Structural insights into parallel strategies for germline antibody recognition of lipopolysaccharide from Chlamydia

Dylan W Evans2,†, Sven Müller-Loennies3,†, Cory L Brooks2, Lore Brade3, Paul Kosma4, Helmut Brade1,3, and Stephen V Evans1,2
1Department of Biochemistry and Microbiology, University of Victoria, PO Box 3055 STN CSC, Victoria, BC, Canada V8P 3P6; 2Research Center Borstel, Leibniz-Center for Medicine and Biosciences, Parkallee 22, Borstel D-23845, Germany; and 3Department of Chemistry, University of Natural Resources and Life Sciences, Vienna A-1190, Austria

Received on November 14, 2010; revised on March 24, 2011; accepted on March 24, 2011

The structure of the antigen-binding fragment from the monoclonal antibody S64-4 in complex with a pentasaccharide bisphosphate fragment from chlamydial lipopolysaccharide has been determined by x-ray diffraction to 2.6 Å resolution. Like the well-characterized antibody S25-2, S64-4 displays a pocket formed by the residues of germline sequence corresponding to the heavy and light chain V gene segments that binds the terminal Kdo residue of the antigen; however, although S64-4 shares the same heavy chain V gene segment as S25-2, it has a different light chain V gene segment. The new light chain V gene segment codes for a combining site that displays greater affinity, different specificity, and allows a novel antigen conformation that brings a greater number of antigen residues into the combining site than possible in S25-2. Further, while antibodies in the S25-2 family use complementarity determining region (CDR) H3 to discriminate among antigens, S64-4 achieves its specificity via the new light chain V gene segment and resulting change in antigen conformation. These structures reveal an intriguing parallel strategy where two different combinations of germline-coded V gene segments can act as starting points for the generation of germline antibodies against chlamydial antigens and show how anti-carbohydrate antibodies can exploit the conformational flexibility of this class of antigens to achieve high affinity and specificity independently of CDR H3.

Keywords: antibody–antigen recognition / carbohydrate antigen / chlamydial LPS / crystal structure / germline antibody

Introduction

Antibodies play a vital role in the adaptive humoral immune response of all jawed vertebrates where they have the potential to bind to a seemingly limitless number of epitopes. The immunoglobulin repertoire in mice and humans is generated first by recombination of different variable region germline gene segments (V and D gene segments for light chains and V, D and J gene segments for heavy chains) in maturing B-cells. Second, there is an increase in the diversity of the repertoire resulting from the differential pairing of heavy and light chains to make fully functional antibodies (Hozumi and Tonegawa 1976). Third, the diversity of this primary antibody repertoire is significantly enhanced by somatic hypermutation (usually induced by Th-cells) during affinity maturation and class switching (Jacob et al. 1991). However, small carbohydrate antigens generally cannot stimulate T-cell help and antibodies raised against carbohydrates are therefore somewhat more dependent on the primary immunoglobulin repertoire, making them an excellent probe of the germline antibody response.

The nature of the germline antibody response to an antigen has been under debate for some time. Antibodies had initially been characterized as possessing “exquisite specificity” toward their antigens (Landsteiner 1962); however, there have now been several reports of antibodies that display polyspecificity and cross-reactivity (Pinilla et al. 1999; Marchalonis et al. 2001; Nguyen et al. 2003; Brooks et al. 2008; Brooks, Blackler, et al. 2010; Brooks, Muller-Loennies, et al. 2010). The very concept of affinity maturation requires that germline antibodies display some measure of polyspecificity or cross-reactivity in the initial encounter with antigen, and there is a clear need to explore this phenomenon at the molecular level. In order to compare selected monoclonal antibodies (mAbs) against lipopolysaccharide (LPS) of Chlamydia, we have determined their relative binding strengths with an enzyme-linked immunosorbent assay (ELISA) and their structures alone and in complex with a variety of structurally related natural and artificial ligands (Muller-Loennies et al. 2000; Nguyen et al. 2003; Brooks et al. 2008; Brooks, Blackler, et al. 2010; Brooks, Muller-Loennies, et al. 2010).

Chlamydiae are Gram-negative obligate intracellular bacterial pathogens. One species, in particular C. trachomatis, causes a number of diseases in humans including trachoma, salpingitis, ectopic pregnancy, infertility, epididymitis, prostatitis and reactive arthritis (reviewed in Wagenerlehner et al. 2006; Bebear and de Barbeyrac 2009). Worldwide, trachoma...
is the most common cause of blindness due to infection (Burton 2007).

Chlamydiae possess a truncated LPS consisting only of an inner core, composed of a few Kdo (3-deoxy-α-D-manno-oct-2-ulopyranonic acid) residues, and lipid A. Similar to most Gram-negative bacteria, the lipid A contains an acylated 1,4’-bisphosphorylated β1→6-linked glucosamine disaccharide (Rund et al. 1999, 2000). The Kdo transferases of all Chlamydiae share the rare ability to generate 2→8 and 2→4 Kdo linkages, giving rise to a number of LPS molecules containing inner-core regions Kdo(2→8)Kdo(2→4)Kdo, Kdo (2→4)Kdo(2→4)Kdo and Kdo(2→8)[Kdo(2→4)]Kdo(2→4) Kdo (Figure 1). Previous investigations have shown that even these very small antigens contain a number of diverse epitopes that can be recognized by antibodies with high affinity.

Characterization of mAbs S25-2, S45-18, S73-2, S54-10, S67-7 and others, through sequencing, SPR, ELISA and x-ray crystallographic studies (Muller-Loennies et al. 2000; Nguyen et al. 2003; Brooks et al. 2008; Brooks, Blackler, et al. 2010; Brooks, Muller-Loennies, et al. 2010) revealed that they all utilize the same set of heavy and light chain V gene segments to form a terminal Kdo binding pocket of a few highly conserved residues. The near-germline sequence of mAb S25-2 prompted the hypothesis that this binding pocket conferred initial specificity to Kdo-containing antigens, while the remainder of the combining site could adapt to antigen variability via conformational changes in existing residues or, when coupled to a protein or peptide carrier, through affinity maturation. Although all of the selected mAbs contained a terminal Kdo binding pocket, their specificities and their abilities to cross-react varied. The largest contributor to the different specificities between the antibodies was determined to be CDR H3, which is encoded by different D and J gene segments.

MAb S25-2 was observed to exhibit significant levels of cross-reactivity to several antigens derived from chlamydial LPS (Muller-Loennies et al. 2000). This cross-reactivity was attributed to CDR L1, which contains specific amino acids that could adapt to different ligands, and to CDR H3, which only contacted the terminal antigen residue and could not impose the selectivity seen in other antibodies with the same V gene segments. Antibodies of the S25-2 family that did display a higher degree of specificity contained longer CDR H3 loops that, for example, could project a single aromatic residue into the combining site that simultaneously provided energetically significant stacking interactions with some ligands while precluding the binding of others (Brooks, Blackler, et al. 2010; Brooks, Muller-Loennies, et al. 2010).

Preliminary investigation of S64-4 suggested that it contained a different light chain V gene segment than the S25-2-type antibodies. To explore how the different light chain V gene segment affects the architecture of the combining site and how the binding strategy compares with that of the S25-2-type antibodies, we characterized the mAb S64-4 using binding studies in combination with single crystal x-ray

Fig. 1. Chemical structure of Chlamydia psittaci LPS which harbors all the antigen fragments listed in Table I. S64-4’s immunogen, the 2→8 pentasaccharide bisphosphate (Table I, h), is shown by red in the online version and grey in the printed version.
diffraction to determine the high-resolution structure of the antibody in complex with antigen.

**Results**

**Immunization of mice and preparation of mAb**

Earlier investigations from our groups on the specificity of polyclonal antibodies found in mice and rabbits after immunization with Chlamydiae indicated that, in addition to antibodies against the family-specific epitope 2→8 Kdo trisaccharide (Table I, c), antibodies exist that require the 2→8 Kdo trisaccharide and the phosphorylated lipid A backbone for binding (Brade et al. 1990). Similar results were found in human sera after natural chlamydial infection (Brade et al. 1990). The isolation of a mAb of such specificity was achieved by immunization of mice with the neoglycoconjugate 2→8 pentasaccharide 4-phosphate-BSA (Table I, g) and screening for antibodies which, in addition to the 2→8 Kdo trisaccharide (Table I, c) oligosaccharide, required parts of the lipid A backbone for binding. MAb S64-4 was identified as a clone that bound to a variety of phosphorylated pentasaccharide antigens with at least 100 times higher avidity than to 2→8 Kdo trisaccharide-BSA (Table I, c).

**Serological characterization of mAb S64-4 by ELISA**

The relative affinities of S64-4 and S25-2 were determined by ELISA at antigen concentrations of 2 and 20 pmol/well (Table I). No reactivity to Kdo monosaccharide-BSA (Table I, a) was observed for antigen concentrations up to 20 pmol/well and antibody concentrations up to 1 μg/mL. Binding to the family-specific 2→8 Kdo trisaccharide (Table I, c) was seen with marginal avidity only with high amounts of antigen and showed somewhat higher binding for the GlcNAc-containing 2→8 tetrasaccharide (Table I, d). High avidity binding was only observed for 2→8 pentasaccharide 4-phosphate (Table I, g) and 2→8 pentasaccharide bisphosphate (Table I, h). Binding to the branched antigen hexasaccharide 4-phosphate (Table I, m) was less than 1% this strength and was only observed in ELISA when large amounts of antigen were used.

Checkerboard titrations against differently phosphorylated 2→8 pentasaccharide antigens covering concentrations between 10 and 0.04 pmol/well (200 and 0.8 pmol/mL) and antibody concentrations between 1 ng and 1 μg/mL revealed a slightly higher affinity for 2→8 pentasaccharide 4-phosphate-BSA (Table I, g) than for 2→8 pentasaccharide bisphosphate-BSA (Table I, h) (Figure 2). Binding to 2→8 pentasaccharide 1-phosphate-BSA (Table I, f) and 2→8 pentasaccharide-BSA (Table I, e) were much weaker where OD values >0.5 were only observed with high concentrations of antigen (10 pmol/well) and antibody (500 ng/mL). No binding was observed for variously phosphorylated tetrasaccharide ligands containing Kdo (2→4)Kdo(2→6)βGlcN(1→6)αGlcN (data not shown).

**Structures of liganded S64-4**

The data collection and refinement statistics for S64-4 bound to Kdo monosaccharide (Table I, a) and 2→8 pentasaccharide bisphosphate (Table I, h) are given in Table II. Excellent electron density was observed for all regions of the polypeptide with the exception of a string of six residues in the heavy chain constant region: Ser 128, Ala 129, Ala 130, Gln 131, Thr 132 and Asn 133. Excellent electron density was also observed for the single monosaccharide observed in the binding site of S64-4 + Kdo monosaccharide (Table I, a) as

---

**Fig. 2.** Binding of mAb S64-4 to Kdo oligosaccharides conjugated to BSA. ELISA plates were coated with graded concentrations of neoglycoconjugates corresponding to 200 (filled circles), 50 (filled triangles), 12.5 (filled squares), 3.2 (filled diamonds), 0.8 (open circles) pmol of ligand per milliliter using 50 μL/well and reacted with mAb S64-4 at the concentrations indicated on the abscissa. The antigens were: 2→8 pentasaccharide bisphosphate (Table I, b) Kdo(2→8)Kdo(2→4)Kdo(2→6)βGlcN-4P(1→6)αGlcN-1P-BSA (A), 2→8 pentasaccharide 4-phosphate (Table I, g) Kdo(2→8)Kdo(2→4)Kdo(2→6)βGlcN-4P(1→6)αGlcN-1P-BSA (B), 2→8 pentasaccharide 1-phosphate (Table I, f) Kdo(2→8)Kdo(2→4)Kdo(2→6)βGlcN(1→6)αGlcN-1P-BSA (C) and 2→8 pentasaccharide (Table I, e) Kdo(2→8)Kdo(2→4)Kdo(2→6)βGlcN(1→6)αGlcN-1P-BSA (D). The average of quadruplicate measurements is shown for A–C. The measurements for D are single data points.
Table I. Relative avidity of S64-4 and S25-2 IgG to various chlamydial LPS fragments as determined by ELISA

<table>
<thead>
<tr>
<th>Code</th>
<th>Terminal linkage</th>
<th>Abbreviation</th>
<th>Antigen</th>
<th>2 pmol/wellb</th>
<th>20 pmol/wellb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S25-2</td>
<td>S64-4</td>
</tr>
<tr>
<td>a</td>
<td>N/A</td>
<td>Kdo monosaccharidec</td>
<td>3-deoxy-o-sallo-oct-2-ulosonic acid</td>
<td>500 &gt;1000</td>
<td>125 &gt;1000</td>
</tr>
<tr>
<td>b</td>
<td>2–8</td>
<td>Kdo disaccharide</td>
<td>Kdo(2→8)Kdo</td>
<td>250 &gt;1000</td>
<td>63 &gt;1000</td>
</tr>
<tr>
<td>c</td>
<td>Kdo trisaccharide</td>
<td>Kdo(2→8)Kdo(2→4)Kdo</td>
<td>32 1000 8</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td>d</td>
<td>Tetrasaccharide</td>
<td>Kdo(2→8)Kdo(2→4)Kdo(2→6)GlcNAc</td>
<td>63 &gt;1000</td>
<td>125</td>
<td></td>
</tr>
<tr>
<td>e</td>
<td>Pentasaccharide1-phosphatee</td>
<td>Kdo(2→8)Kdo(2→4)Kdo(2→6)GlcNAc1→6GlcNAcol</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>f</td>
<td>Pentasaccharide4-phosphate</td>
<td>Kdo(2→8)Kdo(2→4)Kdo(2→6)GlcNAcP1→6GlcNAcol</td>
<td>16 2 4 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g</td>
<td>Pentasaccharide 4-phosphate</td>
<td>Kdo(2→8)Kdo(2→4)Kdo(2→6)GlcNAcP1→6GlcNAcol</td>
<td>63 2 8 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>h</td>
<td>Pentasaccharide bisphosphatef</td>
<td>Kdo(2→8)Kdo(2→4)Kdo(2→6)GlcNAcP1→6GlcNAcol</td>
<td>63 2 8 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i</td>
<td>2–4</td>
<td>Kdo disaccharide</td>
<td>Kdo(2→4)Kdo</td>
<td>500 &gt;1000</td>
<td>125 &gt;1000</td>
</tr>
<tr>
<td>j</td>
<td>Kdo trisaccharide</td>
<td>Kdo(2→4)Kdo(2→4)Kdo</td>
<td>1000 &gt;1000</td>
<td>250 &gt;1000</td>
<td></td>
</tr>
<tr>
<td>k</td>
<td>Tetrasaccharide</td>
<td>Kdo(2→4)Kdo(2→4)Kdo(2→4)Kdo</td>
<td>1000 &gt;1000</td>
<td>125 &gt;1000</td>
<td></td>
</tr>
<tr>
<td>l</td>
<td>Pentasaccharide 4-phosphate</td>
<td>Kdo(2→4)Kdo(2→4)Kdo(2→6)GlcNAc</td>
<td>&gt;1000</td>
<td>500 500 4</td>
<td></td>
</tr>
<tr>
<td>m</td>
<td>Branched</td>
<td>Hexasaccharide 4-phosphate</td>
<td>[Kdo(2→4)]Kdo(2→8)Kdo(2→4)Kdo(2→6)GlcNAcP1→6GlcNAcol</td>
<td>&gt;1000</td>
<td>250 &gt;1000 4</td>
</tr>
<tr>
<td>n</td>
<td>Hexasaccharide bisphosphate</td>
<td>[Kdo(2→4)]Kdo(2→8)Kdo(2→4)Kdo(2→6)GlcNAcP1→6GlcNAcol</td>
<td>&gt;1000</td>
<td>1000 500 32</td>
<td></td>
</tr>
</tbody>
</table>

Antigens which S64-4 bound with measurable avidity are shown in bold letters.

* The ligands were conjugated to BSA by the isothiocyanate or glutardialdehyde method; for details, see Materials and methods.

** mAb conc (ng/mL) yielding an optical density at 405 nm (OD405) > 0.2 in ELISA using 2 or 20 pmol/well of antigen.

*Antigens crystallized with S64-4.

**Antigens measured in a checkerboard titration, see Figure 2.

well as four of the five sugar residues in S64-4 + 2→8 pentasaccharide bisphosphate (Table I, h) (Figure 3). The second GlcN phosphate was completely disordered.

**Germline gene and sequence comparison**

The germline gene and sequence comparisons with antibody S25-2 are presented in Tables III and IV. S64-4 and S25-2 use different light chain V gene segments, where S64-4 utilized IGHV7-3*02 and S25-2 used IGHV8-21*01. The light chain J gene segment used by S64-4 was IGKJ1*01, whereas the light chain J gene segment used by S25-2 was IGKJ2*02.

S64-4 and S25-2 use the same heavy chain V and J gene segments but different D gene segments. The heavy chain V gene segment used by S64-4 and S25-2 was IGHV7-3*02. The heavy chain D gene segment used by S64-4 was IGHD1*1*01, whereas the heavy chain D gene segment used by S25-2 was IGHD2*9*01. The heavy chain J gene segment used by S64-4 and S25-2 was IGJH3*01.

The light chain was determined to have five point mutations in the amino acid sequence away from the germine. One occurs in the framework region (RL18G), three occur in CDR L1 (TL27DS, GL29V and YL30N) and one occurs in CDR L3 (WL69R). The heavy chain has six mutations away from the germine in the V and J genes. One occurs in CDR H1 (TH30I), two in CDR H2 (AH50CG and AH61T), one in CDR H3 (WH100BP) and two in the framework regions (KH3M and LH78V). The CDRs in S64-4 are the same length as those in S25-2 barring CDR L1, which is two residues shorter in S64-4.

**Antigen conformations**

The dihedral angles along the glycosidic linkages in common between the antigens bound to S64-4 and S25-2 are given in Table V.
contact to the third Kdo residue where the guanidinium group of Arg L92 forms a salt bridge with the carboxylic acid. GlcN4P binds to three different residue side chains on S64-4 via its phosphate group. The hydroxyl groups of Ser L27D and Tyr L32 both form hydrogen bonds, while the guanidinium group of Arg L92 forms a salt bridge. There is a single intramolecular hydrogen bond between the terminal Kdo OH-7 and the carboxylic acid group of Kdo3.

Discussion

Binding specificity of mAb S64-4

Earlier investigations demonstrated the existence of natural animal and human antibodies against Chlamydiae that require the 2→8 Kdo trisaccharide (Table I, c) and the phosphorylated lipid A backbone for binding (Brade et al. 1990); however, it was not then possible to determine which components of the lipid A backbone were involved in binding. The generation of murine mAb S64-4 with the same binding specificity as the reported polyclonal antibodies together with chemically defined oligosaccharides representing the carbohydrate backbone of the LPS from C. trachomatis or C. psittaci has allowed the elucidation of the chemical moieties crucial to recognition.

S64-4 utilizes a conserved Kdo monosaccharide binding pocket

We have shown previously that one of the most prevalent combinations of V\textsubscript{L} and V\textsubscript{H} regions (of which antibody S25-2 is the prototype) raised against chlamydial LPS produce a Kdo monosaccharide (Table I, a) binding pocket lined with amino acid residues of conserved sequence that forms the basis for binding a stereochemically diverse range of Kdo-based carbohydrate antigens. S64-4 was one of a small group of antibodies raised against chlamydial LPS immunogens that contained the same heavy chain V gene segment but a different light chain V gene segment as the S25-2 group (Tables III and IV).

Despite an entirely different light chain V gene segment, S64-4 binds the terminal Kdo in a binding pocket that is structurally similar to that observed on S25-2 type antibodies (Figure 4A–D). S64-4’s CDR H1, H2 and part of H3 (encoded by the heavy chain V gene segment) make the same interactions with the terminal Kdo as S25-2 where Tyr H33 and Arg H52 form a hydrogen bond and a charged-residue interaction, respectively, to the carboxylic acid group. Interestingly, although the amino acid sequence encoded by the light chain V gene segment of S64-4 is significantly different compared with the S25-2 type antibodies, it makes a similar number and type of contacts with the terminal residue of the antigen. The hydrogen bond of Arg L96 (CDR L3) to Kdo1 OH-4 in S25-2 is duplicated by Arg L96 in S64-4, while the hydrogen bond from the backbone oxygen of Ser L91 to Kdo OH-5 in S25-2 is replaced with the main chain amide of Glu L93 in S64-4.

S64-4 displays specificity for antigen linkage and length

The ability of S25-2 to cross-react with moderate to high affinity to numerous naturally occurring and synthetic Kdo antigens (Brooks et al. 2008; Brooks, Muller-Loennies, et al. 2010) lies in stark contrast to the relative specificity of S64-4, which shows general requirements first for the terminal residue to have a 2→8 linkage and second for the antigen to be a tetrasaccharide or longer (Table I).

The linkage requirement is apparent given that, with a single exception noted below, no antigens with a 2→4 linked terminal glycosidic bond are observed to bind to S64-4 (Table I). The length requirement can be traced from the complete lack of observable binding for the simple 2→8 Kdo disaccharide (Table I, b) to the weak binding observed for the simple Chlamydiae family-specific 2→8 Kdo trisaccharide (Table I, c) antigen and to significantly higher binding upon the addition of the 2→6 linked GlcNAc. Finally, the highest avidity is seen for the phosphorylated pentasaccharide antigens (Table I, g and h) (Figure 1).

The highest avidities are observed generally for antigens that are phosphorylated on the fourth residue, which is readily

| Table III. Germline gene segment usage of the variable regions of antibodies S64-4 and S25-2 |
|------------------|------------------|------------------|------------------|------------------|
| Light chain      | Heavy chain      |
| V               | J               | V               | D               | J               |
| S64-4            | IGKV3-12*01     | IGKJ1*01        | IGKV7-3*02      | IGHD1-1*01      | IGJ3*01         |
| S25-2            | IGKV8-21*01     | IGKJ2*02        | IGKV7-3*02      | IGHD2-9*01      | IGJ3*01         |

Fig. 3. Stereo view of refined omit electron density map contoured at 3σ for 2→8 pentasaccharide bisphosphate (Table I, h), showing the four ordered residues: the terminal Kdo, Kdo2, Kdo3 and the glucosamine 4-phosphate residues.
understood from the structure as this moiety displays some of the most extensive hydrogen bond interactions with the protein (Figure 4A and B). The phosphate group makes hydrogen bonds to the side chains of amino acid residues Ser L27D and Tyr L32, while the side chain of Arg L92 forms a salt bridge. The importance of the phosphate group to antigen recognition is underlined by the fact that the single observed exception to the requirement for a 2→8 terminal linkage is for 2→4 pentasaccharide 4-phosphate (Table I, l), which exhibits weak binding.

Although S64-4 shows greater general specificity toward chlamydial LPS antigens than S25-2, this does not hold for the branched hexasaccharide antigens (Table I, m and n), which S64-4 binds with significantly higher avidity than S25-2 (Figure 1). This is surprising at first glance, as the branched antigens contain the C. psittaci species specific 2→4 Kdo trisaccharide (Table I, j) epitope for which S64-4 shows no avidity when presented as part of the smaller antigens. However, the branched antigens also contain the 2→8 terminal linkage which S64-4 requires in the smaller antigens. This can be understood by examining the shape of the S64-4 combining site which, unlike that of S25-2, does not exclude the branched terminal 2→4 linked epitope by steric conflict.

The inability of S64-4 to recognize antigens that contain only the 2→4 terminal linkage underscores the importance of the 2→8 terminal linkage in placing the terminal Kdo residue in the conserved binding pocket.

S64-4 promotes a novel conformation for the terminal linkage

Similar to the S25-2 antibodies, S64-4 contains a Kdo monosaccharide binding pocket of conserved sequence. The heavy chain CDRs H1 and H2 are coded by the same heavy chain V gene segment as S25-2, and the interactions made by those
CDRs to the terminal Kdo residue are conserved; however, while the substitution of the light chain (which codes for CDRs L1, L2 and L3) in S64-4 results in a similar environment to S25-2 for the terminal Kdo residue, it leads to significant differences for the remaining residues. Significantly, CDR L1 has an almost entirely different sequence and is two residues shorter than that of S25-2 (Table IV), which results in a shorter loop that bends away from the antigen to give a larger, more open combining site (Figure 5).

CDR H3 is almost completely coded by the D and J genes and so is not conserved even among the S25-2 type antibodies, and H3 consequent has provided the most significant determinant to antigen specificity in this family (Brooks, Muller-Loennies, et al. 2010). The relative promiscuity of
S25-2 has been attributed to its short CDR H3 that provides an open combining site (Brooks et al. 2008). Interestingly, CDR H3 in the more specific S64-4 is not only the same length as that in S25-2 but bends further away from the antigen to again increase the size of the combining site (Figure 5).

The open combining site of the more specific antibody S64-4 lies in contrast to the general trend observed in the S25-2 type antibodies, where greater specificity was often associated with a more confined antibody combining site (Nguyen et al. 2003; Brooks et al. 2008). Unlike the mechanisms observed for the S25-2 antibodies, S64-4 achieves its specificity by allowing more sugar residues to come in contact with the combining site to yield a greater number of interactions by allowing a new low-energy conformation in the terminal linkage (Figures 4A and B and 5B, Table V).

The novel antigen conformation allows the S64-4 to recognize Kdo2 in a manner totally different from S25-2. Although both S64-4 and S25-2 interact with Kdo2 via Asn H53, the interaction is through different hydroxyl groups, where S64-4 binds to OH-5 and S25-2 binds to OH-7 (Figure 4). S64-4 also makes an additional hydrogen bond to Kdo2 OH-4 through Asn H52A.

The Kdo residue is also affected, as it enters the antibody combining site to form a salt bridge with Arg L92. Another intramolecular bond is observed between the same carboxylic acid group and the terminal Kdo OH-8.

Finally, and most significantly, the new conformation allows extensive recognition of the phosphorylated fourth sugar residue, which forces the fifth sugar residue, glucosamine-1-phosphate, of 2→8 pentasaccharide

---

**Fig. 5.** Antigen-binding sites showing size differences between S64-4 and S25-2. (A) Stereo view of an overlay of the antigen-binding sites, see online version for color figures (orange, S64-4; yellow, 2→8 pentasaccharide bisphosphate [Table I, h]; blue, S25-2; grey, 2→8 Kdo trisaccharide [Table I, c]); (B) molecular surface representation of S64-4 with 2→8 pentasaccharide bisphosphate (Table I, h); and (C) molecular surface representation of S25-2 with 2→8 Kdo trisaccharide (Table I, c).
bissaccharide 4-phosphate (Table I, h) to lie outside of the combining site. This results in the fifth sugar showing complete disorder in the electron density map, as it cannot contact the antibody, and is consistent with the lack of increase in relative avidity upon the phosphorylation of the fifth residue of 2→8 penta-

Conclusions

Despite sharing heavy and light chain V gene segments, the S25-2 family of antibodies displays a broad specificity for a wide range of chlamydial LPS antigens. Specificity for LPS is achieved through the conserved Kdo monosaccharide binding pocket formed largely by CDRs L1, L2, L3, H1 and H2, which are coded by the heavy and light chain V gene segments. Antigenic variation is accommodated through somatic hypermutation of these genes and differential use of D and J gene segments that code CDR H3. Although S64-4 has a different light chain V gene segment from the S25-2 type antibodies, it too displays a terminal Kdo binding pocket. At first glance, this may seem to be a parallel strategy for humoral recognition of this class of antigens; however, this second route to a terminal Kdo binding pocket changes the combining site architecture and greatly expands the potential repertoire of antigens that may bind. S64-4 shows higher specificity for a 2→8 terminal linkage than S25-2; however, the S64-4 combining site allows the antigen to assume conformations different from those required by S25-2.

The difference in specificity and relative affinity of S64-4 toward various antigens can largely be attributed to new CDRs in light chain V segment, the last two i.p. Two days after the last injection, the mice were bled from the tail vein and the sera were tested for the presence of antibodies against the immunizing antigen. The mouse with the highest titer received three booster injections of 200 µg each in PBS on days 83, 84 and 85; the quizzes (47%) primary hybridomas were obtained. One aliquot (50 µL) was injected i.p. and four aliquots (50 µL each) were injected s.c. at four different sites. On day 28, again 50 µg of the antigen in PBS (50 µL) emulsified with an equal volume of Freund’s incomplete adjuvant were injected i.p. Seven days later, the mice were bled from the tail vein and the sera were tested for the presence of antibodies against the immunizing antigen. The mouse with the highest titer received three booster injections of 200 µg each in PBS on days 83, 84 and 85; the first one i.

Materials and methods

Generation of isolated oligosaccharides and neoglycoconjugate antigens

Kdo monosaccharide (Table I, a) (3-deoxy-α-D-manno-oct-2-ulosonic acid), 2→8 Kdo disaccharide (Table I, b) (Kdo(2→8)Kdo(2→allyl)), 2→4 Kdo disaccharide (Table I, i) (Kdo(2→4)Kdo(2→allyl)), 2→8 Kdo trisaccharide (Table I, c) (Kdo(2→8)Kdo(2→4)Kdo(2→allyl)), 2→4 Kdo trisaccharide (Table I, j) (Kdo(2→4)Kdo(2→4)Kdo(2→4)Kdo(2→allyl)), 2→8 tetrasaccharide (Table I, d) (Kdo(2→8)Kdo(2→4)Kdo(2→6)βGlcNAc(1→4)allyl) and 2→4 tetrasaccharide (Table I, k) (Kdo(2→4)Kdo(2→4)Kdo(2→6)βGlcNAc(1→4)allyl) were chemically synthesized and conjugated to bovine serum albumin (BSA) as reported (Kosma et al. 1987, 1988, 1989, 1990; Fu et al. 1992). The oligosaccharides 2→8 pentasaccharide (Table I, e) (Kdo(2→8)Kdo(2→4)Kdo(2→6)βGlcNAc(1→4)6GlcNAcol), 2→8 pentasaccharide 1-phosphate (Table I, f) (Kdo(2→8)Kdo(2→4)Kdo(2→6)βGlcN(1→6)αGlcN-1P), 2→8 pentasaccharide 4-phosphate (Table I, g) (Kdo(2→8)Kdo(2→4)Kdo(2→6)βGlcNAc(1→4)6GlcNAcol), 2→8 pentasaccharide bisphosphate (Table I, h) (Kdo(2→8)Kdo(2→4)Kdo(2→6)βGlcN-4P(1→6)αGlcN-1P), 2→4 pentasaccharide 4-phosphate (Table I, l) (Kdo(2→4)Kdo(2→4)Kdo(2→6)βGlcN-4P(1→6)GlcNAcol), branched hexasaccharide 4-phosphate (Table I, m) (Kdo(2→8)Kdo(2→4)Kdo(2→6)βGlcNAc(1→4)6GlcNAcol) and branched hexasaccharide bisphosphate (Table I, n) (Kdo(2→8)Kdo(2→4)Kdo(2→6)βGlcN-4P(1→6)GlcN-1P) were obtained from recombinant Re-mutant strains of Salmonella enterica serovar Minnesota or from Escherichia coli as reported elsewhere (Holst et al. 1994, 1995).

Antigens were conjugated to BSA by the glutardialdehyde or, after cysteamination, by the isothiocyanate method as described (Muller-Loennies et al. 2002, 2003). The amount of ligand present in the conjugates was determined by measuring the amount of protein (Bradford assay, Bio-Rad, Hercules, CA, USA) and Kdo (thiobarbiturate assay). As the penta- and hexasaccharides contain a glucosaminitol at the reducing end, they would more properly be named as penta- and hexasaccharide alditols to avoid confusion.

ELISA using oligosaccharide-BSA conjugates

Neoglycoconjugates were coated onto MaxiSorp microtiter plates (96-well, U-bottom, NUNC) at various concentrations. Antigen solutions were adjusted to equimolar concentrations based on the amount of ligand present in the respective glycoconjugate. Details of the assay have been described elsewhere (Muller-Loennies et al. 2006).

Monoclonal antibodies

Laboratory strain BALB/c mice were immunized by a protocol described by Stabli et al. (1983). Mice (groups of four) were injected on day 0 with 2→8 pentasaccharide 4-phosphate-BSA (Table I, g) (50 µg) in phosphate buffered saline (PBS) (125 µL) emulsified with an equal volume of Freund’s complete adjuvant. One aliquot (50 µL) was injected i.p. and four aliquots (50 µL each) were injected s.c. at four different sites. On day 28, again 50 µg of the antigen in PBS (50 µL) emulsified with an equal volume of Freund’s incomplete adjuvant were injected i.p. Seven days later, the mice were bled from the tail vein and the sera were tested for the presence of antibodies against the immunizing antigen. The mouse with the highest titer received three booster injections of 200 µg each in PBS on days 83, 84 and 85; the first one i.

Downloaded from https://academic.oup.com/glycob/article-abstract/21/8/1049/1988187 by guest on 20 November 2018
Preparation of S64-4 Fab

Fab was prepared by digesting the IgG with papain at a 1:400 papain to IgG ratio. The digestion was carried out at ambient temperature in 20 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES), pH 7.5, with 0.2 M ethylenediaminetetraacetic acid (EDTA) and 0.5 M dithiothreitol (DTT) for 2.5 h. The reaction was quenched by the addition of 10 mM iodoacetamide (Sigma, St. Louis, MO, USA) and the contents were subsequently dialyzed into 20 mM HEPES, pH 7.5, to remove the DTT and EDTA. The Fab was purified using a cation exchange column (CM-825 Shodex) with a linear gradient of 0.0–1.0 M NaCl in 20 mM HEPES, pH 7.5.

Crystallization of S64-4 Fab in complex with Kdo monosaccharide

Puriﬁed Fab was dialyzed into 20 mM HEPES, pH 7.5, and concentrated to 12.5 mg/mL using Amicon Ultra centrifugal filters; 50 mM Kdo monosaccharide (Table I, a) was added to the concentrated Fab, and an initial screen was performed using Hampton Crystal screen I (Hampton Research, Aliso Viejo, CA) via the hanging-drop vapor-diﬀusion method at a ratio of 1:1 well to Fab solution. Small acicular crystals appeared in condition 41 (0.1 M HEPES, pH 7.5, 10% v/v 2-propanol and 20% w/v PEG 4000). Longer and thicker crystals (1.2 × 0.4 × 0.4 mm) formed when the condition was adjusted to 0.1 M HEPES, pH 8.5, 20% PEG 4000, 1:2 well to Fab solution.

Crystallization of S64-4 Fab in complex with 2→8 pentasaccharide bisphosphate

Puriﬁed Fab was dialyzed into 20 mM HEPES, pH 7.5, and concentrated to 12.5 mg/mL; 50 mM 2→8 pentasaccharide bisphosphate (Table I, h) was added to the concentrated Fab, and an initial screen was performed the same as above. Small irregular crystals appeared in condition 40 (0.1 M sodium citrate tribasic dihydrate, pH 5.6, 20% v/v 2-propanol and 20% w/v PEG 4000). Larger crystals (0.5 × 0.3 × 0.3 mm) formed when the condition was adjusted to 0.1 M sodium citrate tribasic dihydrate, pH 5.6, 15% v/v 2-Propanol and 15% w/v PEG 4000, 1:1 well to Fab solution.

Data collection, structure determination and reﬁnement

Crystals were ﬂash frozen to −160°C with an Oxford Cryostream 700 crystal cooler (Oxford Cryosystems, Devens, MA) using mother liquor supplemented with 25% MPD (2-methyl-2,4-pentane-diol, Sigma, St. Louis, MO, USA) as a cryoprotectant. Data were collected on a Rigaku R-AXIS 4++ area detector (Rigaku, Japan) coupled to a MM-002 x-ray generator with Osmic “blue” optics (Rigaku Americas, TX) and processed using Crystal Clear/4trek (Rigaku). The structure of the liganded S64-4 Fab was solved by molecular replacement using PHASER (McCoy et al. 2007) with the liganded S25-2 structure (PDB code 1Q9T) as a model. Manual ﬁtting of sigma A weighted Fo–Fc and 2Fo–Fc electron density maps was carried out with Coot (Emsley and Cowtan 2004) and SetoRibbon (Evans, unpublished). Restrained reﬁnement allowing isotropic thermal motion was carried out with REFMAC5 as implemented in CCP4 (Collaborative Computational Project 1994). The ﬁnal reﬁnement and model statistics are given in Table II.

Germline gene analysis

Putative germine gene segments were determined using IMGT/V-Quest (Brochet et al. 2008), and residues were numbered according to Kabat et al. (1983).

Funding

This work was supported in part by grants from the Natural Sciences and Engineering Research Council of Canada (S.V.E.), the Deutsche Forschungsgemeinschaft grant SFB 470/C1 (S.M.L. and H.B.) and from the Austrian Science Fund FWF-grants P17407 and P19295 (P.K.). S.V.E. is the recipient of a Michael Smith Foundation for Health Research Senior Scholarship.

Acknowledgements

The technical assistance of U. Agge, Ch. Schneider and V. Susott is acknowledged. The atomic coordinates and structure factors (3PHO and 3PHQ) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ, USA (http://www.rcsb.org).

Conﬂict of interest

None declared.

Abbreviations

BSA, bovine serum albumin; CDR, complementarity determining region; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; Ig, immunoglobulin; Kdo, 3-deoxy-α-o-manno-oct-2-ulosylpentosonic acid; LPS, lipopolysaccharide; mAb, monoclonal antibody; OD405, optical density at 405 nm; PBS, phosphate buffered saline.

References


