunochemical behavior is very similar to L1.

L9: Basic structure the same as L3 and L7. Position of PEA group not elucidated.

L4 and L10-L12 Structures not elucidated.

FIG. 1. Primary structures of isolated meningococcal oligosaccharides of various immunotypes. Oligosaccharides were isolated by treatment with 1% acetic acid at 100°C for 2 h. This treatment resulted in the removal of sialic acid from structures with a terminal lacto-N-neotetraose unit (86). O acetylation of the various sugar residues will probably also be a source of microheterogeneity in immunotypes other than L1, L2, L5, and L6. The presented structures are based on the elegant studies of Jennings and coworkers (32, 41, 53, 69, 70, 91, 137). Abbreviations: Galp, galactose; GlcNacP, N-acetylglucosamine; Glcp, glucose; Hepp, heptose; L-glycero-d-manno-heptopyranoside. Adapted from reference 137 with permission.
Immunotypes L1 through L9 are identified within groups B and C meningococci. Studies by Zollinger and Mandrell (150) and Poolman et al. (105) suggest that immunotype L3,7,9 is the most prevalent within these serogroups, followed by immunotypes L2 and L1.8.

Meningococci have also been subdivided by clonal analysis on the basis of the variation in electrophoretic behavior of cytoplasmic isoenzymes (22-24, 42, 121). The technique is used to measure the genetic distance of meningococcal strains and demonstrate differences between meningococcal strains during an epidemic, which are not detected by phenotypic analysis. Large group A pandemics are generally caused by one clone (42).

**PATHOGENESIS AND ENDOTOXIN**

The sole natural habitat and reservoir for *N. meningitidis* is the human upper respiratory mucosal surface, primarily the nasopharynx (6, 99). Meningococci are transmitted by large respiratory droplets or direct contact with respiratory secretions (121). Carriage of the meningococcus in general does not lead to disease. During nonepidemic periods, when disease is rare, 5 to 30% of the adult population are colonized by the meningococcus (39, 99). This suggests that, like so many other bacterial infections, host rather than bacterial factors determine the outcome.

To cause disease, the meningococcus must first breach the mucosal barrier. In a recent review article, Stephens and Farley described a model for meningococcal invasion of the nasopharynx based on studies with human nasopharyngeal organ cultures (128). The meningococcus penetrates the mucus barrier, attaches to the nonciliated epithelial cells, and enters these cells by parasite-directed endocytosis (6, 126). Large numbers of meningococci are subsequently found in the phagocytic vacuoles of these cells. After 24 h of infection, the meningococcus reaches the subepithelium of the nasopharyngeal organ cultures adjacent to the lymphoid follicles. In principle, it could interact with the cellular components of the human immune system, but this has not yet been observed, probably because of the absence of specific antibodies (83). During these events, the meningococcus exerts its influence on the epithelial cells in the mucosa. It causes cytotoxicity, resulting in breakdown of tight junctions of epithelial cells, sloughing of ciliated cells, loss of ciliary activity, and alteration of microvilli of nonciliated cells (128).

Meningococcal pili are the major components that determine the attachment to nonciliated cells; piliated encapsulated meningococci are more capable of damaging the ciliated cells than are nonpiliated meningococci (127). Other surface proteins (e.g., class 5 OMps) may play a role in the adhesion process. Meningococci affect the ciliated cells from a distance, suggesting the release of soluble toxins by the bacteria or the induction of cytotoxic host factors by these components. The presence of both CPS and pili does not seem to be essential for this process, and the ciliary function of nasopharyngeal organ cultures is not influenced by purified LPS. These studies, however, do not exclude a role for meningococcal endotoxin in the invasion of the human upper respiratory mucosal surfaces (128).

When the meningococcus reaches the subepithelium, it is possible for it to gain access to the blood vessels of this mucosa, which can lead to the development of disease. The exact factors governing this access are not known, but it certainly will depend on the "state of readiness" of the immune system of the infected host (presence of specific antibodies, intact complement system, etc.; see the section on host defense and predisposing factors).

The clinical picture of systemic meningococcal disease can range from fulminant sepsisemia with septic shock, multiple-organ failure, and death (this can happen within 6 h after the first symptoms) to self-limiting benign meningococcal disease, but its most common presentation is meningitis (38). Little is known about the underlying pathophysiological mechanisms leading to the different syndromes, but a key role for endotoxin (LPS) in the induction of septic shock is beyond doubt (93, 94). In a recent study, Brandtzaeg et al. (17) demonstrated a relation between the levels of endotoxin present in patients suffering from systemic meningococcal disease and their chance of survival. Meningococcal LPS induces a cytokine cascade (tumor necrosis factor alpha, interleukins 1 and 6) (144, 145), activates the complement system (18), and enhances the plasminogen activator inhibitor levels (44). However, the precise mechanisms leading to induction of septic shock still remain to be elucidated.

**VIRULENCE FACTORS**

The meningococcus can evade the host defense mechanisms. The bacterium contains surface components and secretes molecules and complex vesicle structures (blebs) that modulate or deflect the immune system. It can undergo phase and antigenic variation and is able to use host factors for its own protection and growth. A summary of the most important factors and mechanisms is given in Table 2.

Recently, renewed interest has been drawn to the LPS as an important virulence factor. These molecules probably not only play a role in breaching the mucosal barrier but also are target molecules for sialylation by enzymes (sialyltransferases) (86, 87, 96, 98, 131). For example, gonococcal strains appear to be sialylated in vivo and sialylation of gonococcal LPS results in an increased resistance against lysis (7, 87, 98). Studies by Schneider et al. (119) suggested that the presence of high-molecular-weight LPS molecules (probably containing sialic acid residues) is associated with the development of gonococcal leukorrhoea. Furthermore, terminal sialic acids can down-regulate the alternative-pathway activation, shield underlying protective epitopes, or change the immunogenicity of antigenic determinants (73, 117).

Both gonococci and meningococci contain endogenous sialyltransferases, but only meningococci synthesize the necessary donor molecule cytidine-5'-monophospho-N-acetylenuraminic acid (CMP-NANA) (12, 86, 87). The target of these endogenous sialyltransferases is the terminal lacto-N-neotetrose unit (86, 87), which is found in LPS of *N. meningitidis, Neisseria gonorrhoeae*, and *Neisseria lactamica* (75, 77, 86, 87, 131). This target structure is present on meningococcal immunotypes L2, L3, L5, L7, and L9 LPS (Fig. 1). Mandrell et al. (86) and Tsai and Civin (131) demonstrated that L2, L3, L4, L5, L7, L8, L9, and L10 LPS are sialylated during growth in vitro without the use of exogenous CMP-NANA. It is possible to modulate the amount of sialic acid incorporated by adding exogenous CMP-NANA or using different growth media (86, 131). Fox et al. (48) showed no effect of exogenous CMP-NANA on the serum resistance of groups A, B, Y, and Z and nonencapsulated meningococci. It was, however, not clear whether these results were due to the sialylated sialylated LPS; the immunotypes of the strains and the specificity of the bacterial antibodies in the antisera used were not described (48). Whether meningococcal LPS is sialylated in
vivo and whether it plays a role in pathogenesis are currently not known and are the subjects of further research (86).

HOST DEFENSE AND PREDISPOSING FACTORS

The first and most important line of defense against infection with *N. meningitidis* and other pathogens is the integrity of the mucosal membrane. All factors that diminish the quality of the mucosal barrier can contribute to an enhanced infection rate. The increased incidence at the end of the dry season in Africa and the annual increase in the winter and early spring in countries with a temperate climate have been attributed to the dryness of the air, which probably influences the integrity of the mucosal membranes of the nasopharynx (33, 99). Furthermore, acute respiratory disease and viral infections such as influenza have been suggested as predisposing factors for meningococcal disease (26, 43, 92).

Specific antibodies and the complement system play a key role in host defense against *N. meningitidis*. They can cause lysis of the bacteria, enhance phagocytosis by monocytes or polymorphonuclear leukocytes, or neutralize the effects mediated by endotoxin (31, 36, 57, 61, 65, 67, 83, 88, 112–114). The presence of bactericidal antibody, however, is of the utmost importance (57). Anti-CPS antibodies and anti-OMP antibodies are in general bactericidal and facilitate phagocytosis (57, 65, 67, 88, 112–114). The role of anti-meningococcal LPS antibodies remains unclear. In the literature, conflicting results concerning the bactericidal activity of anti-LPS antibodies in polyclonal antisera have been reported, whereas endotoxin neutralization and opsonophagocytic capacity have not been evaluated (36, 61, 113). Bactericidal epitopes are present on meningococcal LPS, because monoclonal antibodies capable of lysing meningococci have been developed (116).

The serogroups differ in their susceptibility to serum bactericidal activity or phagocytosis (114). Group B meningococci are relatively resistant to serum bactericidal activity and more susceptible to killing by human neutrophils. In contrast, group Y meningococci are relatively susceptible to the bactericidal activity of serum and resistant to killing by neutrophils (114).

People lacking bactericidal antibodies, suffering from a deficiency of the late complement components, or lacking properdin are prone to meningococcal infections (34, 38). The levels of "natural" bactericidal antibodies present in individuals are influenced by carriage of meningococci or colonization by nonpathogenic bacteria (containing cross-reactive epitopes) such as *N. lactamica* (anti-LPS). Colonization with these bacteria can result in the induction of specific bactericidal antibodies or high levels of secretory immunoglobulin A (IgA) in the nasopharynx (38, 55, 59, 99). It is possible to improve the immunological status of these high-risk individuals by vaccination (34, 35, 50, 104, 114, 124).

**MENINGOCOCCAL LPS**

**Immunochemistry and Structure of Core Oligosaccharides**

The 12 known immunotypes of meningococcal LPS are distinguished on the basis of a combination of SDS-PAGE patterns and the use of specific rabbit antisera and specific monoclonal antibodies (78, 105, 132, 150). The LPS of meningococcal strains are composed of one to six components with molecular weights ranging from 3,200 to 5,100 (78, 120, 132). They contain lipid A and a small oligosaccharide of up to 10 monosaccharides but lack an O antigen (78, 120, 132). The expression of the various components depends on the growth conditions, the growth phase, and probably the absence or presence of exogenous CMP-NANA (86, 106, 107, 131). Most strains often express more than one immunotype-specific epitope on their LPS, leading to the classification of strains as L3,7,9, L2,4, or L1,8.

Kim et al. (78) described criteria for the assignment of immunotypes L8, L9, L10, and L11 to meningococcal A strains on the basis of electromorphic profiles of the LPS molecules and their reactivity with monoclonal antibodies (see Table 4). For some of the other immunotypes, however, no specific monoclonal antibodies are yet available, nor can

### Table 2. Virulence factors of *N. meningitidis*

| Category                      | Description                          | Function or mechanism                                                                 | Reference(s) |
|-------------------------------|--------------------------------------|---------------------------------------------------------------------------------------|--------------|
| Secreted                      | IgA proteases                        | Destruction of secretory IgA                                                          | 39, 95       |
|                               | Blebs*                               | Enhanced invasiveness, deregulation, and deflection of defense mechanisms             | 3, 4         |
| Surface structures            | Pili                                 | Attachment to nonciliated cells of the nasopharynx                                    | 127          |
|                               | CPS                                  | Anti-phagocytic, reduced immunogenicity in young children, no memory induction, shielding of epitopes | 34, 38       |
|                               |                                      | Down-regulation of the alternative pathway activation (group B)                      | 46, 67       |
|                               |                                      | Antigenic mimicry (group B)                                                          | 47, 123      |
|                               | OMP                                  | Antigenic variation                                                                  | 52, 134      |
|                               | RIPa                                 | Phase variation                                                                      | 106, 148     |
|                               | LPS                                  | Uptake of iron for growth                                                            | 38, 102      |
|                               |                                      | Phase variation/shielding of bactericidal epitopes                                    | 106, 107     |
|                               |                                      | Antigenic mimicry*                                                                   | 84, 135      |
|                               |                                      | Variable sialylation of LPS/shielding of epitopesa                                     | 86, 96, 133  |
| Use of host defense factors   | IgA                                  | Blocking lytic action of other antibody subclasses                                    | 59, 62       |

* Blebs are vesicle-like structures containing lipids, LPS, OMP, and CPS, which are released during growth (3, 4).
* RIP, regulatory iron proteins (102).
* Lacto-N-neotetraose unit: β-D-Galp-1→4-β-D-GlcNAcp(1→4)-β-D-Galp(1→4)-β-D-Galp(1→4) unit on O1 LPS (84, 85).
* Sialic acid (NeuNAc) derived from CMP-NANA is coupled by sialyltransferase to the LPS molecules (86, 87).
immunotype-specific epitopes be correlated to LPS molecules of a unique molecular weight (see Table 4). Immunotyping of meningococcal strains is therefore difficult and more prone to error than serogrouping or (sub)serotyping is.

Two types of epitopes can be distinguished within meningococcal LPS: (i) immunotype-specific epitopes, and (ii) cross-reactive epitopes (conserved/common epitopes). Immunization with whole bacteria, outer membrane vesicles (OMV), or purified LPS often results in the induction of both immunotype-specific antibodies and cross-reactive antibodies. Studies by Zollinger and Mandrell (150) and Tsai et al. (132) suggest the existence of immunochemoically and immunologically related groups of immunotypes (Table 3). Only immunotypes L1, L5, and L6 possess immunotype-specific epitopes that appear to be immunodominant in rabbits (Table 5).

The differences between the LPS molecules expressed by a strain, or the differences between the 12 immunotypes, can be found in the oligosaccharide part of the LPS molecules (63, 99). In a number of studies, Jennings and coworkers elucidated the complete primary structures of the oligosaccharides isolated from immunotypes L1, L2, L3, L5, L6, L7, and L9 LPS (Fig. 1) (32, 41, 53, 69, 70, 91, 137). These oligosaccharides are bi- or triantennary structures, which are linked to the lipid A part of the LPS molecule, probably via two 2-keto-3-deoxyoctulosonic acid (KDO) molecules (Fig. 1) (32, 41, 53, 69, 70, 86, 91). They differ in monosaccharide composition, type of linkage between sugar residues, amount and location of phosphoethanolamine (PEA) groups, and degree of O acetylation of α(1→2)-linked GlcNAc or other sugar moieties. However, there are also similarities between these oligosaccharide structures: the inner-core structure is common to most if not all immunotypes, whereas the lacto-N-neotetraoside unit is found on immunotypes L2, L3, L5, L7, and L9 (Fig. 1) and probably on L10 and L11 LPS (77, 78, 131, 133).

The complete structures of immunotypes L4, L8, L11, and L12 oligosaccharides remain to be elucidated, but some data are known about partial structures. L10 oligosaccharides contain the β-D-GlcP(1→4)-β-D-GlcP moiety.

### TABLE 3. Cross-reactivities of rabbit antisera evoked with the various immunotype-specific bacteria, OMV, or isolated LPS

| Antiserum | Reactivity with LPS reference strains and percent inhibition with OMV derived from these strains in a RIA |
|-----------|-------------------------------------------------------------------------------------------------|
| L2E (L1,8)| +++++ (98%) | - | - | - | - | - | - |
| 35E (L2)| - | ++++ (87%) | + | + | + | - | - |
| 6275 (L3)| - (60%) | +++ (75%) | + | + | + | - | - |
| 891 (L4)| 4A8-B2 | - | - | - | - | - | - |
| M981 (L5)| - | ++++ (94%) | - | - | - | - | - |
| M992 (L6)| - | - | - | - | - | - | - |
| M978 (L8)| - (70%) | +++ (59%) | - | - | - | - | - |

* Adapted from references 132 and 150 with permission.

** Inhibition of rabbit antisera by OMV was determined by a solid-phase radioimmunoassay (150). Homologous LPS gave an inhibition of 100%. Only inhibitions higher than 30% are shown. Reactivity was based on the intensity of the immunostain: +++, strong; ++, medium; +, weak; -, nonexistent.

c A 4,300-molecular-weight component of 35E LPS had about 10% of the total reactivity of the antisera and was cross-reactive with the same component in all different LPS immunotypes. This cross-reactivity was excluded from the table (132).

### TABLE 4. Specific monosaccharide antibodies available for immunotyping of *N. meningitidis*

| MAb* | Specificity | $M_r$ | Comments | Reference(s) |
|------|-------------|------|----------|-------------|
| Mn14F21-11 | L1 | - | L1 and L8 are often detected on the same strain | 108 |
| 4D1-B3 | L3,7,9 | - | Immunotypes L3,7,9 contain | 139 |
| 4A8-B2 | L3,7,9 | - | cross-reactive epitopes and are often found on the same strain | 139 |
| 3A1-B1 | L3,7,9 | - | | 139 |
| MN15A11 | L3,7,9 | - | | 28, 139 |
| Mn3ABC | L5 | 4,400 | | 116, 132 |
| Mn4C1B | L6 | - | Single component on SDS-PAGE, probably $M_r$ | 78 |
| 2-1-L8 | L8 | 3,600 | L8 strains express the 2-1-L8 and 4C4 epitopes | 78 |
| 1-9C4* | L9 | 4,500 | L9 strains contain multiple components and often express more immunotypes | 78 |
| MCA14.2 | L10 | 4,000 | Single major LPS component | 78 |
| 4C9* | L11 | 3,600 | Single major LPS component | 78 |

* Monoclonal antibodies (MAb) do not always recognize one specific immunotype but often also recognize immunochemoically closely related groups of immunotypes.

** Epitope recognized by monoclonal antibodies is located on an LPS component of a certain molecular weight.

* Immunoype L1, L2, L3, L4, L7, and L8-specific epitopes are recognized on the larger LPS molecules (132).

* These monoclonal antibodies are used for immunotyping of group A meningococci in combination with electromorphic profiles (78).

* 1-9C4 also reacts with other immunotypes. It recognizes immunotype L2, L3, L7, L8, and L9 strains in the whole-cell ELISA (139a).

* The specificity of this monoclonal antibody is probably the α-D-GalP(1→4)-β-D-GalP unit (143).
which is also found in immunotype L5 oligosaccharides (69). When monoclonal antibodies with a known specificity for the terminal lacto-N-neotetraose unit (monoclonal antibodies 3F11, 06B4 and anti-My-28) were used, this unit was detected in L4, L8, L10, and L11 LPS but not in L12 LPS (77, 78, 131, 133).

The studies by Jennings and coworkers were conducted with oligosaccharides isolated by treating LPS with 1% acetic acid for 2 h at 100°C. Terminal lacto-N-neotetraose-bearing immunotypes are sialylated during growth in vitro and probably also in vivo (86, 131). The absence of sialic acid in the structures presented by Jennings and coworkers can be ascribed to the acid lability of the linkage between the sialic acid residue and the underlying galactose moiety (86). When these oligosaccharides are isolated by milder methods, terminal sialic acid residues will be detected in the structures of meningococcal immunotypes containing a terminal lacto-N-neotetraose unit (86).

Correlation between Oligosaccharide Structures and Immunotype-Specific Epitopes

Knowledge about primary oligosaccharide structures of the various immunotypes is an essential step in the process of correlating immunotype-specific epitopes with well-defined oligosaccharide structures. Primary-structure information, however, is not enough because it is not possible to predict the actual three-dimensional conformation of these oligosaccharides on the basis of primary structures alone. The conformation and immunological behavior of the immunotype-specific epitopes may be influenced by other parts of the oligosaccharide molecule (e.g., shielding by sialic acid or the terminal lacto-N-neotetraose unit, presence of other immunodominant epitopes), the lipid A part of the LPS molecule, or the interaction with other meningococcal surface structures (86, 149). The current knowledge about the primary structures in combination with immunoblot analysis of the various immunotypes (e.g., the molecular weight of the immunodominant and immunotype-specific LPS molecules) makes it possible to speculate about, or sometimes to pinpoint, the oligosaccharide structure or location of the various immunotype-specific epitopes (32, 41, 53, 69, 70, 91, 132, 137).

The immunotype L1-specific epitope is probably located in the terminal α-D-Galp(1→4)-β-D-Galp(1→4) unit. This structure is found only in L1 LPS (Fig. 1), and the immunodominant and immunotype-specific LPS molecule of a L1 strain has a molecular mass of about 4.6 to 4.7 kDa, which is large enough to contain this oligosaccharide (41, 132). The L5-specific epitope is probably part of the terminating β-D-GlcP(1→4)-β-D-GlcP(1→4) structure (Fig. 1). The smallest oligosaccharide isolated from L5 LPS possesses this structure, and the immunotype-specific and immunodominant epitope is located on a 4.4-kDa LPS molecule (Fig. 1) (91, 132). The L6-specific epitope is probably found in the terminal β-D-GlcNAcp(1→3)-β-D-Galp(1→4) unit. This oligosaccharide structure is unique for the isolated L6 oligosaccharide, and the immunotype-specific and immunodominant epitope is present on a 4.5- to 4.6-kDa LPS molecule (Fig. 1) (41, 132).

For the other immunotypes whose primary structures are known, the precise immunotype-specific epitopes are less well-defined. The immunotype L3,7,9-specific epitopes will probably consist of a PEA group (1→3) linked to heptose in combination with unknown adjacent sugar moieties (70, 137). The Glc molecule α(1→3) linked to heptose, in combi-
nation with the PEA group linked to the O-6 or O-7 of the same heptose, is probably part of the L2-specific epitope (Fig. 1) (53, 137).

MENINGOCOCCAL VACCINES

Status, Problems, and Some Future Trends

At present, commercial vaccines are available against serogroups A, C, Y, and W-135 meningococci (50). The vaccines consist of the purified CPS of these four sero-
groups. The efficacies of the separate components, combined serogroup A and C CPS, and more recently the tetravalent vaccine have been evaluated in a number of studies and field trials (8, 35, 56, 76, 80, 81, 100, 101, 109, 146). The CPS vaccines are capable of inducing protective immunity, but this protection is age dependent and rather short-lived (no memory induction) (56, 76, 80, 81, 100, 109, 146). Furthermore, the age dependency of the immune response toward these CPS varies with the serogroup (56, 80). The purified group B CPS was not included in these vaccines because it is poorly immunogenic in humans (152).

In the United States, the tetravalent CPS vaccines are routinely administered to the military and are recommended for use by individuals with a deficiency of the late complement components or properdin and by travelers going to areas of hyperendemicity or areas with recent epidemic disease (e.g., the meningitis belt in Africa) (26).

The intrinsic disadvantages of CPS as vaccines can be overcome by the classical approach of coupling CPS or its oligosaccharides to proteins (see reference 29 for an excellent review of all aspects of polysaccharide- or oligosacchar-
ride-protein conjugate vaccines) (45, 54, 111). Coupling of polysaccharides to proteins changes the character of these kinds of antigens from thymus independent to thymus de-
pendent. Polysaccharide- or oligosaccharide-protein conjugates are generally strong immunogens in young children and are capable of inducing memory (5, 29). Meningococcal group A and C polysaccharide-tetanus toxoid conjugates have been prepared and are highly immunogenic in mice and rabbits (10, 11, 72). So far, these conjugates have not been evaluated in humans, but in the near future the World Health Organization will coordinate field trials with meningococcal group A and C conjugate vaccines in areas with periodic epidemic disease (20). On the basis of experience with the H. influenzae type b conjugate vaccines, positive results are to be expected. Therefore, polysaccharide- or oligosaccharide-protein conjugates will (probably) be the vaccines of choice against infection with groups A, C, Y, and W-135 meningococci.

The main problem with respect to development of a vaccine against N. meningitidis is presented by the group B meningococci (49, 50, 99, 104). The CPS is poorly immuno-
genic in humans. The immune response against this CPS is restricted to the IgM class and is of low avidity (79, 82, 152).

Group B CPS consists of α(2→8)-linked sialic acid residues. These structures are present in human brain tissue during fetal and early postnatal periods and on a number of human gangliosides (Fig. 2) (47, 123). The immunogenicity of the group B CPS can be improved by complexing it to OMP. The human anti-group B IgM antibodies that are induced have proven protective in an animal model (82). Chemical modi-
cation of group B CPS (N-propionylation) and coupling to carrier proteins also enhance the immunogenicity of this CPS (68, 74). This conjugate induces an IgG response in mice, and a booster effect has been observed (thymus-
Group B capsular polysaccharide

\[ \text{[\(\alpha-D-NeuNAc(2\to8)\alpha-D-NeuNAc\)]} \]

Lacto-N-neotetraose unit

\[ \beta-D-Galp(1\to4)\beta-D-GlcNacp(1\to3)\beta-D-Galp(1\to4)\beta-D-Glc \]

Terminal oligosaccharide structure L1 LPS

\[ \alpha-D-Galp(1\to4)\beta-D-Gal \]

FIG. 2. Antigenic structures that are present on meningococcal strains and resemble host antigens.

dependent response) (68, 74). Devi et al. (37) were able to evoke group B-specific antibodies by using an Escherichia coli CPS-tetanus toxoid conjugate, which was prepared by a different coupling procedure (the O antigen of E. coli K1 is identical to group B CPS). Furthermore, they also synthesized a highly immunogenic E. coli K92 CPS-tetanus toxoid conjugate by using the same procedure [E. coli K92 has a repeating unit of \(\alpha(2\to8)\) and \(\alpha(2\to9)\)-linked sialic acid residues]. These studies suggest that a group B capsular polysaccharide-based vaccine is feasible. At present, however, it cannot be excluded that anti-group B antibodies (especially IgG) cross-react with host antigens in vivo and, as a consequence, may be a (potential) cause of autoimmune diseases. Therefore, most of the research efforts concerning the development of a group B meningococcal vaccine is directed to the potential use of subcapsular components such as OMP and LPS (49, 50, 104).

OMP have been evaluated as vaccine candidates during the last decade (16, 21, 50, 65, 88, 104, 112, 114, 130). An excellent review of meningococcal OMP vaccines is given in an issue of the Norwegian NIPH Annals (130). This review describes the results of the vaccine trials in Norway, Chile, and Cuba. This type of vaccine is prepared by isolating the OMP in the form of vesicles (OMV) depleted of LPS by treatment with a detergent. To improve solubility and immunogenicity, the OMVs are complexed with one or more meningococcal CPS (50, 104). The OMV vaccines, containing OMP and limited amounts of LPS, have a three-dimensional liposomal structure in which the OMP are stabilized in their native conformation (51). The OMV vaccines are immunogenic in adults and young children, and the antibodies induced are bactericidal and capable of enhancing phagocytosis (50, 65, 88, 112, 114). The protection induced by OMV vaccines is considered to be OMP specific. However, the Cuban vaccine was able to protect against meningococcal strains with OMP types not included in the vaccines, but so far these results have not been confirmed by an independent group (21). It suggests that not all essential factors are known for the preparation of an optimal OMV vaccine.

It has been estimated that to achieve protection in a certain geographical area, 5 to 10 different (sub)serotype OMP are necessary. This problem cannot be solved simply by combining the various OMV vaccines, since that would lead to high levels of LPS per vaccine dose, with the risk of unacceptable side effects (104). Several solutions to this problem are possible. New meningococcal strains can be constructed by recombinant DNA techniques in which more subserotype OMP (class 1 OMP) are expressed (135). Methods such as chemical mutagenesis and genetic transformation techniques can be used to generate strains containing LPS with a diminished toxicity (27). Highly purified OMP preparations can be prepared and subsequently stabilized by addition of detoxified meningococcal LPS (153). Furthermore, the number of OMP needed can be limited by using OMP as a carrier protein for meningococcal LPS-derived oligosaccharides. Of the 12 different immunotypes known, only 3 are prevalent in the western world (105, 150). Such an oligosaccharide-OMP conjugate vaccine combines (sub)serotype determinants with immunotype-specific epitopes and will probably be able to induce better and broader protection against infections with N. meningitidis. This kind of vaccine is potentially capable of inducing neutralizing antibodies against endotoxin, the component responsible for the induction of septic shock during systemic meningococcal disease (17, 18, 44, 93, 94, 144, 145).

A potential disadvantage of using the complete meningococcal oligosaccharides is the presence of the terminal lacto-N-neotetraose unit or the terminal \(\alpha-D-Galp(1\to4)\beta-D-Gal\) moiety, structures which are also found in the human host (Fig. 2). These units are detected on precursors of blood group antigens and human epithelial cells, respectively (85, 141). In rabbits the lacto-N-neotetraose unit is immunodominant and no antibodies are formed against this epitope after immunization with bacteria, LPS, or oligosaccharide-protein conjugates (9, 71, 140, 150). The \(\alpha-D-Galp(1\to4)\beta-D-Gal\) unit is probably the immunotype L1-specific epitope, and rabbits are capable of making immunotype L1-specific antibodies (41, 132, 143, 150). Deletion of these determinants, however, might be preferable, because it may improve the immunogenicity of the underlying epitopes and will avoid the potential risk of autoantibody induction. Progress in the organic synthesis of partial structures of meningococcal oligosaccharides and the availability of suitable enzymes will make such an approach feasible (13-15, 91, 97, 137).

**Detoxified LPS (Outer Membrane) Protein Vaccines**

The toxicity of the lipid A part of meningococcal LPS prevents the use of combinations of OMV vaccines, since high levels of LPS per vaccine dose might lead to undesirable side effects (104). Although complete removal of the LPS is probably possible, too extensive a purification of OMP can influence the conformation of the OMP, resulting in a vaccine that is unable to induce protective antibodies. The toxicity of the lipid A part of LPS can, however, be diminished. Treatment with NaOH removes the ester-linked fatty acid tails (O-acyl groups) of lipid A. The toxicity of, for instance, gonococcal LPS was reduced 100,000- to 1,000-fold by this treatment as determined by the Limulus lysate assay, and the detoxified LPS retained its antigenicity (122).

Zollinger et al. used this approach to prepare a combination of OMP vaccines (153). A group B meningococcal vaccine was prepared as a noncovalent complex of detoxified type L3,7,9 LPS and purified OMP from strains H44/76 (B:15;P1.7,16) and 8047 (B:2b;P1.2). The vaccine was tested in mice, rabbits, and human volunteers. It was highly immunogenic in rabbits and in mice, inducing cross-reactive bactericidal antibodies. The preparations were well tolerated by the volunteers and produced strong bactericidal antibodies in most individuals. The specificities of most of the cross-reactive antibodies were not known, but they seemed
to be directed against proteins other than the serotype or subserotype protein.

Seid et al. also used detoxified LPS for the preparation of meningococcal pilus-LPS conjugates (122). Instead of complexing the detoxified LPS to the protein, they covalently coupled the detoxified LPS to the protein by the following procedure. The carboxyl groups of the KDO residues were treated with \(N\)-hydroxysuccinimide in the presence of 1-ethyl-3-dimethylaminopropylcarbodiimide (EDC) to yield active esters, and the activated LPS were subsequently reacted with the amino groups present in the protein. The chemistry used did not affect the antigenicity of either the LPS or the pilus protein. Immunization studies with mice showed that the immune response to the detoxified LPS and pilus protein was enhanced compared with that of detoxified LPS or pilus protein alone (122).

**LPS-Derived Oligosaccharide-Protein Conjugates**

Since Avery and Goebel conducted their studies with S. pneumoniae type 3 CPS-derived oligosaccharide-protein conjugates in the late 1920s, polysaccharide- or oligosaccharide-protein conjugate vaccines have been prepared against encapsulated pathogens such as *N. meningitidis, Streptococcus* species, *H. influenzae, Pseudomonas aeruginosa*, and, more recently, *Staphylococcus aureus* (5, 10, 11, 29, 45, 54, 74, 111). These conjugates differ with respect to the carrier protein, the kind of carbohydrate used (polysaccharide versus oligosaccharide, length of oligosaccharide), the linkage of antigens (direct coupling or with a spacer chain), the conjugation model (single ended versus cross-linked, e.g., neoglycoprotein [polysubstitution of a protein carrier with oligosaccharides having a single terminal attachment point] versus lattice-like conjugate [three-dimensional matrix generated by multiple attachment points on protein and carbohydrate]), the coupling chemistry, the amount of oligosaccharide incorporated or the degree of cross-linking between carbohydrate and protein, and the presence or absence of free polysaccharide or oligosaccharide (40). At present no definite rules can be given for the preparation of a carbohydrate-protein conjugate vaccine with optimal immunological properties (40).

For specific coupling of carbohydrates to proteins, suitable groups such as an amino (-NH\(_2\)), carboxylic acid (-COOH), thiol (-SH), or aldehyde (-CHO) must be available within the carbohydrate antigen (40). These groups can already be present in the native antigen, but they often have to be introduced by chemical or enzymatic methods (40, 72, 97). Whatever coupling method is used, it always results in the modification of the carbohydrate antigen, leading to the potential destruction of protective epitopes or the generation of unwanted immunodominant neoantigens. The influence of the coupling procedure on the immunological properties of an oligosaccharide-protein conjugate is large, especially when small oligosaccharides are used (66). Most of the existing conjugate vaccines are based on CPS or O antigens consisting of repeating units of one to eight monosaccharides, so that the influence of the coupling procedure can be minimized by using larger oligosaccharides, i.e., those containing more repeating units (5, 40, 45, 66, 111). Meningococcal LPS do not contain repeating units (78, 120, 132), and the choice of the coupling procedure will therefore probably determine the immunological and immunochromatic characteristics of the resulting conjugate.

Within the meningococcal oligosaccharides, two groups are available for coupling these oligosaccharides to carrier proteins: the free amino function of the PEA group and the carboxylic acid function of the KDO moiety (Fig. 3). The PEA group, however, cannot be used for the coupling procedure, since it is (probably) part of the immunotype-specific epitopes of L2, L3, L7, and L9 LPS and should not be modified (Fig. 1). Moreover, the presence of this free amino function prohibits the use of coupling procedures normally applied with oligosaccharides or polysaccharides containing a carboxylic acid function (40, 118, 142). These methods are based on coupling of the carboxylic acid function of the oligosaccharides to the free amino groups of the protein. With regard to the meningococcal oligosaccharides, oligosaccharide can be coupled to oligosaccharide or linked to the carboxylic acid groups of the protein via the PEA group of the oligosaccharide. Jennings et al. tried to solve this problem by removing the PEA groups with hydrogen fluoride (71). Dephosphorylated immunotype L2, L3,9, L5, and L10 oligosaccharides were subsequently coupled to tetanus toxoid by introduction of \(\beta\)-(4-aminophenyl)ethylamine as a spacer at the reducing end by means of reductive amination (loss of the ring structure of KDO) followed by activation of the amino function with thiophosgene and coupling to tetanus toxoid (Fig. 3) (71, 129). The immunotype L2, L5, and L10 conjugates were highly immunogenic in rabbits, inducing antibodies against both the homologous and heterologous immunotypes (Table 5). The antibodies induced, however, were bactericidal only for the homologous immunotype. The endotoxin neutralization and phagocytic capacities of the antibodies evoked were not evaluated (9, 71).

In contrast, the L3,9 dephosphorylated oligosaccharide-tetanus toxoid conjugate was only slightly immunogenic and the evoked antibodies were not bactericidal. These results suggest that the loss of PEA groups and/or ring structure of the KDO residue is of minor importance for immunotype L2, L5, and L10 conjugates, whereas it leads to modification of essential (bactericidal) epitopes on the L3,9 oligosaccharide. It demonstrates the necessity of developing coupling procedures in which the PEA groups are preserved and the meningococcal oligosaccharides are modified as little as possible.

Recently, our group has developed a method for the specific coupling to proteins of oligosaccharides or polysaccharides which contain both a carboxylic acid function and free amino groups (138). The method is based on the selective introduction of a free thiol group (S—H) in the meningococcal oligosaccharides by a two-step procedure (Fig. 4A). First, cystamine is coupled to the carboxylic acid (-COOH) residue of the KDO moiety by using EDC and \(N\)-hydroxysulfosuccinimide. Second, the thiol function is liberated with reduction by dithiothreitol. The free amino groups of the protein are modified by reaction with \(N\)-succinimidyl bromoacetate (Fig. 4B). The thiol-functionalized oligosaccharides are subsequently coupled to the bromoacetylated proteins, giving conjugates with stable thioether linkages (Fig. 4C).

The prepared immunotype L2 and L3,7,9 oligosaccharide-tetanus toxoid conjugates were highly immunogenic in rabbits even without the use of an adjuvant (Table 5). The L3,7,9 oligosaccharide-tetanus toxoid conjugate was able to induce high antibody levels, which could be detected for 9 months after a booster injection (138). These antibodies were directed against the oligosaccharide part of meningococcal LPS and reacted with both homologous and heterologous immunotype LPS (Table 5) (137, 138, 140). Antibody populations with a different epitope specificity were detected in
these antisera by performing extensive inhibition studies with the homologous and heterologous LPS, oligosaccharides, and dephosphorylated oligosaccharides as inhibitors (Table 6). Interestingly, the epitope specificity of the antibody populations induced depended on the use of the adjuvant Quil A (140). The L3,7,9 conjugates alone elicited two different antibody populations: one population which recognized PEA group-containing oligosaccharide-specific epitopes on L1 and L3,7,9 LPS (antibody population A) and a second population which was directed against immunotype

FIG. 3. Coupling of meningococcal oligosaccharides to proteins by means of reductive amination (71, 129). PEA groups present in meningococcal oligosaccharides (structure 1) are removed by treatment with hydrogen fluoride. β-(4-Aminophenyl)ethylamine is incorporated in the dephosphorylated oligosaccharides (structure 2) by means of reductive amination with NaCNBH₄, leading to the loss of the KDO ring structure (structure 3). After activation with thiophosgene (CSCI₂), the oligosaccharides (structure 4) are coupled to the free amino groups of the protein (structure 5).

TABLE 5. Cross-reactivities of antisera evoked with meningococcal LPS-derived oligosaccharide-tetanus toxoid conjugates

| Conjugate | Positive reactions with immunotype LPS* | Other immunotypes studied | Reference(s) |
|-----------|--------------------------------------|--------------------------|--------------|
| L2 (OS*)₁₂⁻⁻TT⁺ | L2 (d), L4, L5 (m) | L3,9, L4, L10 | 71 |
| L5 (OS*)₃⁻⁻TT⁺ | L5 (d), L2 (m) | All except L11 and L12 | 9, 71 |
| L3,9 (OS*)₃⁻⁻TT⁺ | L2 (d), L3 (m) | L4, L5, L10 | 71 |
| L10 (OS*)₂⁻⁻TT⁺ | L10 (d), L2, L4, L5 (m) | L3,9 | 71 |
| L3,7,9 (OS*)₃⁻⁻TT⁺ | L3,7,9 (d), L2, L2 (m), L1 (m)⁺ | 137, 138, 140 |
| L3,7,9 (OS*)₂⁻⁻TT⁺ | L3,7,9 (d), L2, L1 (m)⁺ | 137, 138, 140 |
| L2 (OS*)₂⁻⁻TT⁺ | L2 (d), L3,7,9 (m) | L1 | 137, 138, 140 |

* In the various immunotype-specific ELISAs, purified LPS were used as coating antigens. The specificities of various LPS were checked by using immunotype-specific monoclonal antibodies. d, dominant; m, medium; l, low.

* Conjugates were prepared from dephosphorylated oligosaccharides (OS*) and tetanus toxoid (TT) by the method of Svenson and Lindberg (129) (Fig. 3). It resulted in the destruction of the ring structure of KDO (129). Rabbits were immunized with the conjugates in combination with Freund’s complete adjuvant.

* Conjugates were prepared from PEA group containing (OS) or partially dephosphorylated OS (OS*) and TT by a recently developed coupling method in which the PEA groups and the ring structure of KDO are preserved (138) (Fig. 4). Rabbits were given the conjugates alone or in combination with the adjuvant Quil A (137, 138, 140).

* L1-reactive antibodies were detected only in rabbit antisera evoked with the conjugate in combination with Quil A.

* L1-specific antibodies were detected mainly in class I antisera, in which an antibody population directed against the PEA-group containing epitopes on L3,7,9 LPS is present (137, 140).
A. Oligosaccharide-COOH + NH₂(CH₂)₂S-S(CH₂)₂NH₂ → Cystamine

B. Protein-[NH₃]₄ + Br-CH₂-CO-NHS → Protein-[NH₂CO-CH₂Br]₄

C. [3] + [5] → Protein-[NH₂CO-CH₂-S(CH₂)₂-NH₂]₄

FIG. 4. Reaction scheme of coupling of carboxylic acid- and free amino group-containing oligosaccharides (structure 1) to proteins (structure 2). (A) A thiol (S-H) group is introduced by coupling of cystamine to the carboxylic acid function of the terminal KDO residue (structure 2), followed by reduction with diithiothreitol (structure 3). Coupling of structure 1 to structure 2 by the already present free amino group is prevented by using excess amounts of cystamine. (B) Bromoacetyl groups are introduced into proteins (structure 4) by using N-succinimidy bromoacetate. (C) Thiol-functionalized oligosaccharides (structure 3) are coupled to bromoacetylated proteins (structure 5), giving conjugates with stable thioether linkages (structure 6). Reprinted from reference 138 with permission.

L2 oligosaccharide-specific epitopes (antibody population B). Addition of Quil A to the L3,7,9 conjugates resulted in the predominant induction of an antibody population recognizing the common oligosaccharide epitopes of L2 and L3,7,9 LPS (antibody population C). This antibody population did not react with L1 LPS (Table 5 and 6) (137). The biological functionalities of these antisera were evaluated in a bactericidal and opsonophagocytic (chemiluminescence) assay. None of the antisera displayed a bactericidal activity against the homologous or heterologous strains despite the use of different sources of complement (guinea pig, human, and rabbit complement). Antisera containing antibody population A as the major antibody population, however, were capable of enhancing the chemiluminescence induced by the homologous strain H44/76 (immunotype L3,7,9) (Table 6). These results suggest that for the induction in rabbits of opsonophagocytic meningococcal LPS-specific antibodies by oligosaccharide-protein conjugates, a subtle relation exists between the antigen-adjuvant combination used, the epitope specificity of antibodies raised, and their biological function (Table 6).

The same conjugates induced slightly to moderately LPS-specific antibody levels in mice. Addition of adjuvants such as Quil A and the nonionic blockpolymer L121 enhanced these responses significantly and shifted the IgG isotype distribution from mainly IgG1 toward the complement-activating subclasses IgG2a, IgG2b, and IgG3. However, these antisera were also not bactericidal for homologous and heterologous meningococcal strains (141). Our results and the work of Jennings and coworkers therefore indicate that it will be difficult if not impossible to induce bactericidal antibodies against immunotype L3,7,9 strains in a heterogeneous population by using meningococcal LPS-derived oligosaccharide-tetanus toxoid conjugates (9, 71, 137–141).

For the preparation of meningococcal LPS-derived oligosaccharide-protein conjugates, heterogenous oligosaccharide mixtures were used (9, 71, 138). In addition to the various LPS (oligosaccharide) molecules synthesized by a single strain, this heterogeneity is introduced as a consequence of the isolation method used. Recently, Mandrell et al. (86) and Tsi and Civan (131) demonstrated the presence of terminal sialic acid groups attached to the lacto-N-neotetraose unit of meningococcal LPS. The meningococcal oligosaccharides used in our studies and in the studies of Jennings and coworkers did not contain these terminal sialic acid residues (9, 71, 137–141). These groups could have been removed during the hydrolysis of LPS in 1% acetic acid for 2 h at 100°C (86). Terminal sialic acid residues by themselves are not immunogenic but can influence the conformation of the oligosaccharide or the polysaccharide. Removal of sialic

### TABLE 6. LPS-specific IgG antibody populations identified in various rabbit antisera

| Population | Epitope specificitya | Opsonophagocytosisb | Induced byc |
|------------|----------------------|----------------------|-------------|
| A          | PEA group containing OS-specific epitopes on L1 and L3,7,9 LPS | + | L3,7,9 OS-TT (4/5) |
| B          | L2 OS-specific epitopes, no PEA group present | ? | L3,7,9 OS-TT (3/4) |
| C          | Common OS-specific epitopes on L2 and L3,7,9 LPS | ± | L3,7,9 OS-TT + Quil A (4/5) |

*a Epitope specificities of the antibodies were analyzed by various immunotype-specific inhibition ELISAs with homologous and heterologous inhibitors (137, 138, 140).

*b Opsonophagocytosis was studied by a chemiluminescence assay and a flow cytometry assay. Symbols: +, opsonophagocytosis; ±, doubtful; ?, not known.

*c Numbers in parentheses indicate the number of rabbits that were immunized with the antigen and made the antibody population/total number of rabbits immunized with the antigen. See references 137, 138, and 140 for details of immunization procedures.
acid residues in group B Streptococcus III CPS, for instance, leads to destruction of protective conformational epitopes (73). The consequences of the removal of the sialic acid on meningococcal LPS, however, will probably be minimal. The sialic acids are linked to the lacto-N-neotetraose unit, a structure which is not immunogenic in humans and rabbits (69, 85, 140) (Fig. 2). The absence or presence of terminal sialic acid residues will therefore not affect the immunological characteristics of the directly underlying sugar residues (Fig. 1). Its importance in the overall conformation of the meningococcal oligosaccharides is difficult to predict, but it does not seem to be crucial for the immunological properties of these molecules: desialylated (and dephosphorylated) LPS-derived oligosaccharide-tetanus toxoid conjugates raised bactericidal or opsonophagocytic antibodies against meningococcal strains (9, 71, 141) (Table 6). In these studies, strains containing LPS immunotypes that can be sialylated after growth in culture were used (86, 131). Furthermore, it is not known whether the complete LPS molecules (i.e., those containing terminal sialic acid residues) are synthesized by the meningococcal strains during every stage of colonization or infection. Poolman et al. (106) showed that meningococci in the log phase contained shorter LPS molecules than did meningococci in the stationary phase. However, to address this intriguing issue fully, additional studies must be performed in which the relationship between the growth phase and the presence of protective epitopes is investigated by using (protective) monoclonal antibodies that recognize a distinct set of epitope specificities on the LPS molecules.

Synthetic Oligosaccharide-Protein Conjugates

Vaccines are preferentially prepared by using well-defined components. Recent progress in carbohydrate organic synthesis has made it possible to prepare oligosaccharides of increasing length and complexity (13–15). Oligosaccharide structures that may induce protective immunity against more than one immunotype might be synthesized. Such an approach, however, will be difficult. The differences in oligosaccharide structures of the various immunotypes are relatively small, suggesting that minimal changes in these structures will lead to a shift in immunodominance of epitopes (see the discussion, above, about the correlation between oligosaccharide structures and immunotype-specific epitopes).

As a first step in our research program to define the minimal oligosaccharide structures required for the induction of a protective immune response against the prevalent immunotypes and to identify useful protective B-cell epitopes, three linear and one branched oligosaccharides, all partial structures of the common inner core, were synthesized (Fig. 1 and 5) (13–15). The results demonstrated that the branched β-D-GlcP(1→4)-[L-α-D-Hepp(1→3)]-L-α-D-Hepp is a minimal oligosaccharide structure required for the induction of an L1 and L3, 7, 9 oligosaccharide-specific IgG response and is part of the cross-reactive epitope between these two immunotypes. Structures terminating in β-D-GlcP(1→4), α-D-GlcNAc(1→2), or L-α-D-Hepp(1→3) are capable of raising an L2 oligosaccharide-specific IgG response (137). Although the antisera evoked were not bactericidal and the opsonophagocytic and endotoxin-neutralizing capacity remain to be evaluated, the data suggest that these small, synthetic oligosaccharides might have the potential to function as cross-reactive (protective?) B-cell epitopes in a vaccine against group B meningococci.

CONCLUDING REMARKS

The LPS of N. meningitidis are a complex group of molecules with only minor differences in primary oligosaccharide structures but with distinctly different immunological and immunochemical behaviors. Most of the studies of the structure, immunochemistry, and immunology of these molecules and their role in pathogenesis are conducted with LPS derived from or with bacteria grown in batch culture. Mertola et al. (90) recently demonstrated the profound influence of growth conditions and various animal models on the epitope expression of the LPS of H. influenzae. Therefore, research into the differences in LPS epitope expression of meningococci freshly isolated from patients and meningococci cultured in vitro is necessary. For this purpose monoclonal antibodies recognizing different (protective) epitopes on meningococcal LPS must be developed.

Much research has been devoted to the development of vaccines against infection with N. meningitidis during the last decade. Polysaccharide- or oligosaccharide-protein conjugate vaccines will become available against group A and C meningococci in the very near future, but developing a vaccine against group B meningococci is still a problem. OMV-based vaccines are the most likely candidates. Unfortunately, OMV alone afford protection primarily against strains containing the homologous OMP. This problem cannot be solved by combining the various (sub)serotype-specific OMP preparations since this will lead to high levels of endotoxin, with the risk of unacceptable side effects. Construction of meningococcal strains containing more than one class 1 OMP (135) and preparation of highly purified OMP which are subsequently stabilized by detoxified (NaOH-treated) meningococcal LPS (153) are promising approaches. Furthermore, the use of meningococcal LPS-derived oligosaccharide-outer membrane protein conjugate vaccines, which contain a component (oligosaccharide) potentially capable of inducing endotoxin-neutralizing antibodies, is also under investigation (9, 71, 137, 138, 140). The optimal oligosaccharides, however, still have to be selected. Their selection should be based on their potential to induce cross-reactive protective antibodies. Although it is possible to prepare these kinds of conjugates without extensive modification of the oligosaccharide molecules, antibodies induced against the most prevalent immunotype (L3, 7, 9) were not bactericidal (9, 71, 141). The protective role of LPS-specific antibodies as described in the literature, however, is unclear (36, 61, 113). These antibodies can potentially afford protection against infection with N. meningitidis.
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