Comparative Analysis of Two Meningococcal Immunotyping Monoclonal Antibodies by Resonant Mirror Biosensor and Antibody Gene Sequencing

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Lipooligosaccharide (LOS) is a major surface component of the cell walls of \textit{Neisseria meningitidis}, which is important for its roles in pathogenesis and antigenic variation, as a target for immunological typing, and as a possible vaccine component. Although the structures of many antigenic variants have been determined, routine immunological typing of these molecules remains problematic. Resonant mirror analysis was combined with gene sequencing to characterize two monoclonal antibodies (MAbs) used in typing panels that were raised against the same LOS immunotype, L3,7,9. The two MAbs (MAb 4A8-B2 and MAb 9-2-L379) were of the same immunoglobulin subtype, but while MAb 9-2-L379 was more than a 1,000-fold more sensitive in immunotyping assays of both whole meningococcal cells and purified LOS, MAb 4A8-B2 was more specific for immunotype L3,7,9. The differences in sensitivity were a consequence of MAb 9-2-L379 having a 44-fold-faster association constant than MAb 4A8-B2. Comparison of the amino acid sequences of the variable chains of the MAbs revealed that they had very similar heavy chains (81% amino acid sequence identity) but diverse light chains (54% sequence identity). The differential binding kinetics and specificities observed with these MAbs were probably due to differences in the epitopes recognized, and these were probably a consequence of the different immunization protocols used in their production.

\textit{Neisseria meningitidis}, an etiological agent of meningitis and septicaemia, is a normally commensal bacterium that nevertheless causes significant morbidity and mortality worldwide (3). Lipooligosaccharide (LOS) is an essential glycolipid component of the meningococcal outer membrane that is equivalent to the longer chained LOSs of the enteric bacteria, which is important in strain identification, vaccine development, pathogenesis and host damage (17). Twelve LOS immunotypes have been described in the literature (20); however, the L3, L7, and L9 immunotypes have an identical carbohydrate structure and have therefore been designated L3,7,9 (8). Specific and cross-reactive epitopes are located on the oligosaccharide part of the LOS molecule. Immunotypes L1 to L9 are associated primarily, but not exclusively, with serogroup B and C meningococci, while immunotypes L10 to L12 are mainly associated with serogroup A isolates (18). Immunotype L3,7,9 is often found in strains thought to be particularly virulent (13, 18) and may contribute to the resistance of these meningococci to complement-mediated lysis (14, 16). This is perhaps due to the fact that the oligosaccharide structure invariably terminates in a moiety that is structurally similar to the terminal sequence of human glycosphingolipids (17). Meningococcal LOSs of immunotypes L3,7,9, L2, and L5, in common with that of the related gonococcus, can be further modified in vivo by sialylation or by the addition of cytidine 5'-monophosphate-N-acetylmuraminic acid (7, 15, 17).

Although the complete oligosaccharide structures of LOS molecules corresponding to most immunotypes have been elucidated, making it possible to correlate the immunotype-specific epitopes with defined oligosaccharide structures (20), immunological characterization of the variants for both routine epidemiological and research purposes remains problematic, requiring a relatively complex algorithm based on the reactivity of meningococcal whole cells or purified LOS in enzyme-linked immunosorbent assays (ELISAs) with a panel of monoclonal antibodies (MAbs) (18). For protein antigens it is frequently possible to correlate amino acid sequences of antigenically variable proteins, deduced from gene sequences, with immunological reactivity, and genetic techniques are consequently playing an increasing role in the characterization and study of such molecules. In the case of carbohydrates, including meningococcal LOS, such techniques are unlikely to provide a viable alternative to immunological studies, despite advances in understanding of the biosynthetic genes responsible for their production (10). Consequently, an improved understanding of LOS-antibody interactions is necessary for epidemiological surveillance and studies of the vaccine potential of this antigen.

In the present study, two mouse MAbs that were raised against LOSs of immunotype L3,7,9 were compared. The hybridoma cell lines producing these MAbs were made by immunizing animals with either oligosaccharide-tetanus toxoid conjugate (hybridoma 4A8-B2) (21) or outer membrane complexes (hybridoma 9-2-L379) (24). Unlike MAb 4A8-B2, MAb 9-2-L3,7,9 cross-reacted with the L2, L5, and L8 immunotypes (18), but ascitic fluid produced from this hybridoma appeared to be more sensitive, being usable at a much higher dilution. The use of purified antibodies and LOS in ELISA, together with real-time kinetic analyses with the same reagents, established the relative sensitivities of these MAbs and allowed us to
measure their binding kinetics. These data were correlated with the deduced primary structures of the antibodies and known sugar structures of the relevant LOS molecules.

**MATERIALS AND METHODS**

Preparation of purified L3,7,9 LOS. Purified immunoreactive LOS for use in ELISA and biosensor analysis was prepared from meningococcal isolate K454 (B15:1P17.16L3,7,9) as described previously (6). The LOS was resuspended in distilled water, dispensed into 0.5-ml samples, vacuum dried, and stored at -20°C until required. Each sample contained 30 to 60 ng of LOS, as estimated by the Limulus amoebocyte lysate (LAL) chromogenic assay (23). The purity, number of species, and Mᵣ of the LOSs were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining (6, 19) (data not shown).

**Purification of MAbs.** The MAbs were purified from ascites collected from pristane treated mice after intraperitoneal injection with cells of the appropriate hybridoma cell line. The resultant ascitic fluids were buffer exchanged on a Biogel P4 desalting column (Pharmacia, Ph.). With 20 mM sodium phosphate buffer (pH 7.0), the MAbs captured on a protein G column and eluted with 0.1 M glycine (pH 2.7). The peak fractions were pooled, neutralized with Tris (pH 8.8; 65 mM), and buffer exchanged in phosphate-buffered saline (PBS; pH 7.4). The samples were concentrated to ca. 1 μM and tested for immunoglobulin subclass by using an isotyping kit for mouse MAbs (Serotec, Ltd.). The ascites containing MAb 4A8-B2 gave two protein peaks after elution from the protein G column with 0.1 M glycine (pH 2.7). The first peak contained both immunoglobulin G2a (IgG2a) and IgM antibodies, indicating that this sample was contaminated with serum. A second peak contained antibodies of the IgG2a subclass alone, and only the antibodies from this peak were used. Ascites containing MAb 9-2-L379 had a single peak, comprising antibodies of the IgG2a subclass.

**PCR cloning and sequencing of the immunoglobulin variable regions.** Hybridoma cell lines were grown to confluence in 25-ml flasks at 37°C in a 5% CO₂ atmosphere. Total RNA, prepared with an Isogiquick Kit (Orca Research, Inc.), was used to synthesize cDNA by extension of an oligo(dT) primer by using a reverse transcription kit (Clontech UK, Ltd.). The immunoglobulin variable (V) regions encoding each of the MAbs were rescued by PCR from degenerate primers designed from the immunoglobulin framework regions bordering the V₃₄C₄ and V₃₄ domains as described by Kettleborough et al. (11). The PCR products encoding the V₃₄C₄ domains were cloned between the AatII and SalI sites of both the f⁺ and f⁻ forms of the vector pGEMMSZ, while the sequences encoding the V₃₄ regions were similarly cloned between the NcoI and XhoI sites of both pGEMMSZ vectors. The nucleotide sequences of several independently isolated clones were determined on both strands by “cycle sequencing” with a Taqsequencing kit (Amershams) with M13 forward and reverse primers radiolabeled by T4 polynucleotide kinase with [γ-³²P]ATP. ELISA. For whole-cell ELISA microtiter plate wells were coated with N. meningitidis K454 (L3,7,9) by the method of Abdillahi and Poolman (1). Coating of plate wells with purified LOS was as described previously (21). To avoid interplate variation, assays on both MAbs were carried out on the same microtiter plate. The secondary antibody was anti-mouse IgG conjugated to horseradish peroxidase. The absorbance was read at 450 nm 30 min after the addition of the chromogenic substrate (0.4 mg of 1,2-phenylenediamine dihydrochloride and 0.4 mg of urca hydrogen peroxide per ml in 0.05 M phosphate citrate buffer, pH 5.0). Immobilization of L3,7,9 LOS to the resonant mirror biosensor surface. Purified LOS was biotinylated (5), hophylated and stored in lots of 30 to 60 ng, as estimated by the chromogenic LAL assay, at -20°C. Immobilization of the LOS was carried out in an IAsys resonant mirror biosensor (Affinity Sensors, Cambridge, United Kingdom), essentially according to the manufacturer’s protocol. Streptavidin (Sigma) was captured onto the biotin-coated biosensor cuvette surface in PBST (10 mM sodium phosphate–138 mM NaCl–2.7 mM KCl [pH 7.4] containing 0.05% Tween 20), and unbound streptavidin was removed by washing with PBST after 10 min. Biotinylated LOS (3 to 5 ng) was added and binding was monitored. A response of 100 arc seconds was observed on the addition of LOS to the cuvette. Further additions did not increase the sensitivity of the assay (data not shown). A final bovine serum albumin (BSA) blocking step was performed by reacting the biosensor cuvette with 0.1 mg of BSA per ml in PBST for 5 min. The LOS-coated biosensor surface was treated with 20 mM HCl to remove any weakly bound substances before interaction kinetics were performed and also to regenerate the LOS surface prior to interactions with various MAb concentrations. To obtain comparative kinetic data, the same LOS-coated biosensor cuvette was used with both of the MAbs.

**Resonant mirror biosensor analysis.** Real-time kinetic analyses with the IAsys resonant mirror biosensor were undertaken in PBST at 25°C, according to the method described by the manufacturer. The kinetic data were analyzed by curve-fitting software (FASTfit v2.01), and the binding curves from different MAb concentrations were overlaid and plotted by using FASTplot software (both supplied by Affinity Sensors). Dissociation rates (Kᵣₒᵤₜ) were determined by dilution of unbound MAb in the biosensor cuvette to zero concentration at a relatively high concentrations of antibody (~10 times the dissociation equilib- rium constant, Kᵣₒᵤₜ) and averaged to give the dissociation rate constant Kᵣₒᵤₜ.

**RESULTS**

Comparison of MAbs 4A8-B2 and 9-2-L379 against N. meningitidis by ELISA. The concentration-dependent binding of 4A8-B2 and 9-2-L379 MAbs to whole cells and to purified LOS demonstrated that MAb 9-2-L379 exhibited approximately a 1,000-fold-greater binding to both whole cells and purified LOS than 4A8-B2 (Fig. 1). The binding observed for both MAbs to whole cells was approximately 10-fold weaker than to the purified LOS.

**Interaction kinetics of MAbs 4A8-B2 and 9-2-L379 with L3,7,9 LOS.** The real-time binding interactions of 4A8-B2 and 9-2-L379 to purified LOS (3) and whole cells (4) and MAbs 4A8-B2 to purified LOS (3) and whole cells (4) of the same meningococcal isolate are shown. Error bars represent the standard deviation of triplicate determinations.
than that of MAb 4A8-B2 (Fig. 3; Table 1). Although real-time kinetic data between anti-carbohydrate IgGs and their carbohydrate antigens is limited, the binding kinetic data reported in this study are comparable to the binding kinetics of an anti-carbohydrate IgG against \textit{Salmonella} serogroup B O-polysaccharide as determined by using surface plasmon resonance (12).

DNA sequence analysis and primary sequence comparison of the V regions of MAbs 9-2-L379 and 4A8-B2. The VHV-D-J region of MAbs 4A8-B2 and 9-2-L379 shared 81% overall identity, both being derived from the J558 V region family. The MAb 4A8-B2 and MAb 9-2-L379 heavy chains showed similar levels of identity to the germ line genes VMU3.2 and 186-2 and used JH2 and JH3, respectively. The deduced amino acid sequences of their CDR1s were identical, their CDR2s were 88% identical, and their CDR3s had no sequence identity.

The amino acid sequences of the V\textsubscript{L} regions of the MAbs were 54% identical overall, the CDR1s, CDR2s, and CDR3s exhibiting 47, 14, and 11% identity, respectively (Fig. 4). The V\textsubscript{L} regions of the MAbs were encoded by different Vk gene families, Vk 8 in MAb 4A8-B2 and Vk ARS in MAb 9-2-L379. Comparison of the nucleotide sequences of MAb 4A8-B2 and MAb 9-2-L379 with other murine antibody genes revealed that MAb 4A8-B2 \kappa light chain was 82% identical to the D23 germine and used the J\kappa 1 J segment, whereas the MAb 9-2-L379 \kappa chain was 84% identical to germ line gene 28.4.10A(\kappa) and used J\kappa 2. In addition to the high degree of identity to \kappa light chains, both MAb 4A8-B2 and MAb 9-2-L379 had the highly conserved residues phenylalanine at position 71 and glutamine at positions 90 for MAb 9-2-L379 and 89 for MAb 4A8-B2 (Fig. 4), confirming the VL regions as \kappa light chains.

Significant differences were found between the two MAbs in the VL CDR1, where MAb 9-2-L379 had four positively charged residues and MAb 4A8-B2 had one, and in the V\textsubscript{H} CDR3, where MAb 9-2-L379 had no charged residues but three hydrophilic residues and MAb 4A8-B2 had two negatively charged residues and one positively charged residue, with only one hydrophilic residue (Fig. 4).

**DISCUSSION**

\textit{N. meningitidis} presents carbohydrate structures to its human host that mimic self-antigens and are poor immunogens. During colonization of the nasopharynx, switching between the capsulate and acapsulate forms occurs, exposing the capsule and the outer membrane LOS sequentially (10). Consequently, the interactions between host defences and the bacterial carbohydrate are of central importance in understanding the pathogenicity of, and in the development of vaccines against, the meningococcus. Further, mouse MAbs are important reagents in the immunotyping of this organism (18). The measurement of the binding properties of antibodies to antigens by real-time binding kinetic analysis therefore has potential applications in both the standardization of immunotyping reagents and assays and in the investigation of human responses to bacterial antigens.

The two antibodies investigated in the present work, although originally raised against the same meningococcal LOS immunotype, were produced by distinct immunization protocols and exhibited different apparent sensitivities and specificities in routine immunotyping ELISAs (18). The results ob-
used for their production in different laboratories, the two antibodies shared practically identical heavy chains, with the exception of their CDR3s, but possessed diverse light chains. This observation presents the interesting prospect of using antibody engineering techniques (2) to produce anti-L3,7,9 antibodies with different properties by using different combinations of the various complementarity-determining regions (CDRs) reported here. An MAb with the sensitivity of MAb 9-2-L379 but the specificity of MAb 4A8-B2 would be a particularly useful reagent. Such constructs would also be potentially valuable in improving our understanding of the immunology of LOS immunotypes.

The differences of the specificity and binding kinetics of these two MAbs, together with the differences in their sequences and the differences in the arc second response observed, implied that they recognized distinct epitopes within the LOS of immunotype L3,7,9. Previous studies of the specificity of anti-LOS immunotyping MAbs in whole-cell ELISA demonstrated that MAb 9-2-L379 cross reacted with LOS of immunotypes L2, L3,7,9, L5, and L8, whereas MAb 4A8-B2 was specific for LOS of immunotype L3,7,9 (18). These observations, in combination with the data reported here, suggest that the specificity of MAb 4A8-B2 may be the result of interactions with both the phosphoethanolamine (PEA) group 1-3 linked to the second heptose of the L3,7,9 structure and the terminal disaccharide of the lacto-N-neotetraose moiety. Conversely, MAb 9-2-L379 may interact with the galactose residue, located between the N-acetylglucosamine and glucose residues in the lacto-N-neotetraose moiety, in combination with the same PEA group which are present in both the L3 and L8 immunotypes. The relatively weak cross-reactivity of MAb 9-2-L379 with LOS of immunotypes L2 and L5 suggests that the interaction with this PEA group can be replaced, to a limited extent, by interactions with a glucose moiety in the same position (Fig. 5).

While much remains to be learned concerning antibody-LOS interactions, and particularly human antibody-LOS interactions, this analysis of two mouse MAbs by a combination of resonant mirror and antibody sequencing technologies shows the potential of these techniques in enhancing our understanding of antibody-carbohydrate interactions for both routine typing and research purposes.

| MAb    | $K_{on}$ (M$^{-1}$ s$^{-1}$) | $K_{off}$ (s$^{-1}$) | $K_{D}$ (M) |
|--------|-----------------------------|--------------------|------------|
| 9-2-L379 | 1.33 ($\pm 0.01$) $\times 10^6$ ($n = 7$) | 0.010 ($\pm 0.005$) ($n = 10$) | 133 ($\pm 67$) $\times 10^6$ |
| 4A8-B2  | 0.03 ($\pm 0.002$) $\times 10^6$ ($n = 8$) | 0.007 ($\pm 0.004$) ($n = 8$) | 4.29 ($\pm 2.47$) $\times 10^6$ |

$K_{D}$ is calculated from the $K_{on}/K_{off}$ ratio. $K_{D}$ is calculated from the $K_{on}/K_{off}$ ratio. Figures in parentheses are the standard deviation of the given measurement, and $n$ is the number of replicate $K_{on}$ or $K_{off}$ rates used.

FIG. 4. Primary sequence comparison of the variable regions within MAbs 4A8-B2 and 9-2-L379. The aligned, deduced primary structures of the two antibodies revealed differences responsible for their distinct binding activities. Notwithstanding the different protocols used for their production in different laboratories, the two antibodies shared practically identical heavy chains, with the exception of their CDR3s, but possessed diverse light chains. This observation presents the interesting prospect of using antibody engineering techniques (2) to produce anti-L3,7,9 MAbs with different properties by using different combinations of the various complementarity-determining regions (CDRs) reported here. An MAb with the sensitivity of MAb 9-2-L379 but the specificity of MAb 4A8-B2 would be a particularly useful reagent. Such constructs would also be potentially valuable in improving our understanding of the immunology of LOS immunotypes.
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