Structural characterization and MHCII-dependent immunological properties of the zwitterionic O-chain antigen of *Morganella morganii*

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*Morganella morganii* is a commensal Gram-negative bacterium that has long been known to produce an antigen bearing phosphocholine groups. We determined the structure of this O-chain antigen and found that its repeating unit also contains a free amino group and a second phosphate:

\[
\text{Cho-P-2} \quad (\text{Ac-6})_\text{as} \quad [\text{4-\(\beta\)-D-Gal\(\beta\)-(1→3)-\(\beta\)-D-Gal\(\beta\)NAc-(1→3)-Gro-1-P(O→)}]_\text{h} \quad \alpha-\text{D-GalN-(1→3)}
\]

This alternating charge character places the *M. morganii* O-chain polysaccharide into a small family of zwitterionic polysaccharides (ZPSs) known to induce T-cell-dependent immune responses via presentation by class II major histocompatibility complex (MHCII) molecules. In vitro binding assays demonstrate that this O-chain interacts with MHCII in a manner that competes with binding of the prototypical ZPS antigen PSA from *Bacteroides fragilis* (Cobb et al. 2004; Cobb and Kasper 2008), and there is evidence for similar behavior by other ZPSs such as the teichoic acid from *Streptococcus pneumoniae*, termed C-substance or PnC (Tzianabos et al. 1993), and the *S. pneumoniae* type 1 capsular polysaccharide (Tzianabos et al. 1993; Velez et al. 2009). The structure of PnC includes phosphocholine groups, which are themselves zwitterionic, as well as an amino group and a second phosphate (Kulakowska et al. 1993).

The reactivity of several mouse myeloma proteins with PnC led to the finding that they were recognizing its phosphocholine moiety (Leon and Young 1971), and crystallography of the Fab fragment of one such myeloma protein, M603, with phosphocholine gave the first structure of an antibody with a bound hapten (Satow et al. 1986). The phosphocholine hapten is small when compared with the overall dimensions of the binding site, and hence additional interactions could occur between the M603 binding site and the antigen that bears the phosphocholine epitope. The identity of the original immunogen cannot be established with certainty, but Potter (1971) found that several organisms in the environment and flora of laboratory-raised Balb/c mice carried antigens that...
contained phosphocholine. These included Aspergillus species, the parasite Ascaris suum and a Gram-negative bacterium from the normal mouse flora, Morganella (Proteus) morganii. The M. morganii organism is an opportunistic pathogen in humans causing bladder infections and bacteremia (for hospital surveys, see Kim et al. 2003; Falagas et al. 2006), and several O-antigen serotypes have been recognized by studies of organisms recovered from patients (Penner and Hennessy 1979). Immunization of mice with M. morganii cells led to hybridoma antibodies predominantly arising from the same VH and Vk germline genes as M603 (Claffin et al. 1985), principally varying from it in the second complementarity-determining region of the H-chain (Claffin et al. 1987). However, M603 binds the M. morganii antigen less strongly (Claffin et al. 1985). The restriction to the M603 family was in contrast to immunization experiments with a rough strain of S. pneumoniae, R36A, rich in PnC, which gave hybridomas related to two other phosphocholine myeloma families T15 and M167/M511 as well as M603 (Claffin and Berry 1988); the three families use different Vk germline genes.

The antibody properties found against the M. morganii phosphocholine-containing antigen prompted us to perform a structural analysis of the O-chain polysaccharide. We found not only phosphocholine, but also an amine and a second phosphate, extending its zwitterionic nature and raising the possibility for MHCII presentation and subsequent T-cell recognition as seen with other ZPS molecules. Here, we report its structure, demonstrate MHCII binding and T-cell activation and characterize the binding of repeating unit fragments by hybridoma antibodies. These findings reveal the first known example of an O-chain polysaccharide with the capacity to activate CD4+ T-cells via MHCII presentation, potentially identifying another commensal organism with the ability to promote immune system homeostasis (Mazmanian et al. 2005; Ochoa-Reparaz et al. 2010).

Results

Determination of the polysaccharide structure

The M. morganii strain used in the above studies was serotyped by Dr. J. Penner and it belonged to the most common serotype, O:lab (Penner and Hennessy 1979). Exclusion experiments and tests for quelling reaction with the monoclonal antibody gave no evidence for capsular polysaccharide around organisms grown in liquid culture or on plates. Extraction with phenol in the standard manner for lipopolysaccharides (LPSs) gave only poor yields of antigens compared with extraction with sodium dodecyl sulfate (SDS)-citrate. When the latter extract was ultracentrifuged, both the supernatant and precipitate contained antigens. Fractionation of the supernatant on Sephadex G100 yielded pure polysaccharide antigen, as well as a lower molecular fraction that was predominantly enterobacterial common antigen. This was a linear form with a well-resolved nuclear magnetic resonance (NMR) spectrum (data not shown), which was fully assignable to the reported repeating unit structure of this polysaccharide, in contrast to the circular or lipid-attached forms previously reported (Dell et al. 1984).

NMR experiments on the LPS precipitate dissolved in deuto-SDS/ethylene diamine tetraacetic acid (EDTA) (Risberg et al. 1999) showed that it contained significantly more antigens, and mild acid hydrolysis of the LPS precipitate released antigenically active O-chain, which was separated by gel filtration from a core fraction and free Kdo. The total yield of antigens was approximately 3 mg/g of cells (wet weight). LPS was also isolated from a serotype O:1 reference strain, ATCC 499993, and NMR spectra obtained in deuto-SDS/EDTA were consistent with it having the same polysaccharide structure as the Potter strain (data not shown).

Complete acid hydrolysis of the O-chain fraction afforded D-galactose and D-galactosamine (at an approximate molar ratio of 1:2) together with low amounts of glycerol, as determined by gas–liquid chromatography (GLC) of the derived alditol acetate derivatives. The absolute stereochemistry of the glycoses was determined from the GLC retention times of the trimethylsilylated R-2-butyl glycoside derivatives. Colorimetric analysis for phosphate groups gave 20% (w/w) as PO₄, consistent with the two groups found by NMR (see below).

The 13C NMR spectrum of the O-chain indicated the polymer to be composed of regular repeating units that contained three glycosyl residues (Figure 1). A strong signal corresponding to the choline methyl groups was evident at 55.1 ppm and substitution by O-acetyl groups was indicated by signals at 21.5 ppm (CH3) and 174.6 ppm (C=O). These latter signals were absent in the 13C NMR spectrum of the O-deacetylated O-chain (Figure 1B), which also showed that only one of the galactosamine residues was N-acetylated.

The 1H and 13C NMR spectra of the native and O-deacetylated polymers were fully assigned by 2D homo- and heteronuclear chemical shift correlation techniques (Figure A and B; Tables I and II). From the chemical shifts and 1H-1H vicinal couplings, the subspectra corresponding to α- and β-linked α-D-GalpN residues and a β-D-Galp residue were identified. N-Acetylation of the polymer led to a substantial upfield shift of the α-D-GalpN H-1 resonance (ca. 0.4 ppm) which indicated this residue to be present as the free amino sugar in the native O-chain. Resonances for glycerol and choline spin systems were also identified in the 2D spectra. A comparison of the NMR data from the O-deacetylated and the native O-chains indicated that the 6-position of the β-D-GalpNAc was substituted by O-acetyl groups to the extent of 80%.

The occurrence of transglycosidic NOEs between H-1 of α-D-GalpN and H-3 of β-D-Galp, between H-1 of β-D-Galp and H-3 of β-D-GalpNAc and between H-1 of β-D-GalpNAc and the methylene protons of glycerol (H-3) established the arrangement of the residues within the O-chain repeating unit:

\[
\begin{align*}
[A] & \quad [B] & \quad [C] \\
\alpha-D-GalpN(\rightarrow 3)-\beta-D-Galp(\rightarrow 3)-\beta-D-GalpNAc(\rightarrow 3)-\text{glycerol} \\
\end{align*}
\]

Methylation analysis confirmed the positions of the linkages.

The H-2 and H-4 resonances of the β-D-Galp residue showed additional coupling and were down-field shifted compared with the corresponding resonances of the methyl
glycoside; this indicated phosphate substitution at the corresponding positions. The $^{31}\text{P}$ NMR spectrum showed two resonances at $-2.0$ and $1.5$ ppm. The high-field resonance was assigned to $^{31}\text{P}$ of the phosphocholine since a correlation to the adjacent methylene protons was observed in the $^1\text{H}$–$^{31}\text{P}$ correlation experiment (Figure 2C). In addition, this resonance showed a strong correlation to H-2 of β-d-Galp, which established the site of substitution at the C-2 position. The low-field $^{31}\text{P}$ signal showed connectivities to C-4 of β-d-Galp and the methylene protons (H-1) of the glycerol moiety, indicating the O-chain to have the teichoic acid-like structure shown in Figure 3. This structure is also consistent with the ES-MS spectrum of the intact polysaccharide (Figure 4), which showed prominent ions from the acetylated and free repeating units, together with characteristic fragment ions for the components.

Treatment of the N-acetylated O-antigen with 48% HF afforded the expected glycerol trisaccharide glycoside (1), together with significant amounts of the free trisaccharide. The acid lability of the glycosidic linkage of 3-linked β-d-GalpNAc has been previously observed (Jennings and Lugowski 1980). Heating of the O-antigen with 1% trifluoroacetic acid (TFA) at 80°C for 1 h led to cleavage at the same position, liberating the complete zwitterionic repeating unit.

Circular dichroism spectroscopy of the ZPS

NMR studies have shown that the ZPSs from B. fragilis (Wang et al. 2000) and S. pneumoniae (Choi et al. 2002), PSA and Sp1, respectively, are predominantly helical and that the immunologic properties of PSA depends on this helical content as measured by circular dichroism (CD; Kreisman et al. 2007). The far-UV CD spectra of the M. morganii ZPS and the monomer of its repeating unit were therefore determined. Molar ellipticities were calculated on the basis of the mass of the repeating unit, and these spectra showed only weak features in the 200–230 region (Figure 5). The ellipticities at 212 nm were $-2.4$ and $-2.0 \times 10^4$ deg cm$^2$ dmol$^{-1}$, respectively, which are two orders of magnitude less than the corresponding value for the B. fragilis PSA, calculated from the data of Kreisman et al. (2007) to be $-1.9 \times 10^6$ deg cm$^2$ dmol$^{-1}$ at 225 nm. These data suggest that the M. morganii ZPS is not significantly helical in nature, although a full NMR-based conformational analysis is needed to confirm these findings.

Interaction with a monoclonal antibody

In order to begin assessment of the immunologic properties of the M. morganii ZPS, the O-chain was hydrolyzed with mild acid to fragment the molecule into a mixture of the monomer and higher oligomers of its repeating unit, which were purified by gel filtration. Their binding affinities for an anti-M. morganii hybridoma, 180.7c9, were measured in an enzyme immunoassay inhibition assay, with antigen partially biotinylated on the amino groups of its D-galactosamine residues (Meikle et al. 1990). The oligomer fragments were better inhibitors than glycerophosphocholine (Figure 6 and Table III), but were weaker than phosphocholine.

Interaction with MHCII

Purified recombinant human MHCII (HLA-DR1) was used in binding assays as described by Cobb and Kasper (2008) with 5 kDa M. morganii ZPSs biotinylated on the amino groups of its D-galactosamine residues (Meikle et al. 1990) to approximately 1 mol of biotin per mole of polysaccharide to
determine whether the nature of ZPSs would enable MHCII binding. Binding between M. morganii ZPSs and MHCII was saturable with an apparent $K_D$ value of $3.66 \pm 0.63 \mu M$ (Figure 7A), which is similar to the $K_D$ between HLA-DR1 and B. fragilis PSA ($1.9 \pm 0.4 \mu M$; Cobb and Kasper 2008). Experiments using unlabeled PSA as a binding competitor with the biotinylated M. morganii ZPS showed that these two molecules appear to compete for the same binding site on MHCII (Figure 7B). In contrast, binding experiments with the M. morganii monomer or dimer forms as competitors failed to reduce the 5 kDa M. morganii ZPS binding, reflecting a length requirement for MHCII interactions beyond the basic repeating unit structure, as seen with PSA (Kreisman et al. 2007). However, both acetylated and control (native polysaccharide) effectively competed for binding with the labeled 5 kDa molecule (Figure 7C). Since the acetylated molecule has lost the positive charge on the primary amine, these data suggest that the zwitterionic nature of the phosphocholine group in itself is sufficient to retain the MHCII binding properties.

**T-cell activation assays**

Given the observed ability to bind with MHCII protein in vitro (Figure 7A–C), we sought to determine whether the M. morganii O-antigen could also mediate activation of CD4$^+$ T-cells. Human peripheral blood mononuclear cells (PBMCs) from a hepatitis B peptide antigen (HepB)-immunized individual were used to isolate CD4$^+$ T-cells using positive selection on magnetic beads as well as T-cell-depleted antigen-presenting cells (APCs) using CD3 negative selection. For activation, CD4$^+$ T-cells and APCs were co-cultured at a 1:1 ratio with M. morganii ZPSs or B. fragilis PSA at 50 µg/mL. Controls included media alone (no antigen-negative control), superantigen [Staphylococcus enterotoxin B (SEB); positive control] or hepatitis B peptide (conventional antigen-positive control). On days 5, 6 and 7, culture supernatants were collected and analyzed for interferon-$\gamma$ (IFN$\gamma$) by enzyme-linked immunosorbent assay (ELISA) as a measure of T-cell activation. The M. morganii ZPS activated the CD4$^+$ T-cells to a similar extent as the B. fragilis PSA (3084 pg/mL IFN$\gamma$ vs. 5807 pg/mL IFN$\gamma$ on day 7, respectively; Figure 7D). The stimulation index was calculated to illustrate fold IFN$\gamma$ production over the negative media control (set to unity) and demonstrates a stimulation of 17-fold for the M. morganii ZPS antigen on day 7 (Figure 7E). To confirm that T-cell activation was due to presentation via MHCII, activation was compared with and without blocking antibody specific for HLA-DR (clone L243). We found that INF$\gamma$ production was abrogated in the presence of anti-MHCII antibody (Figure 7F). Collectively, these data show for the first time that a bacterial O-antigen can activate CD4$^+$ T-cells via MHCII presentation.

**Discussion**

Since the discovery that ZPSs stimulate the T-cell-dependent immune system directly (Tzianabos et al. 1993), only a small number of them have had their interactions with the MHC determined by direct assay (Cobb and Kasper 2008; Velez...
et al. 2009). To date, all of the identified ZPS molecules are capsular polysaccharides and the most characterized example is PSA from B. fragilis, commensal Gram-negative organism. The M. morganii antigen is the first example of an O-chain polysaccharide that is zwitterionic in nature and shows similar immunological properties to those of PSA and other ZPS molecules. The structure we have determined was confirmed as the O1 form by examining the O-chain from a second strain, ATCC 49993, which has also been serotyped as O1. In contrast, it does not resemble in any way the M. morganii structure determined by Kilcoyne et al. (2002), whose serotype was not reported.

Investigation of the structure of the M. morganii O-chain not only confirmed the presence of phosphocholine, but also disclosed the presence of another pair of ions in the repeating unit, an amino group and a phosphate diester, adding to the zwitterionic character of the polysaccharide. The backbone phosphate group and the amino group on the α-β-GalNAc are brought together by the conformation of the molecule, close enough for them to form a salt bridge, a most unusual feature in a polysaccharide. The interaction of the polysaccharide with MHCII molecules and the resulting T-cell activation were very similar on a weight basis to those of the prototypic ZPS PSA. However, in the M. morganii case, the CD spectra were not intense enough to suggest a helical structure, a feature of PSA that has been linked to its ability to interact with MHCII molecules (Kreisman et al. 2007).

Table I. ¹H NMR chemical shifts (ppm) and coupling constants (Hz) for O-deacylated M. morganii O-chain

<table>
<thead>
<tr>
<th>Component</th>
<th>H-1 (J1,2)</th>
<th>H-2 (J2,3)</th>
<th>H-3 (J3,4)</th>
<th>H-4 (J4,5)</th>
<th>H-5</th>
<th>H-6 (J6,6)</th>
<th>H-6 (J6,6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A α-β-GalN</td>
<td>5.55 (3.6)</td>
<td>3.56 (10.7)</td>
<td>4.35 (2.8)</td>
<td>4.04 (~1)</td>
<td>4.34</td>
<td>3.77h</td>
<td></td>
</tr>
<tr>
<td>B β-β-Gal</td>
<td>4.75 (7.8)</td>
<td>4.24 (9.8) (10.8)c</td>
<td>4.04 (~3)</td>
<td>4.77 (~1) (10.0)c</td>
<td>3.76</td>
<td>3.77h</td>
<td></td>
</tr>
<tr>
<td>C β-β-GalNAc</td>
<td>4.46 (8.2) [4.48]^</td>
<td>4.01 (10.8)</td>
<td>4.05 (~3) [4.06]</td>
<td>4.15 (~1) [4.18]</td>
<td>3.73 [3.93]</td>
<td>3.83 (7.2) [4.34]</td>
<td>3.79 (2.7, 11.3) [4.31]</td>
</tr>
<tr>
<td>Phosphocholinef</td>
<td>~3.44h (~6)^</td>
<td>~3.66b</td>
<td>4.01 (5.5)</td>
<td>3.67</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>~3.99h (~6) (8.0)c</td>
<td>4.01 (5.5)</td>
<td>3.67</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Values in square brackets correspond to resonances shifted (&gt;±0.01 ppm) in native O-acetylated polysaccharide.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unresolved chemical shift.</td>
</tr>
<tr>
<td>Unresolved coupling constant.</td>
</tr>
<tr>
<td>Values in square brackets correspond to resonances shifted (&gt;±0.01 ppm) in native O-acetylated polysaccharide.</td>
</tr>
<tr>
<td>Unresolved chemical shift.</td>
</tr>
</tbody>
</table>

Table II. ¹³C NMR chemical shifts (ppm) for O-deacylated M. morganii O-chain

<table>
<thead>
<tr>
<th>Component</th>
<th>C-1</th>
<th>C-2</th>
<th>C-3</th>
<th>C-4</th>
<th>C-5</th>
<th>C-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>A α-β-GalN</td>
<td>91.80</td>
<td>51.18</td>
<td>67.64</td>
<td>69.37</td>
<td>72.47</td>
<td>61.69h</td>
</tr>
<tr>
<td>B β-β-Gal</td>
<td>103.57 (5.8)^</td>
<td>74.34 (6.4)⁵</td>
<td>74.34 (~4)⁵</td>
<td>70.32 (5.7)⁵</td>
<td>75.67 (2.8)e</td>
<td>62.38h</td>
</tr>
<tr>
<td>C β-β-GalNAc</td>
<td>103.67</td>
<td>52.47 [52.28]⁸</td>
<td>78.12 [77.99]</td>
<td>69.23</td>
<td>75.85 [73.19]</td>
<td>61.92 [64.68]</td>
</tr>
<tr>
<td>Phosphocholineh</td>
<td>67.19 (~8)⁴</td>
<td>60.58 (5.7)e</td>
<td>70.31 [7.6]f</td>
<td>67.52 (5.7)⁴</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>71.67 [71.86]⁸</td>
<td>70.31 (7.6)²</td>
<td>67.52 (5.7)⁴</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Measured at 320 K in D₂O (p D ~ 4). |
| Assignments may be interchanges. |
| Unresolved ¹J_C coupling constant. |
| NAc ≥C =O, 175.4 ppm; NAc CH₃, 23.35 ppm. |
| Values in square brackets correspond to resonances shifted (>±0.01 ppm) in native O-acetylated polysaccharide. |
| Phosphocholine CH₃ groups, 55.05 ppm. |
PnC is a teichoic acid of the ribitol phosphate type, and the *M. morganii* ZPS would be classified as a teichoic acid of the glycerol phosphate type, if it were not from a Gram-negative organism. The evidence at present indicates that the ZPS is an LPS O-chain, although O-chains with charged groups are uncommon. This evidence includes the (i) NMR spectrum of the ultracentrifuge precipitate, (ii) the “ladder” of bands found by silver-staining of deoxycholate polyacrylamide gel electrophoresis (PAGE) gels of it and by immunoblotting (data not shown), and (iii) the lack of evidence for a capsule. The overall charge on the polysaccharide is zero, since the two phosphate negative charges are counterbalanced by the positive charges on the D-GalN and choline constituents. Similarly, PnC is also neutrally charged, with an amino group on the trideoxy sugar offset by a phosphate group in the backbone, plus two phosphocholine groups.

The *M. morganii* ZPS is acid-labile, cleaving facilely at the β-D-GalNAc glycosidic bond to glycerol, rather than at the backbone phosphate as one might expect. This lability may explain two properties of the polysaccharide: the poor yield of the antigen obtained by phenol extraction, which may be due to the acidity of the extraction conditions, and the appearance of the antigen in both the supernatant and precipitate fractions from the ultracentrifugation. The facile cleavage enabled us to prepare oligosaccharides representing the repeating unit and oligomeric forms of it. Because the phosphate is not the site of cleavage, the charge arrangement within the repeating unit in the polymeric antigen is retained in the monomer. The monomeric fragment has the phosphocholine unit centrally placed, thus the sugar units would fill the center of the binding site around the phosphocholine pocket.

**Fig. 3.** Structure of the *M. morganii* LPS O-chain (R = Ac, 80% or H, 20%) and comparison with the structures of two other ZPS species, *S. pneumoniae* PnC and *B. fragilis* PSA.
The inhibition experiments with antigen fragments show that the phosphocholine group is very much the immunodominant feature in antigen recognition by the hybridoma antibody 180.7c9. The antigen fragments had activities that were intermediate between those of the two simpler ligands, phosphocholine and glycerophosphocholine (Table III). The concentration for 50% inhibition approached $5 \times 10^{-5}$ M, which approximates the dissociation constant in the type of enzyme immunoassay system used here. This affinity for antigens is within the range normally found for anti-carbohydrate IgMs, despite the high affinity for phosphocholine itself, and the antigen’s charged groups do not lead to stronger binding in the manner of Kdo-containing antigens (Müller-Loennies et al. 2000). It appears that whatever new hydrogen bonds or other interactions are formed in the binding of the oligomers, increasing their affinities up to 7-fold compared with glycerophosphocholine, their effects are largely negated by other unfavourable interactions. The affinities of the oligomers are approximately proportional to their phosphocholine contents, suggesting that there are no extended interactions beyond one repeating unit. However, the restriction of the anti-\textit{M. morganii} hybridomas to the M613 family shows that the structure

Fig. 4. ES-MS spectrum of the intact polysaccharide. The annotated series of ions assigned to fragmentation of the repeating unit is paralleled by a second set with masses 18 Da lower, arising from initial loss of a water molecule. The major peaks to the left of the dimer ion can be assigned to successive losses of two GalN and one phosphoglycerol substituents. The peak at $m/z$ 346.2 indicated with an arrow is from GalNAcOAc.

Fig. 5. CD spectra of \textit{M. morganii} ZPS (solid line) and its monomer fragment (dashed line). The spectra were calculated as molar ellipticities per monomer unit.

Fig. 6. Inhibition enzyme immunoassays of the monoclonal antibody 180.7c9 and biotinylated \textit{M. morganii} antigen. The antigen fragments and other haptens tested were, from the left, phosphocholine (♦), tetramer of the repeating unit (●), trimer (●), dimer (□), monomer (●) and glycerophosphorylcholine (●).
that presents the phosphocholine moiety does have an important role, despite its limited effect on the binding constant, and there is an interdependence of this dominant epitopic feature and the presenting polysaccharide. It is interesting that the sequence differences in the CDR H2 regions of the M. morganii antibodies compared with M603 (Claffin et al. 1989) lead to a very different distribution of positive and negative sidechains around this loop, i.e., -53KNTHD- in 180.7c9 compared to -53NKGNK- in M603. This may allow more charge interactions with the phosphate/GalN region of the antigen compared with M603, which binds only weakly to the M. morganii antigen (Claffin et al. 1985).

M. morganii is a component of the normal human flora that can cause nosocomial infections, usually following surgery, much like Bacteroides. Remarkably, B. fragilis, through PSA and other polysaccharide components, has been postulated to play a role in the development of the mucosal immune system (Mazmanian et al. 2005). Recent studies have also shown that T-cells activated by ZPS molecules result in anti-inflammatory regulatory T-cells that can prevent the onset of inflammatory bowel disease (Mazmanian et al. 2008) and even central nervous system demyelinating disease (Ochoa-Reparaz et al. 2010) in animal models, suggesting that commensal bacteria that carry ZPS molecules can directly contribute to immune homeostasis and protect against inappropriate inflammation through T-cell activation. The occurrence of a T-cell-activating ZPS in the LPS of M. morganii, itself a commensal bacterium, not only suggests it could also contribute to the overall balance of the immune system, but also highlights the importance of including the O1 serotype M. morganii in the sequencing efforts to understand the human microbiome community.

**Materials and methods**

**Growth of the organism**

The M. morganii strain originally isolated by Potter (1971) and a monoclonal antibody to the phosphocholine antigen, 180.7C9.1, were kindly provided by Dr. L. Claffin. This strain is no longer available, but NMR experiments confirmed that the serotype O1 reference strain ATCC 49993 bears the same polysaccharide antigen. The organism was grown in 20 L batches in Bacto brain-heart infusion for 20 h at 37°C. The cells were harvested by centrifugation and extracted with 0.05% SDS, 0.1 M sodium citrate for 1 h at 56°C (Williams and Claffin 1980). The extract was centrifuged and the supernatant was extensively dialysed and then freeze-dried.

**Purification of the polysaccharide**

The extract was re-suspended in phosphate-buffered saline (PBS) and treated with nuclease and ribonuclease, followed by proteinase K. After dialysis and freeze-drying, it was re-suspended in PBS and ultracentrifuged at 100,000 × g for

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**Table III.** Inhibition of antigen binding by the hybridoma 180.7c9 in ELISA, by phosphocholine hapten and oligomers of the repeating unit of the ZPS.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration for 50% inhibition (M)</th>
<th>Inhibition ratio (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphocholine</td>
<td>7.0 × 10^-6</td>
<td>156</td>
</tr>
<tr>
<td>Glycerophosphocholine</td>
<td>4.5 × 10^-4</td>
<td>1</td>
</tr>
<tr>
<td>Monomer</td>
<td>2.2 × 10^-4</td>
<td>2.3</td>
</tr>
<tr>
<td>Dimer</td>
<td>1.1 × 10^-4</td>
<td>3.5</td>
</tr>
<tr>
<td>Trimer</td>
<td>6.3 × 10^-5</td>
<td>7.5</td>
</tr>
<tr>
<td>Tetrmer</td>
<td>5.6 × 10^-5</td>
<td>9</td>
</tr>
</tbody>
</table>

\(^a\)Compared with glycerophosphorylcholine.

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**Fig. 7.** Analyses of M. morganii ZPS interactions with MHCI and downstream T-cell activation. (A) Binding curve of M. morganii ZPS with HLA-DR1, showing saturable interactions with a Kd value of 3.66 ± 0.63 μM. The dotted lines represent the 95% confidence interval of the curve fit. (B) Binding competition experiments using unlabeled B. fragilis PSA with the biotinylated M. morganii ZPS, showing that these molecules utilize the same MHCI binding site. (C) Unlabeled monomeric and dimeric fragments of the M. morganii ZPS failed to inhibit the 5 kDa ZPS binding, although N-acetylation of the ZPS had no effect on the ability to inhibit, indicating that the acetylated ZPS still bound well to MHCI. (D) T-cell activation studies using a variety of positive (PSA, HepB and SEB) and negative (media alone) controls revealed that the M. morganii ZPS induced a strong T-cell response as measured by IFNγ production (3084 pg/mL). By day 7, the stimulation of the T-cells was 17-fold over background (E). Activation on day 6 was also compared in separate assays with and without anti-MHCI monoclonal antibody (clone L243) to confirm the MHCI dependence of activation. IFNγ production was abrogated in the presence of blocking mAb for both PSA and M. morganii O-Ps (F).
16 h. The supernatant was fractionated by gel filtration on a 2.5 × 90 cm column of Sephadex G100 (GE Biosciences, Inc., Baie D’Urfé, Quebec, Canada) in 0.1 M ammonium acetate. Fractions were tested for the antigen by immunodiffusion in gel (Ouchterlony procedure) with the above monoclonal antibody. The LPS precipitate was re-suspended in 2% acetic acid and hydrolyzed at 100°C for 1.5 h. Insoluble material was removed by centrifugation and the supernatant was freeze-dried. The O-chain was separated from LPS core material and free Kdo by gel filtration on Sephadex G50, in pyridine-acetic acid buffer, 0.05 M, pH 4.7. The fractions were assayed for hexose/heptose (Dubois et al. 1956), hexosamine (Gatt and Berman 1965) and Kdo (Aminoff 1965).

Composition analyses

Hexose composition was determined by GLC separation of alditol acetate derivatives of the products of hydrolysis with 3 N HCl at 100°C for 3 h (Gunner et al. 1961). The absolute stereochemistry was determined by GLC of trimethylsilylated R-2-butyl derivatives (Gerwig et al. 1978). Phosphate content was measured colorimetrically (Chen et al. 1956). O-Acetyl groups were estimated from the NMR spectra and were removed by treatment with 5% NH₃ at 37°C for 3 h, and free amino groups were acetylated by treatment with acetic anhydride at pH 10. Methylation analysis was done by the method of Hakomori (1964).

Oligosaccharide preparations

The core trisaccharide was obtained in dephosphorylated form by treatment with 48% aqueous HF. Hydrolysis with 1% trifluoroacetic acid led to production of the intact repeating unit, while mild treatments with 0.2% trifluoroacetic acid at 100°C for 1 h produced the di-, tri- and tetra-oligomers of this structure. The fragments were separated by gel filtration on a column (1 × 57 cm) of BioGel P4, 100–200 mesh (Bio-Rad, Mississauga, Ont., Canada) run in water. The sizes of the fragments and their structures were checked by mass spectrometry and proton NMR.

NMR spectroscopy

NMR spectra were obtained with a Bruker AMX 500 spectrometer using standard Bruker software. All measurements were made on solution at 320 K in 0.5 mL of D₂O, subsequent to several lyophilizations with D₂O. Proton spectra were obtained by using a spectral width of 10.6 KHz and a 90° pulse. Acetone was used as the internal standard, and chemical shifts were referenced to the methyl resonance (δH, 2.225 ppm; δC, 31.07 ppm). 31P NMR spectra were measured at 202 MHz using spectral width of 12.1 and 41.6 KHz and phosphoric acid (85%) was used as the external standard (δp, 0.0 ppm). Correlation spectroscopy (COSY) and nuclear overhauser effect spectroscopy (NOE Sy) experiments were performed as described earlier (Masoud et al. 1994); a mixing time of 400 ms was used for NOE Sy. Heteronuclear 2D 13C–1H and 31P–1H heteronuclear multiple quantum correlation (HMQC) correlation experiments were performed as previously described (Masoud et al. 1994). LPS samples were solubilized for NMR by adding perdeutero-EDTA (2 mM) and perdeutero-SDS (10 mg/mL) to the D₂O solutions (Risberg et al. 1999). These deuterated compounds were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA).

Mass spectrometry

CE-MS was performed using a Prince CE system (Prince Technologies, Emmen, The Netherlands) coupled to a 4000 QTrap mass spectrometer (Applied Biosystems/Scienx, Concord, Ont., Canada). CE separation was obtained on a 90 cm length of bare fused silica capillary (365 μm OD × 50 μm ID) with CE-MS coupling using a liquid sheath-flow interface and isopropanol:methanol (2:1) delivered at a flow rate of 2 μL/min. An aqueous buffer consisting of 10 mM ammonium acetate, pH 7.0, was used for all experiments. For analysis of intact polysaccharide, in-source collision-induced dissociation was used to afford fragments representing repeating units of the O-chain in the intact polymer. This was accomplished by increasing the declustering potential in the skimmer region of the electrospray ionization source to 400 eV, thus applying extra internal energy to ionic species entering the mass spectrometer and generating fragments where signals corresponding to multiply-charged intact molecules would otherwise not be observed (Li et al. 2005).

MHCIıı binding assays

Purified recombinant human MHCIıı (HLA-DR1 allele) was used in binding assays as previously described for the B. fragilis antigen PSA (Cobb and Kasper 2008). M. morganii ZPS was reacted with aminohexyl-biotin-N-hydroxysuccinimide ester (Invitrogen Canada, Inc., Burlington, Ont., Canada) according to the manufacturer’s directions, to attach biotin to the free amino groups of its D-galactosamine residues (Meikle et al. 1990). Its biotin content, determined with a FluoReporter Biotin Quantitation Kit (Invitrogen Canada, Inc.), was ~0.7 mol of biotin per mole of ZPSs, assuming that its molecular weight was 5 kDa. Binding of the labeled antigen to MHCIıı was compared with that of the B. fragilis antigen using detection by europium-conjugated streptavidin and time-resolved fluorescence (Cobb and Kasper 2008). Molar quantities and Kd values were calculated based on an average molecular weight of 5 kDa.

T-cell activation assays

Human PBMCs were isolated on a Lymphoprep cushion as previously described. CD4+ T-cells were first isolated from PBMCs by positive selection using anti-CD4 magnetic beads according to the manufacturer’s protocol (Miltenyi Biotec, Inc., Auburn, CA). The remaining CD4+ cells were then depleted using anti-CD3 magnetic beads according to the manufacturer’s protocol (Miltenyi Biotec, Inc.) to yield a T-cell-negative population of cells that were used as APCs. APCs were then treated with Mitomycin C to prevent proliferation. For activation, APCs and CD4+ T-cells were co-cultured at a 1:1 ratio (1 × 10⁵ APCs and 1 × 10⁵ T-cells) in 200 μL of RPMI1640 media supplemented with 10% fetal bovine serum. Antigens were added at the following concentrations: SEB (superantigen control) = 50 ng/mL, PSA (ZPS control) = 50 μg/mL, HepB (conventional protein antigen control;
volunteer was previously hepatitis B virus vaccinated) = 5 µg/mL and *M. morganii* ZPS = 50 µg/mL. “Media” assays were performed without added antigens as a negative control. For antibody-blocking experiments, 25 µg of purified anti-HLA-DR monoclonal antibody (clone L243, purified from HB-55 hybridoma cells; ATCC, Manassas, VA) was added to each well at the same time as the antigens. On the indicated days, culture supernatants were collected and analyzed for IFNγ by ELISA as a measure of T-cell activation.

**Enzyme immunoassays**

Optimum amounts of monoclonal antibody 180.7c9 (provided by Dr. L. Claflin) for coating the plates, biotinylated antigens, prepared as above, and streptavidin-horseradish peroxidase conjugate were established in preliminary experiments, to obtain an approximate absorbance at 414 nm of 1.0 after 30 min development with the substrate 2,2-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid), di-ammonium salt (Sigma-Aldrich Canada Ltd., Oakville, Ont., Canada). Antibody solutions in PBS, 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.2, were coated for 3 h, the plates were washed with 0.2% Tween 20 in PBS, blocked for 1 h with 0.1% skim milk in PBS-Tween and washed again with PBS-Tween. Biotinylated antigen in PBS-Tween milk and serial dilutions of milk in PBS-Tween and washed again with PBS-Tween. Biotinylated antigen in PBS-Tween milk and serial dilutions of the test inhibitors in the range 10⁻³ to 10⁻⁷ M were added simultaneously and the plates were incubated at room temperature overnight. After washing as before, the streptavidin conjugate and substrate were added. Colour development was measured with a Titertek Multiscan MC plate reader (Flow Laboratories, Mississauga, Ont., Canada).

**CD spectroscopy**

Spectra were obtained with a Jasco J-815 spectrometer (Jasco, Inc., Easton, MD), for 2.5 mg/mL solutions of the intact antigen and the monomer fragment, in PBS. The pathlength of the cell was 0.1 cm. The spectra were calculated as molar ellipticities per monomer unit.

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**Conflict of interest statement**

None declared.

**Abbreviations**

APC, antigen-presenting cell; CD, circular dichroism; CE-MS, capillary electrophoresis - mass spectrometry; COSY, correlation spectroscopy; EDTA, ethylene diamine tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ES-MS, electrospray mass spectrometry; HepB, hepatitis B peptide antigen; HMQC, heteronuclear multiple quantum correlation; IFNγ, interferon-γ; LPS, lipopolysaccharide; MHCII, class II major histocompatibility complex; NOESY, nuclear Overhauser effect spectroscopy; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; PBMCs, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PnC, *Streptococcus pneumoniae* C-substance; PSA, *Bacteroides fragilis* polyanasaccharide A; SDS, sodium dodecyl sulfate; SEB, staphylococcus enterotoxin B; Spi, type I *Streptococcus pneumoniae* capsular polysaccharide; TFA, trifluoroacetic acid; ZPS, zwitterionic polysaccharide.

**References**


