Structural studies of two anti-carbohydrate antibodies.

By

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BSc, University of Victoria, 2010

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of
Masters of Science
in the Department of Biochemistry and Microbiology

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Abstract

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This thesis is focused on determining the structures of two anti-carbohydrate antibodies to understand how they achieve their specificity toward antigen.

First, the structure of the antigen-binding fragment from the monoclonal antibody S64-4 in complex with a pentasaccharide bisphosphate fragment from chlamydial lipopolysaccharide (LPS) 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 (3-deoxy-α-D-manno-oct-2-ulopyranosonic acid) 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 avidity, 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 avidity and specificity independently of CDR H3.

Second, the structure of a rabbit, single chain variable fragment against terminal mannose-6-phosphate (Man6P) residues, termed scFv M6P-1, has been determined by x-ray diffraction to 2.7 Å resolution with Man6P in the binding site. The Man6P pathway is the predominant pathway that transports acid hydrolases from the trans-Golgi to endosomes. Newly synthesized hydrolases first require the generation of Man6P markers before they can be transported. Maintaining a full complement of hydrolases within lysosomes is essential as failure to do so results in a number of different lysosomal storage diseases. Due to its specificity, scFv M6P-1 is able to diagnose lysosomal storage diseases mucolipidosis II and mucolipidosis III. scFv M6P-1 is also able to purify Man6P containing proteins which may be useful for enzyme replacement therapies. Additionally, scFv M6P-1 is one of the first structures of an antibody fragment that exhibits high specificity for a single carbohydrate residue and is one of the first structures of a rabbit antibody fragment. The specificity of scFv M6P-1, which gives it these unique attributes, is revealed in the structure where multiple hydrogen bonds are seen between the antibody’s heavy chain and the mannose ring while two salt bridges are observed between the antibody’s light chain and the phosphate moiety. Finally, scFv M6P-1 binds in such a way as to allow binding to proteins possessing terminal Man6P residues. Crystallographic challenges that arose during this research included poor crystal growth as well as twinning and these are explored while the structure of scFv M6P-1 complex with Man6P is analysed.
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<tbody>
<tr>
<td>BCR</td>
<td>B-cell receptor</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin;</td>
</tr>
<tr>
<td>CDR</td>
<td>Complementarity determining region;</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EB</td>
<td>Elementary Body</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid;</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>Kdo</td>
<td>3-deoxy-α-d-manno-oct-2-ulopyranosonic acid</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>mAbs</td>
<td>Monoclonal antibodies</td>
</tr>
<tr>
<td>Man6P</td>
<td>Mannose-6-phosphate</td>
</tr>
<tr>
<td>MPR</td>
<td>Mannose-6-phosphate receptor</td>
</tr>
<tr>
<td>OD&lt;sub&gt;405&lt;/sub&gt;</td>
<td>Optical density at 405 nm</td>
</tr>
<tr>
<td>OH</td>
<td>Hydroxyl</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline.</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyle fluoride</td>
</tr>
<tr>
<td>RB</td>
<td>Reticulate Body</td>
</tr>
<tr>
<td>scFv</td>
<td>Single chain fragment variable</td>
</tr>
<tr>
<td>UCE</td>
<td>Uncovering enzyme</td>
</tr>
<tr>
<td>V&lt;sub&gt;H&lt;/sub&gt;</td>
<td>Variable heavy domain</td>
</tr>
<tr>
<td>V&lt;sub&gt;L&lt;/sub&gt;</td>
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Dedication

To Erin, my family, and my friends.
1.1 **Antibody Diversity**

Antibodies, or immunoglobulins (Igs), are proteins that play an essential role in the adaptive humoral immune response of all jawed vertebrates. Antibodies are involved in multiple functions of the immune system including antigen binding, opsonization, complement activation, and antibody dependent cell mediated cytotoxicity (ADCC), (Ravetch and Bolland 2001). The term “antibody” was first used in a paper by Behring and Kitasato in 1890 where they demonstrated that antibodies could extinguish the effects of diphtheria and tetanus toxins, (Lindenmann 1984; von Behring and Kitasato 1991). Since then an extensive amount of research has gone into understanding these crucial molecules. From determining their structure, functions, and roles in the immune system, to figuring out how they are generated and more recently, developing them as clinical and experimental tools, research into antibodies remains an exciting endeavour.

1.1.1 **Antibody Structure**

Antibodies consist of four polypeptide chains, two identical heavy chains and two identical light chains, Fig.1. The heavy chains, roughly 50kDa each, join together to form a ‘Y’ shaped molecule. The light chains are half the size at 25kDa each and they complex with the heavy chains to fashion one Ig molecule of approximately 150kDa. Heavy chains are composed of four domains, three constant and one variable. Light chains contain only one constant and one variable domain however; they exist as two types termed lambda (λ) and kappa (κ). The variable domains from the heavy and light chain (VH and VL respectively) combine to form the variable
region of the antibody which contains the antigen binding site. Each variable domain contains three complementarity determining regions (CDR) loops termed CDR H1, H2, and H3 for the heavy chain and CDR L1, L2, L3 for the light chain. While there exist consensus amino acid sequences for the constant domains, the CDRs have hypervariable sequences and possess the residues that bind to a given antigen. Five isotypes of Igs exist (IgM, IgD, IgG, IgA, and IgE) which are distinguished based on their constant regions and differ in biological properties.

**Figure 1: Structure of an IgG**

Schematic of the structure of an IgG and a scFv. Two variable domains (\(V_L\) and \(V_H\)) combine to form the antigen binding site. Each variable domain contains 3 CDR polypeptide loops which possess most of the residues that contact antigens. Domains and CDR loops are color coded to show their corresponding gene segments, Fig. 2. Black bands represent disulfide bonds.
1.1.2 Primary Antibody Repertoire

While the genomic space for antibody genes is limited, there appear to be antibodies available to bind a seemingly limitless number of foreign antigens. The primary immunoglobulin repertoire is generated first by genetic rearrangement of a small number of inherited germline gene segments, (Hozumi and Tonegawa 1976). The gene segments are termed variable (V gene), diversity (D gene), joining (J gene), and constant (C gene). This genetic rearrangement occurs while B-cells are developing in primary lymphoid tissue and is referred to as V(D)J recombination.

For heavy chains, during the first recombination event, one randomly selected D gene segment and one randomly selected J gene segment are joined, deleting any DNA that lies between the two segments to form a DJ segment, Fig. 2. Next, a recombination event occurs between the DJ segment, and a randomly selected V gene segment to produce a VDJ segment. Finally, a primary mRNA transcript is generated containing the recombined VDJ gene segment as well as the C genes. Recombination for light chains is a similar process however, the light chain loci lack D gene segments. For light chains, one randomly selected V gene segment and one randomly selected J gene segment are joined forming a VJ complex. Next, a primary mRNA transcript is generated containing the recombined VJ gene segment as well as a C gene. The primary transcripts for heavy and light chains are translated and, during B-cell maturation, the diversity of the primary immunoglobulin repertoire is increased by the differential pairing of heavy and light chains to make fully functional antibodies that exist on the surface of B-cells, termed B-cell receptors (BCR), (Hozumi and Tonegawa 1976). B-cells that fail to produce a functional IgM after this genetic recombination undergo apoptosis, (Janeway et al 2001).
Figure 2: V(D)J Recombination
Schematic of V(D)J recombination of germline gene segments for heavy and light chains, occurring in developing B-cells.
1.1.3 Affinity Maturation

Once mature, B-cells migrate to the periphery where they are able to contact and bind antigens to potentially become activated. The activation and subsequent proliferation can be accomplished in a T-cell dependent or T-cell independent manner. T-cell dependent activation requires signals from T-cells and can result in affinity maturation. In T-cell dependent activation protein antigens are internalized by antigen-presenting cells (including B-cells), cleaved, and peptides are presented on the cell surface via a MHC class II protein. T-cells interact with this MHC-peptide complex and produce signalling cytokines. When a B-cell both binds to an antigen via its BCR, and presents the processed antigen to a T-cell it becomes activated in a T-cell dependant manner. T-cell independent activation of B-cells does not require T-cell help and involves carbohydrate antigens such as lipopolysaccharide or other highly repetitive antigens such as bacterial capsular polysaccharides. T-cell independent activation generally does not result in affinity maturation.

Affinity maturation results in increases of both the diversity of the primary antibody repertoire and the affinity of antibodies to a given antigen. Affinity maturation is accomplished through somatic hypermutation, where the variable region genes of immunoglobulins in B-cells undergo mutations at a rate $10^6$ fold greater than the normal mutation rate observed across the genome, (Teng and Papavasiliou 2007). Somatic hypermutation introduces point mutations into the $V_H$ and $V_L$ genes resulting in potentially enhanced affinity for a particular antigen in daughter B-cell generations. B-cells with BCRs that have increased affinity are selected in a process known as ‘clonal selection,’ where progeny with the highest affinities for antigen reproduce more quickly than progeny with lower affinities.
1.2 Antibodies in Crystallography

The structures of antibodies have been studied through the use of X-ray crystallography for some time now but not without problems. Due to the flexible nature of the hinge regions between the antibody domains and because of the large heterogeneous glycosylation of the heavy chain, fully intact antibodies do not readily crystallize, (Saphire et al 2001).

Another problem that can arise in any crystallographic study is the occurrence of a crystal growth defect known as twinning, (Yeates and Fam 1999).

1.2.1 Antibody Fragments

The flexible hinge regions and heterogeneous glycosylation of antibodies can inhibit crystal growth however, for crystallographic studies of antibody-antigen complexes, only the structure of the antigen binding site is important. Therefore, it is beneficial to cleave the antibody in question at its hinge. The major hinge region can be digested with the protease papain to yield three fragments of roughly equal weight (approximately 50kDa), two antigen binding fragments (Fab) and a fragment crystallizable (Fc), Fig. 3, named so due to the relative ease with which it was originally observed to crystallize. Fab and Fc fragments typically differ in pI values where Fabs are generally more acidic and Fcs more basic, therefore purification of Fabs for crystallization experiments can easily be accomplished by cation exchange chromatography. Another option to obtain smaller antibody molecules is to express single chain fragment variable (scFv) antibodies. scFvs are molecules constructed by expressing both V_H and V_L domains of an antibody joined together by a short peptide linker. scFvs maintain the same specificity as the original Ig because they possess the intact antigen combining site with all six of the original CDR loops.
scFvs can oligomerize through their linker peptide where the V\textsubscript{H} domain of one chain associates with the V\textsubscript{L} domain of a different chain. To prevent this, a subtilisin digestion step can be performed to cleave the linker before crystallization trials, (Essig et al 1993).

1.2.2 Twinning

Twinning is a crystal growth phenomenon which yields crystals with two or more domains, (Yeates and Fam 1999). The lattices of the domains have similar crystal symmetry and they exist at different orientations which can be either spatially distinct, or intertwined with one another. The twins share some lattice points which results in a special symmetry between the domains however, this symmetry is different to the existing symmetry of the crystal. Twinning does not refer to crystal growth disorders where multiple crystals grow together with no apparent symmetry. Epitaxial twinning is observed when lattices of twin domains can align in two, but not three dimensions whereas merohedral twinning exists when lattices of twin domains are completely superimposable in three dimensions. Epitaxial twinning results in a diffraction pattern of two distinct lattices and is easily recognized. Epitaxial twinning is therefore normally able to be overcome when processing data, (Yeates and Fam 1999). Merohedral twinning, on the other hand, results in a diffraction pattern that appears to be from a single lattice but the intensity of each spot contains contributions from multiple twin-related reflections. Merohedral twinning is generally only observed in the high-symmetry tetragonal, trigonal, hexagonal and cubic crystal systems whereas pseudo-merohedral twinning can occur in lower symmetry systems (Brooks et al 2008a; Yeates and Fam 1999). Pseudo-merohedral twinning can give rise to diffraction patterns that have symmetry that exceeds the symmetry of the crystal, which makes assigning the correct space group problematic.
This thesis is focused on determining the structures of two anti-carbohydrate antibodies to understand how they achieve their specificity toward antigen. In chapter two, the structure of a monoclonal antibody in complex with a fragment of chlamydial LPS is explored and in chapter three, the structure of a scFv in complex with a single carbohydrate residue is investigated.
Figure 3: IgG digestion and purification. scFv digestion.

A) Reducing SDS-PAGE showing the digestion of a typical IgG with papain. Heavy chains exist in the band at ≈50 kDa whereas light chains exist at ≈25 kDa. Reduced Fab and Fc fragments show up after digestion at ≈25 kDa. B) Reducing SDS-PAGE showing the digestion of a typical scFv with subtilisin. Undigested scFv exists at ≈32 kDa while digested scFv migrates to ≈16 kDa. C) Chromatogram from the purification of Fab via cation exchange chromatography. Negatively charged Fc flows through while positively charged Fab binds. Elution is accomplished with a salt gradient.
Chapter 2: Structural Insights into Parallel Strategies for Germline Antibody Recognition of LPS from *Chlamydia*

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Contributors to work in this chapter included Dylan W. Evans, Cory L. Brooks, Sven Müller-Loennies, Lore Brade, Helmut Brade, Paul Kosma and Stephen V. Evans. Paul Kosma synthesised the various Kdo antigens. Sven Müller-Loennies, Lore Brade, and Helmut Brade prepared the monoclonal antibodies, ran the ELISAs and revised the manuscript. Cory L. Brooks was the graduate student at the time that provided direct supervision and introduction to laboratory techniques. Dylan W. Evans purified and crystallized Fab, determined and analysed Fab structure, and was the primary manuscript author. Stephen V. Evans provided supervision and directed the research. Some portions of the Material and Methods and the Results sections that were performed by other individuals have been included for clarity, and clearly marked as such in the thesis.
2.1 Abstract

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 avidity, 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 avidity and specificity independently of CDR H3.
2.2 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 genes (V and J genes for light chains, V, D, and J genes 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 T_h-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 (Brooks et al 2010a; Brooks et al 2010b; Brooks et al 2008b; Marchalonis et al 2001; Nguyen et al 2003; Pinilla et al 1999). 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 (mAb) against LPS of Chlamydia we have determined their relative binding strengths
with ELISA, and their structures alone and in complex with a variety of structurally related natural and artificial ligands (Brooks et al 2010a; Brooks et al 2010b; Brooks et al 2008b; Muller-Loennies et al 2000; Nguyen et al 2003).

2.2.1 Chlamydiae

Chlamydiae are Gram-negative obligate intracellular bacterial pathogens whose intracellular life cycle often hinders treatment by antibiotics. 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 (Bebear and de Barbeyrac 2009; Wagenlehner et al 2006)). Worldwide, trachoma is the most common cause of blindness due to infection (Burton 2007).

Chlamydiae have a unique life cycle; they exist as two morphologically distinct particles: a small, dense infective particle called an elementary body (EB) and larger, less dense, non-infective but metabolically active particle called a reticulate body (RB). EBs, being stable in the extracellular environment, attach to host cells and are taken in via endocytosis, Fig 4. In the cell they exist in a membrane-lined phagosome that does not fuse with lysosomes. Within the phagosome, EBs differentiate into the metabolically active RB, which replicate via binary fission. After approximately 36 hours, the RBs differentiate back into EBs. Between 48 and 72 hours post infection, the host cells lyses and releases EBs which go on to infect new cells, (Hammerschlag 2002).
Figure 4: The Chlamydial Life Cycle

A) EBs attach to target host cell and are taken up by endocytosis. B) In a membrane-lined phagosome, EBs differentiate into RBs. C) RBs replicate via binary fission. D) After approximately 36 hours, the RBs differentiate back into EBs. E) Approximately 48 – 72 hours after the host cell was initially infected, it lyses and EBs are able to infect other cells.
2.2.2 Chlamydial Lipopolysaccharide

Lipopolysaccharide molecules extrude from the outer membrane of most Gram negative bacteria. To date the only group of Gram negative bacteria reported not to possess LPS are from the genus *Sphingomonas*, (Kawahara *et al* 1991). LPS functions to maintain the structural integrity of the outer membrane as well as protect bacteria from chemical attack. LPS is generally composed of a lipid A disaccharide, an inner core, an outer core, and a repeating O antigen. The Lipid A is an endotoxin, the cause of septic shock during bacterial infection and functions to anchor the LPS in the outer membrane, (Raetz and Whitfield 2002). The O antigen generally is composed of up to 50 repeating units of 3-8 linked monosaccharides. Presence of the O antigen determines whether an organism’s LPS is rough or smooth where full length O antigens are said to be smooth, and the absence of O antigen is termed rough.

Chlamydiae possess a truncated LPS consisting only of lipid A and an inner core composed of a few Kdo residues. Similar to most Gram-negative bacteria, the lipid A backbone is composed of 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 trisaccharides Kdo(2→8)Kdo(2→4)Kdo and Kdo(2→4)Kdo(2→4)Kdo and the tetrascarhide Kdo(2→8)[Kdo(2→4)]Kdo(2→4)Kdo, Fig. 5. 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.
Figure 5: Chlamydial Lipopolysaccharide.
The *Chlamydia psittaci* LPS harbours all the antigen fragments listed in Table 1. S64-4’s immunogen, the 2→8 pentasaccharide bisphosphate [Table 1, h], is shown in red.
2.2.3 The S25-2 Family of Antibodies

Characterization of mAbs S25-2, S45-18, S73-2, S54-10, S67-7, and others, through sequencing, SPR, ELISA, and x-ray crystallographic studies, (Brooks et al 2010a; Brooks et al 2010b; Brooks et al 2008b; Muller-Loennies et al 2000; Nguyen et al 2003) revealed that they all utilize the same set of heavy and light chain V genes to form a terminal Kdo binding pocket of a few highly conserved residues. The near-germline sequence of mAbS25-2 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 cross-reactivity varied. The largest contributor to the differential specificities among the antibodies was determined to be CDR H3, which is encoded by different D and J genes.

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. 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 significant stacking interactions with some ligands while precluding the binding of others through steric interference (Brooks et al 2010a; Brooks et al 2010b).
Preliminary investigation of S64-4 showed 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 diffraction to determine the high-resolution structure of the antibody in complex with antigen.
Table 1: Relative avidities of S64-4 and S25-2 IgG to various antigens.

Relative avidity of S64-4 and S25-2 IgG to various chlamydial LPS fragments as determined by ELISA. Antigens which S64-4 bound with measurable avidity are shown in bold letters. This work was done by Sven Müller-Loennies, Lore Brade, and Helmut Brade at Research Center Borstel, Leibniz Center for Medicine and Biosciences, Borstel, Germany.

<table>
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<th>#</th>
<th>Terminal Linkage</th>
<th>Abreviation</th>
<th>Antigen&lt;sup&gt;a&lt;/sup&gt;</th>
<th>2 pmol/well&lt;sup&gt;b&lt;/sup&gt;</th>
<th>20 pmol/well&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>N/A</td>
<td>Kdo monosaccharide&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3-deoxy-α-D-manno-oct-2ulosonic acid</td>
<td>500</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>b</td>
<td>Kdo disaccharide</td>
<td>Kdo(2→8)Kdo</td>
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</tr>
<tr>
<td>c</td>
<td>Kdo trisaccharide</td>
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<td></td>
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<td>1000</td>
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<td>d</td>
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<td></td>
<td>63</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>e</td>
<td>Pentasaccharide&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Kdo(2→8)Kdo(2→4)Kdo(2→6)GlcNAc(1→6)GlcNAc&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
<td>N/A</td>
<td>8</td>
</tr>
<tr>
<td>f</td>
<td>Pentasaccharide 1-phosphate&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Kdo(2→8)Kdo(2→4)Kdo(2→6)GlcN(1→6)GlcN&lt;sup&gt;1&lt;/sup&gt;AcP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g</td>
<td>Pentasaccharide 4-phosphate&lt;sup&gt;d&lt;/sup&gt;</td>
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<td></td>
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<td>2</td>
</tr>
<tr>
<td>h</td>
<td>Pentasaccharide bisphosphate&lt;sup&gt;c, d&lt;/sup&gt;</td>
<td>Kdo(2→8)Kdo(2→4)Kdo(2→6)GlcN4P(1→6)GlcN&lt;sup&gt;1&lt;/sup&gt;AcP</td>
<td></td>
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<tr>
<td>i</td>
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<td></td>
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<td>&gt;1000</td>
</tr>
<tr>
<td>j</td>
<td>Kdo trisaccharide</td>
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<td>1000</td>
<td>&gt;1000</td>
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<tr>
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</tr>
<tr>
<td>l</td>
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<td></td>
<td>&gt;1000</td>
<td>500</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)GlcN4P(1→6)GlcNAc&lt;sup&gt;ol&lt;/sup&gt;</td>
<td>&gt;1000</td>
<td>250</td>
</tr>
<tr>
<td>n</td>
<td>Hexasaccharide bisphosphate</td>
<td>[Kdo(2→4)][Kdo(2→8)]Kdo(2→4)Kdo(2→6)GlcN4P(1→6)GlcN&lt;sup&gt;1&lt;/sup&gt;AcP</td>
<td></td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

<sup>a</sup>The ligands were conjugated to BSA by the isothiocyanate or glutardialdehyde method; for details see Material and Methods.

<sup>b</sup>mAb conc (ng/mL) yielding OD<sub>405</sub> > 0.2 in ELISA using 2 or 20 pmol/well of antigen.

<sup>c</sup>Antigens crystallized with S64-4.

<sup>d</sup>Antigens measured in a checkerboard titration, see Fig. 6.
2.3 Materials and Methods

*Generation of isolated oligosaccharides and neoglycoconjugate antigens* – This work was done by Paul Kosma, Department of Chemistry, University of Natural Resources and Applied Life Sciences, Vienna, Austria. Kdo monosaccharide (Table 1, a) (3-deoxy-α-d-manno-oct-2-ulosonic acid), 2→8 Kdo disaccharide (Table 1, b) (Kdo(2→8)Kdo(2→)allyl), 2→4 Kdo disaccharide (Table 1, i) (Kdo(2→4)Kdo(2→)allyl), 2→8 Kdo trisaccharide (Table 1, c) (Kdo(2→8)Kdo-(2→4)Kdo(2→)allyl), 2→4 Kdo trisaccharide (Table 1, j) (Kdo(2→4)Kdo(2→4)Kdo(2→)allyl), 2→8 tetrasaccharide (Table 1, d) (Kdo(2→8)Kdo(2→4)Kdo(2→6)βGlcNAc(1→)allyl) and 2→4 tetrasaccharide (Table 1, k) (Kdo(2→4)Kdo(2→4)Kdo(2→6)βGlcNAc(1→)allyl) were chemically synthesized and conjugated to bovine serum albumin (BSA) as reported (Fu et al 1992; Kosma et al 1990; Kosma et al 1987; Kosma et al 1988; Kosma et al 1989). The oligosaccharides 2→8 pentasaccharide (Table 1, e) (Kdo(2→8)Kdo(2→4)Kdo(2→6)βGlcNAc-(1→6)GlcNAcol), 2→8 pentasaccharide 1-phosphate (Table 1, f) (Kdo(2→8)Kdo(2→4)-Kdo(2→6)βGlcN(1→6)αGlcN-1P), 2→8 pentasaccharide 4-phosphate (Table 1, g) (Kdo(2→8)-Kdo(2→4)Kdo(2→6)βGlcNAc-4P(1→6)GlcNAcol), 2→8 pentasaccharide bisphosphate (Table 1, h) (Kdo(2→8)Kdo(2→4)Kdo(2→6)βGlcN-4P(1→6)αGlcN-1P), 2→4 pentasaccharide 4-phosphate (Table 1, l) (Kdo(2→4)Kdo(2→4)Kdo(2→6)βGlcNAc-4P(1→6)GlcNAcol), branched hexasaccharide 4-phosphate (Table 1, m) (Kdo(2→8)[Kdo(2→4)]Kdo(2→4)Kdo-(2→6)βGlcNAc-4P(1→6)GlcNAcol) and branched hexasaccharide bisphosphate (Table 1, n) (Kdo(2→8)[Kdo(2→4)]Kdo(2→4)Kdo(2→6)βGlcNAc-4P(1→6)GlcN-1P) were obtained from recombinant Re-mutant strains of *Salmonella enteric* serovar Minnesota or from *E. coli* as reported elsewhere (Holst et al 1995; Holst et al 1994).
Antigens were conjugated to BSA by the glutardialdehyde or, after cysteamination, by the isothiocyanate method as described (Muller-Loennies et al 2003; Muller-Loennies et al 2002). The amount of ligand present in the conjugates was determined by measuring the amount of protein (Bradford assay, Bio-Rad) and Kdo (thiobarbiturate assay). Note: 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** — This work was done by Sven Müller-Loennies, Lore Brade, and Helmut Brade at Research Center Borstel, Leibniz Center for Medicine and Biosciences, Borstel, Germany. Neoglycoconjugates in carbonate buffer (50 mM, pH 9.2) were coated onto MaxiSorp microtiter plates (96-well, U-bottom, Nunc, Wiesbaden, Germany) at 4°C over night. Antigen solutions were adjusted to equimolar concentrations based on the amount of ligand present in the respective glycoconjugate. If not stated otherwise, 50 µL of volumes were used. Plates were washed twice in PBS supplemented with Tween 20 (0.05%, Bio-Rad) and thimerosal (0.01%, PBS-T) and were then blocked with PBS-T supplemented with casein (2.5%, PBS-TC) for 1 h at 37°C on a rocker platform followed by two washings. Appropriate antibody dilutions in PBS-TC supplemented with 5% BSA (PBS–TCB) were added and incubated for 1 h at 37°C. After two washings, peroxidase-conjugated goat anti-mouse IgG (heavy- and light-chain specific; Dianova, Hamburg, Germany) or IgM (µ-chain specific; Dianova) was added (both diluted 1:1000 in PBS–TCB), and incubation was continued for 1 h at 37°C. The plates were washed three times with PBS-T. Substrate solution was freshly prepared and was composed of azino-di-3-ethylbenzthiazolin sulfonic acid (1 mg) dissolved in substrate buffer (0.1 M sodium citrate, pH 4.5; 1 mL) followed by the addition of hydrogen peroxide (25 µL of a 0.1% solution). After 30 min at 37°C, the reaction was stopped by the addition of aqueous oxalic acid (2%), and
the plates were read by a microplate reader (Tecan Sunrise, Crailsheim, Germany) at 405 nm. Tests were run twice in quadruplicates with confidence values not exceeding 10% (Muller-Loennies et al 2006).

**Monoclonal antibodies** — This work was done by Sven Müller-Loennies, Lore Brade, and Helmut Brade at Research Center Borstel, Leibniz Center for Medicine and Biosciences, Borstel, Germany. BALB/c mice were immunized by a protocol described by Stähli et al. (Stahli et al 1983). Mice (groups of four) were injected on day 0 with 2→8 pentasaccharide 4-phosphate-BSA (Table 1, 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.v., the last two i.p. Two days after the last injection, the animal was exsanguinated and the spleen was removed. Spleen cells (1.2 × 10⁸) were fused and seeded into 720 primary wells; 338 (47%) primary hybridomas were obtained, 7 (2.1%) of which produced specific antibody. Hybridoma supernatants were screened by ELISA using 2→8 pentasaccharide 4-phosphate-BSA (Table 1, g), 2→8 Kdo trisaccharide-BSA (Table 1, c) or purified elementary bodies of Chlamydia. MAb S64-4 was determined as the most relevant clone; it was cloned thrice by limiting dilution, isotyped as an immunoglobulin (Ig) G1 class antibody with a commercially available isotype kit (Bio-Rad) and purified by affinity chromatography using an affinity support of AH-Sepharose
4B to which the ligand $2\rightarrow 8$ pentasaccharide bisphosphate (Table 1, h) was conjugated by the glutardialdehyde method.

*Preparation of S64-4 Fab* – Fab fragment was prepared by digesting the IgG with papain at a 1:400 papain to IgG ratio. The digestion was carried out at ambient room temperature in 20 mM HEPES pH 7.5 with 0.2 M EDTA and 0.5 M DTT for 2.5 hours. The reaction was quenched by the addition of 10 mM iodoacetamide (SIGMA) and the contents were subsequently dialyzed into 20 mM HEPES pH 7.5 to remove the DTT and EDTA. The Fab fragment was purified using a cation exchange column (CM-825 Shodex) with a linear gradient of 0.0 to 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 1, a) was added to the concentrated Fab and an initial screen was performed using Hampton screen I (Hampton Research, Aliso Viejo, CA) via the hanging drop vapour 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 mm x 0.4 mm x 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 1, 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 mm × 0.3 mm × 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, SigmaUltra) 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, Texas) and processed using Crystal Clear/d*trek (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 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 2.

Germline gene analysis – Putative germline gene segments were determined using IMGT/V-Quest (Brochet et al 2008) and residues were numbered according to Kabat, (Kabat et al 1983).
Figure 6: Binding of mAb S64-4 to Kdo oligosaccharides.

Binding of mAb S64-4 to Kdo oligosaccharides conjugated to BSA. ELISA plates were coated with graded concentrations of neoglycoconjugates corresponding to 200 (○), 50 (▲), 12.5 (■) 3.2 (◇), 0.8 (○) pmol of ligand per ml using 50 µl per well and reacted with mAb S64-4 at the concentrations indicated on the abscissa. The antigens were: 2→8 pentasaccharide bisphosphate [Table 1, h] Kdo(2→8)Kdo(2→4)Kdo(2→6)βGlcN-4P(1→6)αGlcN-1P-BSA (A), 2→8 pentasaccharide 4-phosphate [Table 1, g] Kdo(2→8)Kdo(2→4)Kdo(2→6)βGlcNAc-4P(1→6)GlcNAcol-BSA (B), 2→8 pentasaccharide 1-phosphate [Table 1, f] Kdo(2→8)Kdo(2→4)Kdo(2→6)βGlcN(1→6)αGlcN-1P-BSA (C) and 2→8 pentasaccharide [Table 1, e] Kdo(2→8)Kdo(2→4)Kdo(2→6)βGlcNAc(1→6)GlcNAcol-BSA (D). The average of quadruplicate measurements are shown for A through C. The measurements for D are single data points. This work was done by Sven Müller-Loennies, Lore Brade, and Helmut Brade at Research Center Borstel, Leibniz Center for Medicine and Biosciences, Borstel, Germany.
Table 2: Data collection and refinement statistics for liganded S64-4.

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<th>PSBP</th>
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<td><strong>Data Collection</strong></td>
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<tr>
<td>Resolution (Å)</td>
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<td>20-2.6 (2.69-2.60)</td>
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<td>c (Å)</td>
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<td>1</td>
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<tr>
<td>R_sym</td>
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<td>0.076 (0.28)</td>
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<td>I/σ(I)</td>
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<td>11.7 (3.1)</td>
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<td>95.2 (89.1)</td>
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<tr>
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<td><strong>Refinement</strong></td>
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<td>R_free (%)</td>
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<tr>
<td>Mean B-factor (Å²)</td>
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<td>31.88</td>
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Values in parentheses represent the highest resolution shell.

Table 3: Germline gene segment usage of the variable regions of S64-4 and S25-2.

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<th>Heavy Chain</th>
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<td>J</td>
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<td>IGKJ1*01</td>
</tr>
<tr>
<td><strong>S25-2</strong></td>
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<td>IGKJ2*02</td>
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2.4 Results

Immunoization of mice and preparation of monoclonal antibodies — Earlier investigations from our groups on the specificity of polyclonal antibodies from mice and rabbits after immunization with Chlamydiae indicated that, in addition to antibodies against the family-specific epitope 2→8 Kdo trisaccharide (Table 1, 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 1, g) and screening for antibodies which, in addition to the 2→8 Kdo trisaccharide (Table 1, 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 1, 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 1, 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 1, 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 1, d). High avidity binding was only observed for 2→8 pentasaccharide 4-phosphate (Table 1, g) and 2→8 pentasaccharide bisphosphate (Table 1, h). Binding to the branched antigen
hexasaccharide 4-phosphate (Table 1, 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 avidity for 2→8 pentasaccharide 4-phosphate-BSA (Table 1, g) than for 2→8 pentasaccharide bisphosphate-BSA (Table 1, h) (Fig. 6). Binding to 2→8 pentasaccharide 1-phosphate-BSA (Table 1, f) and 2→8 pentasaccharide-BSA (Table 1, 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 1, a) and 2→8 pentasaccharide bisphosphate (Table 1, h) are given in Table 2. 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 1, a) as well as four of the five sugar residues in S64-4 + 2→8 pentasaccharide bisphosphate (Table 1, h) (Fig. 7). 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 gene segments, where S64-4 utilized \textit{IGKV3-12*01} and S25-2 used \textit{IGKV8-21*01}. The light
chain J gene segment used by S64-4 was \textit{IGKJ1*01} whereas the light chain J gene segment used by S25-2 was \textit{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 \textit{IGHV7-3*02}. The heavy chain D gene segment used by S64-4 was \textit{IGHD1-1*01} whereas the heavy chain D gene segment used by S25-2 was \textit{IGHD2-9*01}. The heavy chain J gene segment used by S64-4 and S25-2 was \textit{IGHJ3*01}.

The light chain was determined to have five point mutations in the amino acid sequence away from the germline. One occurs in the framework region (RL18G), three occur in CDR L1 (TL27DS, GL29V, and YL30N), and one occurs in CDR L3 (WL96R). The heavy chain has six mutations away from the germline 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.

\textit{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 5.

\textit{Contacts to antigen} – S64-4 makes five contacts with the terminal Kdo residue in 2→8 pentasaccharide bisphosphate (Table 1, h), Fig. 8a, b. The side chain of Tyr H33 hydrogen bonds to the carboxylic acid group, while Arg H52 makes a bifurcated salt bridge to the carboxylic acid. The side chain of Arg L96 forms a hydrogen bond with OH-4 and the backbone amide group of Glu L93 binds to OH-5.
S64-4 forms two hydrogen bonds to the second Kdo residue as the carboxamide groups of Asn H52A and Asn H53 bind to OH-4 and OH-5, respectively. S64-4 also makes a single 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.

**Figure 7: Electron Density Map for 2→8 pentasaccharide bisphosphate**

Stereo view of refined omit electron density map contoured at 3σ for 2→8 pentasaccharide bisphosphate [Table 1, h], showing the four ordered residues: the terminal Kdo, Kdo2, Kdo3 and the glucosamine 4-phosphate residues.
Table 4: S64-4, S25-2, and germline gene variable region sequence alignment.

Amino acid sequence comparison of the variable regions for antibodies S64-4, S25-2, and putative germline gene sequence from which S64-4 was derived. The CDR regions are shown in grey highlights and mutations or deletions from the germline sequence are shown by the bold, underlined characters.

### Light Chain Variable Region

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<th>Germline</th>
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<th>S25-2</th>
</tr>
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<tbody>
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<td>DIVMSQSPSS LAVSAGEKVT MSCKSS</td>
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<tr>
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<td>KSVST--SGY SYMHWYQQKP GQPPKLIY</td>
<td>KSVSS--SVN SYMHWYQQKP GQPPKLIY</td>
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<tr>
<td></td>
<td>CDR L1</td>
<td>CDR L1</td>
<td>CDR L2</td>
</tr>
<tr>
<td></td>
<td>Germline</td>
<td>S64-4</td>
<td>S25-2</td>
</tr>
<tr>
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<td>LASNLES GVPA RFSGGSGTDT FTNLIHPVEE EDAATYY</td>
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<td>CQHSRELPŴFT GGGTKLEIK</td>
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### Heavy Chain Variable Region

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<td>EVMLVESGGG LVQPGGSLRL SCATS</td>
<td>EVILVESGGG LVQSGGSLRL SCATS</td>
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<td>CDR H1</td>
<td>CDR H2</td>
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<tr>
<td></td>
<td>Germline</td>
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<td>S25-2</td>
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<td>FINKNGGTYTEYSST̂S VKGRFTIS RDNSQSL̂YL QM̂NTLRAEDSATY</td>
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<td>CDR H2</td>
<td>CDR H3</td>
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<td>YC ARDIGYNСПF̂A YWGQGTLVTVS</td>
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Table 5: Dihedral angles for the terminal linkage of antigen in S64-4 and S25-2.

Dihedral angles observed in the terminal Kdo(2→8)Kdo linkage for S64-4 with 2→8 pentasaccharide bisphosphate [Table 1, h] and S25-2 with 2→8 Kdo trisaccharide [Table 1, c] (PDB entry 1Q9Q) (Nguyen et al 2003).

<table>
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<th>Atoms (Kdo residue)</th>
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<th>S25-2 (°)</th>
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<td>68.4</td>
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<td>C8(2) - C7(2) - C6(2) - O6(2)</td>
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<td>173.3</td>
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</tbody>
</table>

Figure 8: Antibody-antigen interactions of S64-4 and S25-2

Antigen binding sites showing key interactions of S64-4 and S25-2. a) Stereo view of S64-4 with 2→8 pentasaccharide bisphosphate [Table 1, h]; b) 2D view of S64-4 with 2→8 pentasaccharide bisphosphate [Table 1, h]; c) stereo view of S25-2 with 2→8 Kdo trisaccharide [Table 1, c] (PDB entry 1Q9Q) (Nguyen et al 2003); and d) 2D view of S25-2 with 2→8 Kdo trisaccharide [Table 1, c].
Figure 8 continued...

B)

C)

D)
2.5 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 1, 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 monoclonal antibody 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* have 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_L* and *V_H* regions (of which antibody S25-2 is the prototype) raised against chlamydial LPS produce a Kdo monosaccharide 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 (Table 1, a). 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 (Fig. 8A–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 avidity to numerous naturally occurring and synthetic Kdo antigens (Brooks *et al* 2010b; Brooks *et al* 2008b) 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 *Chlamydiaceae* 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) (Fig. 5). The highest avidities are observed generally for antigens that are phosphorylated on the fourth residue, which is readily understood from the structure as this moiety displays some of the most extensive hydrogen bond interactions with the protein (Fig. 8A 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 1, 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 1, m and n), which S64-4 binds with significantly higher avidity than S25-2 (Fig. 5). This is surprising at first glance, as the branched antigens contain the *C. psittaci* species specific 2→4 Kdo trisaccharide (Table 1, 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 that 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 4, which results in a shorter loop that bends away from the antigen to give a larger, more open combining site, Fig. 9.
CDR H3 is almost completely coded by the D and J gene segments and so is not conserved even among the S25-2 type antibodies, and H3 consequently has provided the most significant determinant to antigen specificity in this family (Brooks et al 2010b). The relative promiscuity of S25-2 has been attributed to its short CDR H3 that provides an open combining site (Brooks et al 2008b). 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, Fig. 9.

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 (Brooks et al 2008b; Nguyen et al 2003). 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 utilizing a new conformation in the terminal linkage, Fig. 8a, b, 9b, Table 5.

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 while S25-2 binds to OH-7, Figs. 8. S64-4 also makes an additional hydrogen bond to Kdo2 OH-4 through Asn H52A.

The Kdo3 residue is also affected, as it enters the antibody combining site to form a salt bridge with Arg L92 via it’s carboxylic acid group. An 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 bisphosphate (Table 1, 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 pentasaccharide 4-phosphate (Table 1, g).
Figure 9: Spatial comparison of antigen binding sites in S64-4 and S25-2.
Antigen binding sites showing size differences between S64-4 and S25-2. a) Stereo view of an overlay of the antigen binding sites (Orange = S64-4, Yellow = 2→8 pentasaccharide bisphosphate [Table 1, h]; Blue = S25-2, Grey = 2→8 Kdo trisaccharide [Table 1, c]; b) molecular surface representation of S64-4 with 2→8 pentasaccharide bisphosphate [Table 1, h]; and c) molecular surface representation of S25-2 with 2→8 Kdo trisaccharide [Table 1, c].
2.6 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 just 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 avidity of S64-4 toward various antigens can largely be attributed to new CDR L1 as its distinct sequence and shorter length allows a greater number of antigen residues into the combining site and positions the GlcN4P residue in such a way that the phosphate moiety makes extensive contacts with the antibody, resulting in tight binding and a novel conformation of the terminal 2→8 linkage.
Chapter 3: Structure Determination of a Novel Rabbit scFv Against Mannose-6-phosphate.

Dylan W. Evans¹§, Sven Müller-Loennies²§, and Stephen V. Evans¹‡

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²Research Center Borstel, Leibniz Center for Medicine and Biosciences, Parkallee 22, D-23845 Borstel, Germany

Contributors to work in this chapter included Dylan W. Evans, Sven Müller-Loennies, and Stephen V. Evans. Sven Müller-Loennies expressed and partially purified scFv. Dylan W. Evans purified and crystallized scFv, determined and analysed scFv structure, and was the primary manuscript contributor. Stephen V. Evans provided supervision and directed the research. Some portions of the Material and Methods and the Results sections that were performed by other individuals have been included for clarity, and clearly marked as such in the thesis.
3.1 Abstract

The structure of a rabbit, single chain variable fragment against terminal mannose-6-phosphate (Man6P) residues, termed scFv M6P-1, has been determined by x-ray diffraction to 2.7 Å resolution with Man6P in the binding site. The Man6P pathway is the predominant pathway that transports acid hydrolases from the trans-Golgi to endosomes. Newly synthesized hydrolases first require the generation of Man6P markers before they can be transported. Maintaining a full complement of hydrolases within lysosomes is essential as failure to do so results in a number of different lysosomal storage diseases. Due to its specificity, scFv M6P-1 is able to diagnose lysosomal storage diseases mucolipidosis II and mucolipidosis III. scFv M6P-1 is also able to purify Man6P containing proteins in which may be useful for enzyme replacement therapies. Additionally, scFv M6P-1 is one of the first structures of an antibody fragment that exhibits high specificity for a single carbohydrate residue and is one of the first structures of a rabbit antibody fragment. The specificity of scFv M6P-1, which gives it these unique attributes, is revealed in the structure where multiple hydrogen bonds are seen between the antibody’s heavy chain and the mannose ring while two salt bridges are observed between the antibody’s light chain and the phosphate moiety. Crystallographic challenges that arose during this research included poor crystal growth as well as twinning and these are explored while the structure of scFv M6P-1 in complex with Man6P is analysed.
3.2 Introduction

In 1975 Cesar Milstein and Georges J. F. Köhler developed a method to fuse an antibody producing B-cell with an immortal cancerous B-cell. The fusion was later called a hybridoma and it has the ability to indefinitely produce antibodies that share the same specificity, termed monoclonal antibodies (mAbs), (Milstein 1999). Since then, mAbs have been used in numerous applications including diagnostic tests, therapeutic treatments and research experiments. MAbs have several shortcomings including their relatively large size, structurally crucial disulfide bonds, and glycosylation of the heavy chains (Gebauer and Skerra 2009). Due to these drawbacks recombinant antibody techniques have been pursued.

Single-chain fragment variable (scFv) antibodies are molecules constructed by expressing both variable domains of a mAb joined together by a short peptide linker. scFvs maintain the same specificity as the original mAb and can be used for the majority of applications for which mAbs are used. scFvs have some advantages over their larger counterparts including size, relative cost, production speed, the relative ease of sequence manipulation to alter affinity, and the relative ease of crystallization, (Ahmad et al 2012). For whatever application a scFv is used, diagnostic tests, therapeutic treatments or research experiments, it is beneficial to obtain a structure of the antibody-antigen complex in order to determine which residues are making the contacts to allow rational design to manipulate scFv structure and specificity.
3.2.1 The Mannose-6-phosphate Pathway

The mannose-6-phosphate (Man6P) pathway is the predominant pathway that transports acid hydrolases from the trans-Golgi to endosomes, (Coutinho et al 2012). Transport is facilitated by two Man6P-specific receptors (MPR46 and MPR300). Newly synthesized hydrolases first require the generation of Man6P markers at specific N-linked oligosaccharides before they can be recognized by MPRs on the luminal side of the Golgi, (Coutinho et al 2012). 5-20% of hydrolases possessing the Man6P recognition marker escape transport and become secreted however, cell surface MPR300s are able to partially re-internalize these extracellular hydrolases and ship them back to lysosomes via the endocytic pathway, (Muller-Loennies et al 2010).

Mannose residues substituted on hydrolases acquire phosphate groups in a two-step process to become Man6P markers, (Muller-Loennies et al 2010). In the first step, a GlcNAc-1-phosphotransferase catalyzes the transfer GlcNAc-1-phosphate from UDP-GlcNAc to mannose residues. Subsequently, the uncovering enzyme (UCE), a phosphodiester α-N-acetylglucosaminidase removes GlcNAc, and thus exposes Man6P monoesters. GlcNAc-1-phosphotransferase is a 540 kDa hexameric transmembrane enzyme composed two α, β, and γ subunits. The α and β subunits are encoded by the gene GNPTAB, whereas the γ subunit is encoded by the gene GNPTG. The α/β subunits possess the substrate binding sites and function as the catalytic portion of the enzyme. Recently it has been shown that the γ subunit is involved in mediating correct folding of GlcNAc-1-phosphotransferase subunits, maintaining their conformation and regulating the enzyme’s activity, (Pohl et al 2010). The UCE is a 272 kDa tetrameric transmembrane enzyme which is encoded by the NAGPA gene.
3.2.2 Lysosomal Diseases

Lysosomes are cellular organelles containing over sixty soluble enzymes that are responsible for the degradation of many different substrates in the cell, (Coutinho et al. 2012). Maintaining a full complement of hydrolases within lysosomes is essential, and failure to do so results in a number of different lysosomal storage diseases, (Neufeld 1991; Vellodi 2005). Two examples include I-cell disease, or mucolipidosis II (ML II α/β), and pseudo-Hurler polydystrophy, or mucolipidosis III (ML III α/β or ML III γ). In ML II α/β, a mutation in the \textit{GNPTAB} gene causes a complete loss of activity of GlcNAc-1-phosphotransferase which terminates the mannose-6-phosphate pathway and halts transport of hydrolases to endosomes. These hydrolases are hypersecreted from cells resulting in a build up of cellular debris within lysosomes, (Hieber et al. 1975). Individuals with ML II α/β exhibit skeletal abnormalities, enlargement of certain organs including liver, spleen and heart, and gradual psychomotor retardation, (Kornfeld and Sly 2001). Children with ML II α/β generally die within their first decade of life. In ML III α/β or ML III γ, a mutation in the \textit{GNPTAB} or \textit{GNPTG} gene respectively, causes a partial loss of activity of GlcNAc-1-phosphotransferase. This results in similar but less severe symptoms which progress more slowly than those seen in ML II α/β.
3.2.3 **Recognition of small carbohydrates: scFv M6P-1**

scFv M6P-1 is a novel rabbit scFv generated *via* phage display that is highly specific for Man6P molecules, (Muller-Loennies *et al* 2010). ScFv M6P-1 was shown to be able to bind to glycoproteins containing Man6P residues, allowing for the direct demonstration of Man6P residues on proteins, and the indirect determination of GlcNAc-1-phosphotransferase activity. Due to its specificity, scFv M6P-1 is able to successfully diagnose ML II and ML III (and most-likely other lysosomal storage diseases) through western blotting, (Muller-Loennies *et al* 2010). scFv M6P-1 coupled to beads was also able to purify Man6P containing proteins in a one-step purification procedure, (Muller-Loennies *et al* 2010), which may be useful for the purification of lysosomal hydrolases for enzyme replacement therapies.

Aside from its clinical and experimental applications, scFv M6P-1 is also fundamentally and structurally significant as it is one of the first structures of an antibody that exhibits high specificity for a single carbohydrate residue. Structures of antibodies in complex with a single carbohydrate residue exist however, the complexes are normally accomplished by saturating the antigen binding site with a sugar monomer. Binding studies of these complexes reveal that the antibodies exhibit reduced affinity and/or avidity for single carbohydrate residues compared their more complex oligosaccharide immunogen, (Brooks *et al* 2010b; Brooks *et al* 2008b; Evans *et al* 2011). To explore this anti-carbohydrate scFv’s specificity we have used single crystal x-ray diffraction to determine the high-resolution structure of the antibody in complex with Man6P.
3.3 Materials and Methods

**Immunization, Phage Display, Expression and Purification of Soluble scFv** - Previously described, (Muller-Loennies et al 2010). This work was done by Sven Müller-Loennies at Research Center Borstel, Leibniz Center for Medicine and Biosciences.

**Preparation of scFv M6P-1** – To prevent oligimerization, scFv monomers were prepared by digesting the linker with subtilisin at a 1:300 subtilisin to scFv ratio. The digestion was carried out at ambient room temperature in 20 mM HEPES pH 7.2 with 150 mM NaCl for 3 hours. The reaction was quenched by the addition of 1 mM PMSF (G-biosciences). scFv monomers were purified via size exclusion chromatography (PhenomenexBioSepnSEC-s3000 column) using HEPES pH 7.2 with 150 mM NaCl as the mobile phase.

**Crystallization of scFv M6P-1 in complex with Man6P** – Purified scFv was concentrated to 14 mg/ml using Amicon Ultra centrifugal filters. An initial screen was performed by an Art Robbin ‘Gryphon’ robot at Dr Martin Boulanger’s lab, University of Victoria, using Hampton Index (Hampton Research, Aliso Viejo, CA) via the hanging drop vapour diffusion method at ratios of 1:1 and 1:2, well to scFv solution. Small needle-like crystals appeared in condition A6 (0.1 M Tris pH 8.5 with 2 M ammonium sulphate) after approximately 4 days.

**Data collection, structure determination and refinement** – Crystals were soaked in mother liquor with 2 mM Man6P and then flash frozen to -160°C with an Oxford Cryostream 700 crystal cooler (Oxford Cryosystems, Devens, MA) using CryoOil (MiTeGen, Ithaca, NY) as a cryoprotectant. Data were collected on beamline CMCF-ID at the Canadian Light Source synchrotron (Saskatoon, SK) and processed using HKL2000 (HKL Research, Charlottesville, VA). Data quality was analysed using PHENIX.xtriage, (Adams et al 2010). The structure of the
liganded scFv M6P-1 was solved by molecular replacement using PHASER (McCoy et al 2007) with the $V_H$ and $V_L$ domains from the structure of a rabbit Fab (PDB code 4HBC) as a model. Manual fitting of Fo-Fc and 2Fo-Fc electron density maps was carried out with Coot (Emsley and Cowtan 2004) and SetoRibbon (Evans, unpublished). Restrained, amplitude based twinned refinement (law $-h$, l, k) allowing isotropic thermal motion twin was carried out with REFMAC5 as implemented in CCP4 (COLLABORATIVE COMPUTATIONAL PROJECT 1994) and PHENIX.refine (Python-based Hierarchical ENvironment for Integrated Xtallography), (Adams et al 2010). The final refinement and model statistics are given in Table 6.
### Table 6: Data collection and refinement statistics for liganded scFv M6P-1.

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</table>

Values in parentheses represent the highest resolution shell.
3.4 Results

*Analysis of data with PHENIX.xtriage* – Exhaustive attempts to solve the structure using conventional molecular replacement methods failed to yield a convincing solution. Analysis of Patterson function revealed a significant off-origin peak at coordinates 0.000, 0.473, 0.473, with a height 28.44% of the origin indicating possible translational pseudosymmetry. Twinning analysis found 0 merohedral twin operators and 1 pseudo-merohedral twin operator, \(-h, l, k\), with a 2-fold axis. Twin law tests estimated twinned fraction to be 0.419 (Britton analyses), 0.437 (H-test) and 0.435 (Maximum likelihood method with \(R_{\text{obs.}} = 0.081\)), and so fitting and refinement proceeded under the assumption the crystal was twinned.
Structure of scFv M6P-1 in complex with Man6P – The data collection and refinement statistics for scFv M6P-1 bound Man6P are given in Table 6. Excellent electron density was observed for all four scFv molecules. Excellent electron density was also observed for the four Man6P molecules, Fig. 10.

**Figure 10: Electron Density Map for Man6P.**
Stereo view of refined omit electron density map contoured at 3σ for one Man6P observed in the antigen combining site of antibody molecule 1.
Contacts to antigen – scFv M6P-1 makes numerous contacts to the mannose ring of Man6P through CDR H1 and H3, Fig 11. The side chain of Thr H32 forms a hydrogen bond with the ring oxygen. The backbone amide of Thr H32, and both the backbone carbonyl and the side chain of Asp H98 form hydrogen bonds to OH-2. The side chain of Asp H98 and the backbone amide of Asn H101 hydrogen bond to OH-3. scFv M6P-1 binds to the phosphate group on Man6P through CDR L1 and CDR L3 where both Arg L33 and Arg L99 form salt bridges.

Figure 11: Antibody-antigen interactions of svFv M6P-1
Stereo view of the binding site of scFv M6P-1 showing hydrogen bonds and salt bridges to the antigen.

Co-ordinated Sulphates – Density is observed for a sulphate ion bound to Gln L43 and Arg L44 in each molecule in the asymmetric unit.
3.5 Discussion

Challenges – Many obstacles presented themselves during the process of obtaining a structure for scFv M6P-1. The initial obstacle, a familiar one in crystallography, was acquiring suitable crystals for data collection. Proteins are often unstable when not in their native environments and increasing their concentration frequently causes aggregation and precipitation, (Golovanov et al 2004). scFv M6P-1 has a tendency to precipitate in solutions without a moderate concentration of salt. Additionally, crystals were only observed in conditions that have high concentrations of ammonium sulphate. Within the structure, we witness a sulphate molecule co-ordinated with Gln L43 and ArgL44. The fact that crystals only grew in conditions with high concentrations of ammonium sulphate and that a sulphate ion exists in the structure indicates that sulphate ion is probably necessary to stabilize the protein and allow for crystal growth. Crystals are frozen before data collection to slow degradation in the x-ray beam and cryoprotectants are used to prevent crystals from cracking during the freezing process. Cryopreservation of crystals grown in high salt can be problematic due to crystals cracking or dissolving with the introduction of cryoprotectants however, this was overcome with the use of CryoOil (MiTeGen, Ithaca, NY) for protection during the freezing step.

Ambiguity in space group assignment – There existed a single structure of an unliganded rabbit Fab in the protein data bank (PDB code 4HBC). The variable domains from this Fab were used as an initial model for molecular replacement where the second obstacle to structure determination arose. Data were initially indexed in the primitive tetragonal Bravais lattice. After integration and scaling, systematic absences indicated space group P4_2_1_2 or P4_1_2_1_2. Cell content analysis predicted two molecules in the asymmetric unit however, molecular replacement
initially failed to locate both molecules. An extensively refined partial solution was used as a model to eventually yield a full solution in P4₁2₁2 however, refinement of this solution stalled with R factor values above 40% and electron density that did not match the entire lengths of the polypeptide chains. The assumption was made that the space group was incorrect and that the data needed to be reprocessed.

Twinning is a crystal growth phenomenon which yields crystals with two or more domains, (Yeates and Fam 1999). The lattices of the domains have similar crystal symmetry but slightly different orientations. The twins share some lattice points, which gives rise to even more symmetry different to the symmetry of the crystal. Epitaxial twinning is observed when lattices of twin domains can align in two, but not three dimensions whereas merohedral twinning exists when lattices of twin domains are completely superimposable. Epitaxial twinning results in a diffraction pattern of two distinct lattices and is easily recognized. Epitaxial twinning is therefore normally able to be overcome when processing data (Yeates and Fam 1999). Merohedral twinning, on the other hand, results in a diffraction pattern that appears to be from a single crystal lattice but the intensity of each spot contains contributions from multiple twin-related reflections. Merohedral twinning is generally only observed in the high-symmetry tetragonal, trigonal, hexagonal and cubic crystal systems however, pseudo-merohedral twinning can occur in lower symmetry systems, (Brooks et al 2008a; Yeates and Fam 1999). Pseudo-merohedral twinning can give rise to diffraction patterns with symmetry that exceeds that of the crystal, making space group assignment problematic. Pseudo-merohedral twinning in an orthorhombic space group with two axes of similar lengths can mimic a tetragonal space group, (Yeates and Fam 1999).
Assuming that the crystal was twinned, the data were reindexed into the primitive orthorhombic Bravais lattice which produced a unit cell with axis lengths 60.6, 128.05, 127.30 Å. After integration and scaling, the space group was determined to be P2₁2₁2₁ due to systematic absences. Analysis of data by PHENIX.xtriage revealed that the data were consistent with pseudo-merohedral twinning with the operator –h, l, k. Cell content analysis predicted four molecules in the asymmetric unit and molecular replacement found a full solution in P2₁2₁2₁. Two molecules showed average density while the other two molecules showed suboptimal density. Model building followed a cyclic process whereby one molecule was manually built, refined, and then used as a new model for molecular replacement. Multiple rounds of this process yielded a structure that produced acceptable R factors (23.4 R<sub>work</sub> and 27.2 R<sub>free</sub>) and good electron density for all four polypeptides chains and all four Man6P molecules.

**Crystals of M6P contain four independent molecules** – Superposition of the four molecules in the asymmetric unit show that all four molecule’s CDR loops adopt the same conformation and they all show a Man6P molecule clearly bound in the active site, Fig. 12. The four scFv molecules also interact with the Man6P molecules in the same manner.

**Specificity to Man6P** – It has been demonstrated that scFv M6P-1 binds specifically to Man6P and is able to bind many glycoproteins possessing terminal Man6P residues, (Muller-Loennies et al 2010). Due to these properties, scFv M6P-1 is able to successfully diagnose ML II, ML III, (Muller-Loennies et al 2010), and potentially other lysosomal storage diseases resulting from a disruption in the Man6P-pathway. From the structure we can see how scFv M6P-1 accomplishes its specificity and how it is able to bind a number of proteins containing Man6P, giving it diagnostic abilities. An abundance of interactions are made between the heavy chain and the mannose ring however, these alone aren’t enough to achieve specificity as shown in past
experiments by the fact that scFv M6P-1 does not bind mannose, (Muller-Loennies et al 2010). The importance of the phosphate group becomes evident in the structure. Residues from CDR L1 and L2 form a large positively charged surface where two salt bridges are observed between scFv M6P-1 and the phosphate moiety, Fig. 11, 13. Interestingly, scFv M6P-1 doesn’t bind glucose-6-phosphate (Glu6P) (Muller-Loennies et al 2010) which shares a similar structure with Man6P, differing by the position of a single hydroxyl (OH) group, Fig 14. Man6P possess an OH in the axial position on carbon two (C2) whereas the same OH in Glu6P sits equatorially. In the structure, we see that the C2 OH needs to be in the axial position for scFv M6P-1 to form three hydrogen bonds with it. Similarly, the C3 OH group is required to sit equatorially because it is also contacted three times in that position. Additionally, the C1 OH needs to be in the axial position. This requirement comes from the fact that in nature Man6P is covalently joined to glycoproteins through the OH on C1 and, as scFv M6P-1 is able to bind a number of proteins possessing Man6P residues, there needs to be sufficient room at that attachment point. The structure shows adequate space around the C1 OH, which explains how scFv M6P-1 is able to bind a number of different proteins containing Man6P. The structure highlights the importance of both the conformation of the mannose ring as well as the phosphate group. Interactions with the mannose ring along with two salt bridges to the phosphate moiety are how scFv M6P-1 achieves specificity to Man6P.

Significance – Due to its specificity, scFv M6P-1 is able to diagnose ML II and ML III through the use of western blots, (Muller-Loennies et al 2010). Looking at the structure, one can assume that this unique scFv is likely able to diagnose other lysosomal storage diseases that resulted from inadequate transport of hydrolases to endosomes due to defects in the Man6P-pathway. Examination of the binding site reveals that scFv M6P-1 could potentially bind to most proteins
containing Man6P residues. Furthermore, scFv M6P-1 when conjugated to beads was shown to be able to purify Man6P containing proteins in a one-step purification procedure. This could potentially enhance current enzyme replacement therapies, (Ballabio and Gieselmann 2009). These therapies involve cellular uptake of the missing enzymes and is accomplished through cell surface MPR300s. Missing enzymes are subsequently transported to lysosomes where they can degrade the build up of disease-related materials, (Ballabio and Gieselmann 2009; Muller-Loennies et al 2010).

Aside from its clinical and experimental applications, scFv M6P-1 is also fundamentally and structurally significant as it is one of the first structures of an antibody that exhibits high specificity for a single carbohydrate residue. Structures of antibodies in complex with a single carbohydrate residue exist however, the complexes are normally accomplished by saturating the antigen binding site with sugar monomer. Binding studies of these complexes reveal that the antibodies exhibit reduced affinity and/or avidity for single carbohydrate residues compared their oligosaccharide immunogen(s), (Brooks et al 2010b; Brooks et al 2008b; Evans et al 2011). ScFv M6P-1 displayed similar $K_d$ values as those determined for MPR32 to Man6P, (Muller-Loennies et al 2010; Tong and Kornfeld 1989). In the structure, we can see how this affinity is achieved; multiple hydrogen bonds are seen between the antibody and the mannose ring while two salt bridges are observed between the antibody and the phosphate.
Figure 12: Superposition of scFv M6P-1 molecules.
Superposition of the four scFv molecules in the asymmetric unit showing the similarity between the orientations of the CDR loops and the Man6P molecules.
Figure 13: Charged molecular surface representation of scFv M6P-1.
Charged molecular surface representation of scFv M6P-1 with Man6P showing a large positive area, formed by residues on CDR L1 and L2, which interacts with the phosphate group. The surface of positively charged residues are coloured blue, negatively charged residues are red.
Figure 14: Structure of Man6P and Glu6P.
Comparison of the structures of α-D-mannose-6-phosphate and α-D-glucose-6-phosphate showing that the two molecules are epimers that differ only in the stereochemistry of a single hydroxyl group. Man6P has an axial hydroxyl group whereas Glu6P has an equatorial hydroxyl group on carbon 2.
3.6 Conclusions

The novel rabbit scFv M6P-1, which binds a single Man6P molecule, has a number of unique attributes due to its specificity. ScFv M6P-1 is able to diagnose lysosomal storage diseases mucolipidosis II and mucolipidosis III and is able to purify Man6P containing proteins in which may be useful for enzyme replacement therapies. Additionally, scFv M6P-1 is one of the first structures of an antibody fragment that exhibits high specificity for a single carbohydrate residue and is one of the first structures of a rabbit antibody fragment. The specificity of scFv M6P-1 is revealed in the structure. ScFv M6P-1 forms multiple hydrogen bonds between a few residues on the antibody’s heavy chain and the carbohydrate ring of Man6P. Furthermore, two salt bridges form between two arginine residues on the antibody’s light chain and the phosphate moiety of Man6P. Finally, scFv M6P-1 binds in such a way as to leave sufficient space around the Man6P molecule, giving it the ability to bind to proteins possessing Man6P residues.
Chapter 4: Conclusions

This thesis is focused on determining the structures of two anti-carbohydrate antibodies to understand how they achieve their specificity toward antigen.

First, the structure of the antigen-binding fragment from the monoclonal antibody S64-4 in complex with a pentasaccharide bisphosphate fragment from chlamydial lipopolysaccharide was 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 avidity, 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 the same chlamydial antigens and show how anti-carbohydrate antibodies can exploit the conformational flexibility of this class of antigens to achieve high avidity and specificity independently of CDR H3.

Second, the structure of a rabbit, single chain variable fragment against terminal mannose-6-phosphate (Man6P) residues, termed scFv M6P-1, was determined by x-ray diffraction to 2.7 Å resolution with Man6P in the binding site. The Man6P pathway is the
predominant pathway that transports acid hydrolases from the trans-Golgi to endosomes. Newly synthesized hydrolases first require the generation of Man6P markers before they can be transported. Maintaining a full complement of hydrolases within lysosomes is essential as failure to do so results in a number of different lysosomal storage diseases. Due to its specificity, scFv M6P-1 is able to diagnose lysosomal storage diseases mucolipidosis II and mucolipidosis III. scFv M6P-1 is also able to purify Man6P containing proteins in which may be useful for enzyme replacement therapies. Additionally, scFv M6P-1 is one of the first structures of an antibody fragment that exhibits high specificity for a single carbohydrate residue and is one of the first structures of a rabbit antibody fragment. The specificity of scFv M6P-1, which gives it these unique attributes, is revealed in the structure where multiple hydrogen bonds are seen between the antibody’s heavy chain and the mannose ring while two salt bridges are observed between the antibody’s light chain and the phosphate moiety. Finally, scFv M6P-1 binds with sufficient space around the carbon 1 attachment point of the Man6P molecule, to allow it to bind proteins possessing terminal Man6P residues.
References


