Structural Basis for Antibody Recognition of Lipid A

INSIGHTS TO POLYSPECIFICITY TOWARD SINGLE-STRANDED DNA

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Background: Lipid A-specific antibodies are not effective in sepsis treatment; infection by Gram-negative bacteria can induce autoimmune disease.

Results: Antibody-combining sites orient lipid A to bury the LPS attachment point and can cross-react with ssDNA through terminal nucleotides.

Conclusion: Anti-lipid A antibodies cannot bind full-length LPS; phosphates play a crucial role in polyspecificity.

Significance: Antibody recognition of lipid A illuminates the genesis of autoimmunity.

Septic shock is a leading cause of death, and it results from an inflammatory cascade triggered by the presence of microbial products in the blood. Certain LPS from Gram-negative bacteria are very potent inducers and are responsible for a high percentage of septic shock cases. Despite decades of research, mAbs specific for lipid A (the endotoxic principle of LPS) have not been successfully developed into a clinical treatment for sepsis. To understand the molecular basis for the observed inability to translate in vitro specificity for lipid A into clinical potential, the structures of antigen-binding fragments of mAbs S1–15 and A6 have been determined both in complex with lipid A carbohydrate backbone and in the unliganded form. The two antibodies have separate germ line origins that generate two markedly different combining-site pockets that are complementary both in shape and charge to the antigen. mAB A6 binds lipid A through both variable light and heavy chain residues, whereas S1–15 utilizes exclusively the variable heavy chain. Both antibodies bind lipid A such that the GlcN-O6 attachment point for the core oligosaccharide is buried in the combining site, which explains the lack of LPS recognition. Longstanding reports of polyspecificity of anti-lipid A antibodies toward single-stranded DNA combined with observed homology of S1–15 and A6 and the reports of several single-stranded DNA-specific mAbs prompted the determination of the structure of S1–15 in complex with single-stranded DNA fragments, which may provide clues about the genesis of autoimmune diseases such as systemic lupus erythematosus, thyroiditis, and rheumatic autoimmune diseases.

Bacterial Gram-positive and Gram-negative infections are a leading cause of septic shock, with over 750,000 cases annually in the United States and with a mortality rate between 28 and 50% (1–3). It is the third most common cause of death in Germany where it claims 60,000 lives a year (2). The inflammatory cascade at the onset of septic shock can be caused by the presence of bacterial lipopolysaccharide (LPS), which is shed from the outer membrane of Gram-negative bacteria (4).

The inflammatory cascade is initiated by the formation of a signaling complex of the lipid A moiety of LPS with Toll-like receptor 4 (TLR4) and co-receptor myeloid differentiation factor 2 (MD-2) (5–8). One heavily investigated therapeutic route has been focused on the potential of monoclonal antibodies (mAbs) to sequester LPS (9–11) to prevent formation of the LPS-MD-2-TLR4 signaling complex. Although antibodies specific for the lipid A, core, and O-polysaccharide components of LPS have been reported (10–19), the diversity of structures together with the rapid onset of septic shock have hindered the introduction of O-polysaccharide-specific antibodies into clinical use (4, 11, 20–22). In contrast to O-polysaccharide, there is strong structural conservation among the inner cores of relevant serovars (23); however, generation of broadly cross-reactive antibodies has proved challenging. To date, only mAb WN1 222-5 has been reported to be successful in neutralizing a wide range of Gram-negative bacteria, including Escherichia, Salmonella, Shigella, and Citrobacter (9, 10, 24).

Although lipid A structure is relatively conserved among pathogenic species, none of the numerous reported antibodies claimed to be specific for lipid A have led to successful clinical implementation (13–15, 25). Specific binding to lipid A was observed upon acid treatment of bacterial LPS, thereby liberating the lipid A fragment acting then as neoantigen, when embedded into erythrocytes or liposomes (25, 26). Of particular significance were mAbs A6 (IgG2b) isolated from mice immunized with heat-killed Escherichia coli J-5 cells (27) and S1–15, also referred to as S1 (25). Binding studies showed a preference of mAb A6 for the bisphosphorylated (i.e. native) lipid A, with weak binding to the monophosphorylated lipid A.

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(25), although S1–15 displays high avidity for both the mono- and bisphosphorylated lipid A (25, 28). Neither antibody was observed to recognize intact LPS.

Our earlier work showed that such antibodies bind as well to the bisphosphorylated glucosamine (GlcN) disaccharide backbone (BBP) without the acyl chains (28), indicating that S1–15 and A6 recognize the carbohydrate backbone exclusively. Significantly, binding studies of the C6 methyl-capped disaccharide showed that neither antibody bound lipid A lacking a free primary hydroxyl at C6 on the β-GlcN, which serves as the attachment point for inner core residues of the LPS (29).

Interestingly, a number of anti-lipid A antibodies have been reported to display significant polyspecificity to a range of antigens, including cardiolipin (30, 31), I antigen on B-lymphocytes (30), double-stranded DNA (dsDNA) (13), and single-stranded DNA (ssDNA) (14, 30), with reports of mice immunized with bacterial LPS resulting in mAbs cross-reactive to DNA (32, 33). Antibodies against ssDNA are noteworthy, as their induction has been implicated in autoimmune diseases such as lupus (34–37), thyroid disease (38), and rheumatic disorders (31, 39). Furthermore, studies have implicated a specific variable heavy chain family gene V(H)4–21 in dual recognition of lipid A and ssDNA, demonstrating a possible link between infection with Gram-negative bacteria and development of systemic lupus erythematosus and arthritis (31, 34).

To date there are no reports of crystal structures of any anti-lipid A mAb either unliganded or in complex. We now report binding data and crystal structures of unliganded and liganded antigen-binding fragments (Fabs) for two anti-lipid A mAbs, S1–15 and A6, to elucidate the reason these antibodies do not bind intact LPS. Furthermore, we present a structure of S1–15 in complex with ssDNA fragments that supports the polyspecific potential of anti-lipid A antibodies toward ssDNA.

Experimental Procedures

ELISA—ELISA against the immobilized neo-glycoconjugates was performed as described earlier (29). For the binding assay, BBP and B4P were conjugated in a 5 m excess to BSA, which had been activated by divinylsulfone, were then immobilized at concentrations of 2.5, 5.0, 10, and 20 pmol of ligand per well, and reacted with mAbs A6 and S1–15, which were titrated 1:1 over 12 steps starting from 2 μg/ml concentration.

Germ Line Gene Usage Analysis—The nucleotide sequences of the variable regions were analyzed with the IMGT/V-quest and junctional analysis web applications (40, 41) to determine the murine germ line gene segments from which the lipid A-specific antibodies were derived.

Fab Preparation and Crystallization—Generation of mAbs S1–15 (IgG2b) (25) and A6 (IgG2b) (27) was described earlier. For large scale preparation, mAb S1–15 was isolated from ascites by affinity chromatography on BBP conjugated to AH-Sepharose (80 mg of ligand/2.5 ml of packed beads) followed by elution with 0.1 M glycine-HCl, pH 3.2 (29). Fractions were adjusted to pH 4 by addition of 1 M NaHCO3. Antibody A6 was isolated and purified in the same manner as S1–15.

Fab fragments of each antibody were prepared by digestion of the intact immunoglobulin with papain (Sigma). IgG was dialyzed into 25 mM HEPES (Sigma), pH 7.5, diluted to a concentration of 1.0–0.8 mg/ml, 2 mM EDTA (Sigma), and 5–6 mM DTT (Sigma). The digestion reaction was carried out at room temperature using a papain/IgG ratio of 1:200 molar eq for 2–3 h. The reaction was quenched by the addition of 10 mM iodoacetamide (Sigma) and dialyzed overnight into 25 mM HEPES buffer, pH 7.4. Fab fragment purification was by cation-exchange chromatography on a Shodex CM-825 column (Phenomenex, Torrance, CA) using a linear gradient of 0.0 to 0.5 M NaCl in 20 mM HEPES, pH 7.4. Purified Fab was concentrated to 10–12 mg/ml stock and stored at 4 °C.

The Fabs of A6 and S1–15 mAbs were mixed with 3 mM lipid A backbone bisphosphate: βGlcN4P/(1→6)αGlcN1P, designated BBP (28), for liganded crystallization screening. Several crystals (0.25 × 0.35 × 0.30 mm3) were obtained for S1–15 in the presence of BBP after 2 months in a 16 °C room from hanging drop setup in PEG II suite crystal screen (Qiagen, Toronto, Canada), under condition 13 (0.1 M sodium chloride, 0.1 mM bicine, pH 9.0, and 30% (w/v) PEG 550 methyl ether) using a 1:1 reservoir-to-protein ratio. Approximately cubic crystals (0.1 × 0.15 × 0.15 mm3) of A6 were grown initially at a hanging drop setup in a 16 °C room in the presence of BBP using Index crystal screen (Hampton Research, Aliso Viejo, CA) under condition 44 (0.1 M HEPES, pH 7.5, and 25% (w/v) PEG 3350) in 1:1 reservoir-to-protein ratio. Larger crystals (0.4 × 0.4 × 0.2 mm3) were obtained using a 1:1.5 reservoir-to-protein ratio.

Fresh papain digests of intact S1–15 and A6 were used by applying the protocol as above, for crystallization screening of unliganded Fabs and S1–15 Fab in the presence of oligonucleotides. Conditions were set using an Art Robbins Gryphon Xtalization Robot (San Jose, CA), with each 96-well sitting drop plate incubated in a 16 °C room. S1–15 unliganded crystals (0.4 × 0.3 × 0.25 mm3) were grown under condition 21 (25% (w/v) PEG 2000 MME) of the PEG II suite screen. Initial crystals of variable sizes for unliganded A6 Fab were grown under condition 16 (0.1 M Tris-HCl, pH 8.5, and 30% (w/v) PEG 1000) of PEG II suite crystal screen, and subsequently optimized (for diffraction quality) using 35% (w/v) PEG 1000. S1–15 Fab (12 mg/ml) was mixed with the deoxythymidine oligonucleotides of sequence 5′p–TTTTT–3′P (both ends phosphorylated) referred to as p5(dT)p and 5′–TTTTT–3′P (lacking the 5′-phosphate) referred to as 5(dT)p, in a 1:2 molar ratio. Crystals in presence of p5(dT)p oligonucleotides were initially obtained after a few days using the sitting drop method under condition 55 (0.075 M MgCl2, 0.1 M HEPES, pH 7.5, 28% (v/v) PEG 550 MME) of the index screen (Hampton Research). Larger crystals (0.35 × 0.3 × 0.3 mm3) were obtained via the hanging drop method and using 1:2–1:3 reservoir-to-protein ratios. A large crystal (0.7 × 0.4 × 0.3 mm3) was obtained in presence of 5(dT)p antigen, after a few days under condition 22 (0.1 M Tris-HCl, pH 8.5, 25% (v/v) PEG 550 MME) of the PEG suite crystal screen using sitting drop method.
Data Collection, Molecular Replacement, and Structure Refinement—Crystallization conditions for S1–15 liganded and unliganded, as well as A6 unliganded structures already contained levels of cryoprotectant suitable to form a glass upon freezing. Liganded A6 crystals were carefully dehydrated in a 16 °C room until the concentration of cryoprotectant (PEG 3350) reached appropriate levels. All crystals were flash-frozen to −160 °C using an Oxford Cryostream 700 crystal cooler (Oxford Cryosystems, Oxford, UK). The x-ray diffraction data sets were collected at the Canadian Macromolecular Crystallography Facility on beamline 08ID-1 of the Canadian Light Source (Saskatoon, Saskatchewan, Canada) at 0.979 Å wavelength, with a Marmosiac CCD300 detector, and processed using HKL2000 (HKL Research Inc., Charlottesville, VA).

The S1–15 structure in complex with lipid A was solved by molecular replacement using Phaser (42) with the constant domain and Fv fragments of unliganded Fab S55–3 (Protein Data Bank code 4ODS) as search models. The liganded structure of S1–15 was used subsequently as a search model for the unliganded S1–15 dataset and the ssDNA liganded datasets. The A6 liganded structure was also solved using the Fv and constant domains of the S1–15 liganded structure as search models. The liganded Fv and constant domains of A6 Fab were used as search models for the unliganded A6 structure.

Manual fitting of α-A-weighted Fc – F, and 2Fobs – F, electron density maps was carried out with Coot (43). Restrained refinement of translation, libration, and screw (TLS) refinement was carried out using REFMAC5 (44, 45). All stereo figures and r.m.s.d. calculations presented in this paper were made using SetoRibbon (available upon request from author S. V. E.). Electrostatic surface potential figures were made using Chimera molecular visualization software (46). Marvin Sketch Version 5.7.0 from ChemAxon was used for drawing chemical structures.

Results

Characterization of Lipid A–specific Antibodies through ELISA—The quantitative conjugate ELISA for mAbs S1–15 and A6 to BBP and B4P at concentrations of 2.5, 5, 10, and 20 pmol/well are shown in Fig. 1. Both antibodies bound to the immobilized BBP conjugate and much weaker to the B4P conjugate. Against BBP, the affinity of S1–15 was ~4-fold weaker than A6. However, S1–15 showed stronger binding to the B4P conjugate confirming the weaker dependence on the recognition of the phosphate at the anionic position (25). Binding data against synthetic lipid A and derivatives thereof have been reported earlier (15, 25, 29).

X-ray Diffraction Data Collection and Refinement—Data collection and refinement statistics for liganded and unliganded structures of the Fabs from lipid A–specific mAbs S1–15 (IgG2b) and A6 (IgG2b) are given in Table 1. Decacylated and purified lipid A (BBP) was used during crystallization trials (Fig. 1C).

A6—Data were collected to 2.15 Å resolution from a crystal grown in the presence of BBP, which were consistent with orthorhombic space group P212121 with an Rsym of 0.085 and containing one molecule per asymmetric unit. Superb electron density was observed for the antigen (Fig. 2A) and for the remainder of the protein, with the exception of the solvent-exposed residues 128–136 and 157–161 on the H chain constant domain, which is often observed to be disordered in IgG2b Fab structures (47–50). Data were collected on crystals of the unliganded Fab of A6 to 2.75 Å resolution with Rsym of 0.041 in triclinic space group P1. The structure was solved with two molecules in the asymmetric unit, and it exhibited relatively higher thermal motion that manifested as poor electron density in solvent-exposed regions, particularly residues 127–134 and 157–161 on the constant domain of the H chain, some of which were excluded from the final model.

S1–15—Data of crystals of S1–15 grown in the presence of BBP collected to 1.95 Å resolution fit a tetragonal unit cell in Lue group 422 with an Rsym of 0.088 and with systematic absences corresponding to space groups P42122 and P42121. The structure was solved in space group P42121 by molecular replacement with one molecule in the asymmetric unit. Excellent electron density was observed for the antigen (Fig. 2B) and for the remainder of the protein with the exception of residues in the corresponding region as A6 structures.

Data for two additional liganded S1–15 Fab structures were collected as follows: one in complex with 5-stranded deoxythymidine (dT) oligonucleotide with both ends phosphorylated or p5(dT)p and a 5-oligothymidine nucleotide with no 5’–terminal phosphate called 5(dT)p. The data were collected to 1.75 and 1.79 Å resolution with Rsym of 0.059 and 0.053, respectively (Table 1). Both data sets were solved in the same space group and near-identical unit cells as the structure of S1–15 in complex with the lipid A analogue. The ssDNA ligand with both terminal phosphates showed density for two separate oligonucleotide fragments, one at each phosphate-binding site of lipid A. A dinucleotide with the 3′–terminal phosphate occupies the 1P lipid A recognition pocket, and on the 4P lipid A pocket, a trinucleotide could be confidently modeled, although the terminal 5′–phosphate base was largely disordered. The two oligonucleotide fragments stabilized one another through stacking interactions via symmetry-related Fab molecules, showing electron density corresponding to a total of five oligonucleotides (Fig. 2C). In the second data set with 5(dT)p as ligand, density could only be observed for the 3′–phosphate group (Fig. 2D) as the rest of the oligonucleotide could not be modeled due to dynamic disorder.

Data were collected on the unliganded S1–15 Fab to 2.29 Å resolution with Rsym of 0.063 and were solved in the monoclinic space group P21, containing two molecules in the asymmetric unit. As with the liganded structures, residues 128–132 on the H chain were disordered with some residues excluded from the final model.

Germ Line Gene Usage and Sequence Comparison—The primary sequence comparisons for the variable loops of mAbs S1–15 and A6 are given in Table 2. IMGT germ line database analysis revealed that the nucleotide sequences of mAbs S1–15 and A6 arise from closely related V- and J-genes for the VL and VH chain. The A6 VL chain was found to share 292/294 nucleotide identity with the V-gene IGKV10–96*01 and J-gene IGKJ1*02, respectively (the * denotes the allele group). The S1–15 VL chain was found to share 261/279 nucleotide identity with the V-gene IGHV10–1*02, resulting in a single mutation
of a Ser(H)-94 to Arg (residues are identified as H or L to denote the heavy chain and light chains, respectively) at the base of CDR H3. The D- and J-genes of S1–15 are identical in nucleotide sequence to their respective germ line genes IGHD2–1*01 and IGHJ3*01. The A6 VH chain V-, D-, and J-genes belong to IGHV5–6-2*01, IGHD2–1*01, and IGHJ4*01, respectively. The V-, D-, and J-genes of A6 share 280/288, 40/42, and 43/50 in nucleotide identity with their respective germ line genes. Overall, there are 16 amino acid mutations from the germ line gene in A6, six of which lie in the CDRs;

FIGURE 1. Quantitative conjugate ELISAs coated with graded concentrations of neoglycoconjugates corresponding to 2.5 (blue triangle), 5 (green triangle), 10 (red circle), and 20 (black square) pmol of ligand per well and reacted with mAbs (A) S1–15 and (B) A6 at concentrations indicated on the x axis are shown. Ligands used were biphosphorylated lipid A backbone conjugated to BSA (BBP-divinyl sulfone BSA, left panel) and 4′-monophosphorylated lipid A backbone conjugated to BSA (right panel), and absorbance was measured at 405 nm. The chemical structure of the carbohydrate backbone of lipid A (BBP) used for crystallization trials (C). 1P and 4P refer to the position of the phosphates on the pyranose ring.
there were only five amino acid mutations away from the germ line in S1–15 with only one in the combining site (Table 2).

**A6 Recognition of Lipid A**—There are 11 direct hydrogen bonds to the bispophorylated lipid A backbone BBP and two bridging water interactions from one water molecule (Fig. 2E and Table 3). There are three salt bridge interactions as follows: a bidentate salt bridge between 4-phosphate (4P) and Arg(H)-100A and a bifurcated salt bridge between 1P and Arg(H)-100A. There are also two additional weak electrostatic interactions as follows: one between 4P and Arg(H)-52 and salt bridge between 1P and Arg(L)-30. An additional hydrogen bond to the ring oxygen on the α-GlcN. The 1P group forms two hydrogen bonds with a side chain of Tyr(H)-32 and Tyr(H)-100B residues. Finally, there is a hydrophobic interaction (~3.7 Å) between the Tyr(H)-32 phenyl ring and the C6 methyl group on the α-GlcN (Fig. 2F, dashed lines).

**Comparison with DNA-specific Antibodies**—The V-genes of numerous DNA-specific antibodies have been determined (35, 51–53), many of which belong to five subgroups of the J558 family as follows: Vλ1,238 (IGHV5-9-03 or 01)*, 133.16VH (IGHV1–82–01*), Vλ1,31 (IGHV15-19–01*), 6G6VH (IGHV7–3–04*), and pH3–6a (IGVH9–1–02*). Various antibodies utilizing these genes have been reported to be involved in polyspecificity to *p*-azophenylarsenate (54), cardiolipin (52), and other autoantigens (55). The V-genes of mAb A6 are closely related to several characterized anti-DNA mAbs (51, 52). The Vλ chain genes of A6 and S1–15 share over 90% nucleotide identity with several anti-DNA antibodies (52). One of the closest examples include an antibody isolated from hybridoma cell line 73.23 (52) with 96/108 amino acid sequence identity. Although the Vλ1 chain V-gene of A6 is not involved in the recognition of lipid A antigen, it is closely related to known anti-DNA Vλ-gene known as Vλ1,238 (51), sharing 273/291 nucleotides and 85/98 amino acids.

S1–15 also shares Vλ1 genes with an ssDNA-specific autoantibody, BV04-01 (56), and is closely related to antibody Dna-1 (57). S1–15 shares 92% amino acid sequence identity with BV04-01 on the Vλ1 chain, although they do not share Vλ1 chain.

### TABLE 1

Data collection and refinement statistics for liganded and unliganded structures of lipid A-specific antibodies, A6 and S1–15

| Crystal | S1–15 BBP | S1–15 unliganded | A6 BBP | A6 unliganded | S1–15 T5 | S1–15 T5 no 5°P |
|---------|-----------|------------------|--------|--------------|----------|-----------------|
| PDB codes | 4ODT | 4ODU | 4ODV | 4ODW | 4Z8F | 4Z95 |
| Resolution (Å) | 25.0-1.95 (2.02-1.95) | 30.00-2.29 (2.34-2.29) | 25.0-2.15 (2.23-2.15) | 25.0-2.70 (2.80-2.70) | 25.0-1.75 (1.81-1.75) | 30.00-1.79 (1.85-1.79) |
| Wavelength (Å) | 0.979 | 0.979 | 0.979 | 0.979 | 0.979 | 0.979 |
| Volume Å³ | 937500 | 420390 | 372247 | 180558 | 940639 | 946473 |
| Ramachandran outliers | 0 | 1 | 3 | 0 | 0 | 0 |
| Protein atoms | 3375 | 6662 | 3338 | 6534 | 3375 | 3375 |
| Heterogen atoms | 64 | 10 | 38 | 16 | 208 | 278 |
| Nucleic acids | 0 | 0 | 0 | 0 | 0 | 0 |
| Ramachandran outliers | 0 | 0 | 0 | 0 | 0 | 0 |
| Refinement | | | | | | |
| Rwork (%) | 19.7 | 18.7 | 20.4 | 23.1 | 20.8 | 20.3 |
| Rfree (%) | 22.4 | 23.3 | 24.9 | 25.7 | 24.0 | 23.6 |
| r.m.s. bond lengths (Å) | 0.0091 | 0.010 | 0.011 | 0.011 | 0.0093 | 0.010 |
| r.m.s. bond angles (°) | 1.33 | 1.42 | 1.56 | 1.56 | 1.61 | 1.28 |
| Z | 40.3 | 31.0 | 48.1 | 53.30 | 38.9 | 39.0 |
| Unique reflections | 35,329 | 34,731 | 21,015 | 18,489 | 46,712 | 43,755 |
| Mean B-factor (Å²) | 8.8 (78.6) | 6.3 (55.6) | 6.1 (40.4) | 4.0 (11.4) | 9.9 (10.0) | 3.7 (3.7) |
| Sym (%) | 0.00 (100.0) | 0.00 (100.0) | 0.00 (99.1) | 0.00 (98.3) | 0.00 (100.0) | 0.00 (100.0) |
| Protein atoms | 265 | 300 | 94 | 16 | 208 | 278 |
| Solvent atoms | 265 | 300 | 94 | 16 | 208 | 278 |
| Heterogen atoms | 64 | 10 | 38 | 16 | 208 | 278 |
| Nucleic acids | 0 | 0 | 0 | 0 | 0 | 0 |
| Ramachandran outliers | 0 | 0 | 0 | 0 | 0 | 0 |
| Solvent content (%) | 49.0 | 44.9 | 36.8 | 37.3 | 48.4 | 49.9 |

*Antigen T5 represents the DNA oligonucleotide 5′-TTTTTTT-3′P.

*Heterogen atoms describe nonstandard residues, such as prosthetic groups, inhibitors, carbohydrates, solvents, and ions.

*The gene designations shown in parentheses are based on the IMGT/V-quest database (Monod et al. 41) and Brochet et al. 40) and are used throughout this paper.
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genes nor do their heavy chain V-genes match any of the five known anti-DNA V-gene subtypes. Conversely, S1–15 only shares 51% sequence identity with Dna-1 VH chain, but many of these differences are conservative substitutions. Only one of the critical residues contacting lipid A is not present in Dna-1, namely Arg(H)-96.

S1–15 Recognition of ssDNA—The crystal structure of S1–15 Fab in complex with p5(dT)p showed two different oligonucleotide fragments bound at each phosphate-binding site, with a total of 14 hydrogen bonds between ssDNA ligands (Table 3) and residues of the combining site. These include five salt bridges to the terminal phosphates, and 12 hydrogen bonds mediated by eight water molecules (Fig. 2G). There are three salt bridges to the 5′P, formed via Arg(H)-50, Arg(H)-52, and Arg(H)-96. The two latter residues form a bidentate salt bridge. There are also two additional electrostatic interactions to the 3′-terminal phosphate as follows: a bidentate salt bridge from Arg(H)-94 and salt bridge from Arg(L)-55. Tyr(H)-100B and Tyr(H)-102 residues are involved in the hydrogen bonding to the terminal 3′-phosphate. There are only two hydrogen bonds formed between thymidine bases of the ssDNA fragments and the antibody-combining site, both from Thr(H)-28 to the thymidine on the 3′-terminal end. One hydrogen bond is also formed between the ring oxygen of the deoxyribose moiety on

**TABLE 2**

| Clone   | Variable Light Chain (V_L) | Variable Heavy Chain (V_H) |
|---------|-----------------------------|----------------------------|
| S1–15  | GVPSSGSGSGSTG YSLTISNLQ EDTAFY | VGRHAPLGYGGAAW--A YWDGGLTVTS |
| GL1    | GVPSSGSGSGSTG YSLTISNLQ EDTAFY | VSRHAPLGYGGAAW--A YWDGGLTVTS |
| A6     | GVPSSGSGSGSTG YSLTISNLQ EDTAFY | ARLYGNYRIH---D YWDGGLTVTS |
| GL2    | GVPSSGSGSGSTG YSLTISNLQ EDTAFY | ARLYGNYRIH---D YWDGGLTVTS |

FIGURE 2. Stereo diagram 2F_o – F_c electron density map (blue) contoured at 1.0σ for the BBP lipid A analogue was observed in the combining sites of A6 (A) and S1–15 mAbs (B) and two single-stranded DNA fragments, p2(dT)p (top) and p3(dT)p (bottom) were observed in the combining site of S1–15 (C). To show the base stacking interaction, the trinucleotide (bottom) is bound to a symmetry-related Fab. Although the original antigen used was p5(dT)p, only two and three nucleotides could be modeled with confidence. Stereo diagram of S1–15 in complex with 5(dT)p lipid showing showing hydrogen bonds (orange dashed spheres) between residues of S1–15 and to the 3′P (D). The remainder of the antigen was disordered. Indirect hydrogen bonds mediated by waters (cyan spheres) are also shown. CDR loops of the light and heavy chain are colored white and gray, respectively. Color scheme is as follows: carbon, green; oxygen, red; nitrogen, blue; phosphorus, orange; water, cyan. Density (blue) for the phosphate is contoured at 1.0σ. Stereo view of A6 (E) and S1–15 (F) in complex with lipid A analogues BBP shows hydrogen bonds and water bridges between the antigen and S1–15. CDR loops of the light and heavy chain are colored white and gray, respectively. Strong hydrophobic contacts are shown as dashed lines (black). C6 hydroxyl group of second GlcN is the attachment point to inner core residues of LPS. Stereo diagram of S1–15 Fab in complex with two p5(dT)p ssDNA fragments is shown (G). Stereo diagram of S1–15 Fab in complex with two p5(dT)p ssDNA fragments is shown.
the 5’-terminal end and Asn(H)-53. Finally, there are several hydrophobic interactions between Pro(H)-99 ring carbons and the 5’-terminal thymidine base (data not shown).

Crystallization of S1–15 with an ssDNA oligomer lacking the 5’-terminal phosphate resulted in the occupancy of only one phosphate-binding site. Density could only be observed for the terminal thymidine base (data not shown).

Hydrogen bond interactions between mAbs A6 and S1–15 to lipid A

| Antibody and PDB code | Residue | Atom(s) | Residue | Atom | CDR | Distance Å |
|-----------------------|---------|---------|---------|------|------|------------|
| A6 4ODV GlcN4P       | O6      | His(H)-100C | NEZ  | H3  | 3.04 |
|                      | O5      | Asn(H)-98   | ND2  | H3  | 3.16 |
|                      | 4PO4    | Tyr(H)-96   | OH   | H3  | 2.64 |
|                      | Lys(L)-53 | NZ        | L2   | 3.33*|
|                      | Tyrl-49  | OH         | L2   | 2.46 |
|                      | 1PO4    | Tyr(H)-32   | OH   | H1  | 3.15 |
|                      | Arg(H)-100A | NE | H3  | 3.24 |
|                      | Arg(H)-100A | NH2 | H3  | 2.87*|
|                      | Arg(L)-30 | NH1 | L1   | 2.41*|
| S1–15 4ODT GlcN4P   | O6      | Ala(H)-33   | N    | H1  | 2.95 |
|                      | O5      | Asp(H)-98   | O    | H3  | 2.85 |
|                      | 4PO4    | Arg(H)-52   | NE   | H2  | 2.81*|
|                      | Arg(H)-52 | NH2 | H2   | 2.70*|
|                      | Arg(H)-30 | NH1 | H3   | 3.09*|
|                      | GlcN1P  | O5      | Ala(H)-98   | N    | H3  | 3.06 |
|                      | 1PO4    | Tyr(H)-32   | OH   | H1  | 2.62 |
|                      | Tyr(H)-100B | OH  | H3  | 2.52 |
| S1–15 428F d’T chain E 3’PO4 | Arg(H)-94 | NH1 | H3 | 2.96*|
|                      | Arg(H)-94 | NH2 | H3 | 2.69*|
|                      | Tyr(H)-100B | OH | H3 | 2.68 |
|                      | Tryl(H)-102 | OH | H3 | 3.12 |
|                      | Arg(L)-55 | NH2 | L2 | 2.71*|
|                      | O5      | Thr(H)-28   | N    | H1  | 2.85 |
|                      | N3      | Thr(H)-28   | OG1  | H1  | 2.88 |
|                      | 4PO4    | Arg(H)-50   | NH1  | H3 | 2.30*|
|                      | Arg(H)-50 | NH2 | H3 | 2.91*|
|                      | Arg(H)-52 | NE | H3 | 2.92*|
|                      | Arg(H)-52 | NH2 | H3 | 2.87*|
|                      | Arg(H)-96 | NH2 | H3 | 3.05*|
|                      | Arg(H)-96 | NH2 | H3 | 2.93*|
|                      | O5      | Asn(H)-53   | ND2  | H2  | 2.99 |
|                      | O2      | Ser(H)-52   | OG   | H2  | 2.06*|

* Poor electron density was observed for the terminal thymidine of chain A, and this hydrogen bond therefore cannot be assigned with confidence.

The alignment does not include the Fv structure of S1–15 in complex with oligonucleotides, because no significant changes in conformation were observed in comparison with the structure in complex with lipid A. The root mean square deviation (r.m.s.d.) between the liganded and unliganded S1–15 Fv structures are presented in the order of V_{L} to V_{H}, respectively: 0.12 and 0.31 Å for the entire Fv, including a maximum of 2.55 and 1.63 Å and a minimum of 0.02 and 0.06 Å. The only conformational change between the liganded and unliganded occurred for Ser(L)-7, which is responsible for the ~2.55 Å shift (Fig. 3A). The alignment of A6 resulted in a mean r.m.s.d. of 0.16 and 0.52 Å, a maximum of 1.07 and 10.7 Å, and a minimum of 0.03 and 0.12 Å for the V_{L} and V_{H}, respectively. The three different Fv molecules of A6 showed a large conformational change around Lys(H)-64 on framework region 3 (FR3), resulting in 10.7 Å r.m.s.d. observed. A significant structural shift was also observed between liganded and the two unliganded Fab molecules on CDR H3 with an r.m.s.d. of 5.02 Å (Fig. 3B).

Discussion

**Steric Hindrance Prevents S1–15 and A6 from Binding LPS**—The liganded structures for mAbs S1–15 and A6 demonstrate the structural basis for the failure of these antibodies to recognize LPS-bound lipid A (29, 58), which is the steric hindrance surrounding the C6 hydroxyl of the second GlcN residue that forms the attachment point of the inner core. Furthermore, mAbs S1–15 and A6 possess a pocket specific to the OH-6 (Fig. 3, C and D) and form a hydrogen bond to this hydroxyl. Both factors are consistent with the observed loss of binding to lipid A upon the formation of the 6-O-methyl derivative (29).

**S1–15 and A6 Lipid A Recognition Strategy**—The binding mechanism of antibodies is often described as “lock and key” or “induced fit” based on the degree of dislocation of the CDRs when comparing bound and unbound states of the antibody (16, 59–64). Comparison between the unliganded and liganded structure of S1–15 showed that lock and key type, with its associated minimum entropic penalty upon binding (Fig. 3A). However, mAb A6 displayed a significant degree of induced fit, particularly in the FR3 of the V_{H} where a maximum r.m.s.d. of 10.7 Å was observed (Fig. 3B).

An antibody-combining site can form a pocket or groove, or a combination of both depending on the nature of antigen recognition. Antibodies specific for proteins and larger structures often utilize grooves, although antibodies specific for oligosaccharides and hapten often utilize pockets. As expected, each of the lipid A-specific antibodies uses a pocket with high structural and charge complementarity to lipid A (Fig. 3, C and D). Carbohydrate-specific antibodies are usually observed to bind antigen along the V_{L}/V_{H} interface, which explains the difficulty that has been reported in attempts to use the phage display technique to isolate single domain antibodies (i.e. composed solely of a heavy or light chain variable domain) specific for carbohydrates larger than a monosaccharide (65, 66). Interestingly, the lipid A-specific antibody S1–15 Fab binds lipid A with no involvement of the light chain (Figs. 2F and 3C). Although antibodies have been observed to recognize monosaccharides in pockets formed exclusively by heavy CDRs (67), this is the only structural example to date of an antibody using a heavy chain binding pocket to bind a carbohydrate moiety larger than a monosaccharide.

**Comparison between S1–15 and A6 Binding to Lipid A**—Although there are similarities, the combining sites utilize strikingly different mechanisms to recognize the lipid A backbone. In contrast to S1–15, mAb A6 binds to the lipid A antigen with both V_{L} and V_{H} chain residues, despite sharing V_{L} chain genes with S1–15. Most of the hydrogen bonds in the S1–15-lipid A complex generate the 4P of the second
GlcN (Fig. 2F), complementing ELISA data, where binding toward the 4P-monophosphorylated lipid A antigen is largely retained (Fig. 1A). A6 forms multiple contacts through both \( V_L \) and \( V_H \) chain residues to both phosphates of the lipid A analogue BBP, with the majority of these contacts directed toward the 1P (Fig. 2E), and thus there is a significant loss of avidity to the 4P-monophosphorylated lipid A (Fig. 1B). One feature common to both antibodies is a strong hydrophobic contact between the C6 carbon of the first GlcN (Fig. 2, E and F, dashed lines) and Tyr residues His-32 and Leu-50.
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From the conjugate ELISA, we determined the relative strength of binding, showing a 64-fold increase in avidity toward the bisphosphorylated lipid A backbone when comparing A6 to S1–15 at 2 pmol/well concentration of antigen (Fig. 1, A and B). This trend is consistent with the number of contacts and salt bridges formed between these antibodies and lipid A. A6 forms 11 hydrogen bonds, three of which are charged interactions. In contrast, S1–15 forms eight hydrogen bonds between lipid A and residues of S1–15, two of which are charged residue interactions.

Sequence Comparison to Anti-nucleotide Antibodies and the Basis for Polyspecificity—The structure of A6 and S1–15 in complex with lipid A carbohydrate backbone revealed distances of 11.9 and 12.1 Å, respectively, between the 1’- and 4’-phosphates, which approximate the distance (11.1 Å) observed in the trinucleotide in complex with antibody BV04-01 Fab.

Similarities in sequence had pointed toward potential polyspecificity of A6 and S1–15 toward ssDNA, as antibodies produced by immunization with lipid A carbohydrate backbone often share V-genes with DNA-specific antibodies. For example, the antibody belonging to the hybridoma cell line 73.23 possesses a related Vγ gene to A6 mAb, which not only binds ssDNA but dsDNA with lower avidity (52). Of the residues contacting lipid A for mAb A6, only Arg(L)-30 differs from anti-DNA antibody 73.23 (replaced by Ser(L)-30). Although the heavy chain V-region (coded by the Vγ-gene) of A6 is not involved in recognition of lipid A, the regions corresponding to the D and J genes of CDR H3 play a crucial role. DNA-specific antibodies have been shown to have CDR H3 loops dominated by Arg and Tyr residues (51), which is a feature shared with the CDR H3 of A6 (Table 2).

Structural Basis for S1–15 ssDNA Polyspecificity—Crystals were observed growing in number of different conditions, and several structures determined where the oligonucleotide was present, with the most similar structure being 11032 (Fig. 3). The arrangement was stabilized by base stacking between symmetry-related oligonucleotides (Figs. 2C and 3E). The phosphate binding pocket on S1–15 that recognizes 1P of lipid A binds the terminal 3’-phosphate, although the 4P lipid A pocket binds the 5’-phosphate. Interestingly, higher temperature factors were observed for the 5’-terminal phosphate-binding site when refined at full occupancy, despite the fact that there are more interactions with the 5’P. This appears to be due to two reasons. First there are two base-specific hydrogen bonds out of the total 14, both occurring at the 3’-terminal end. Second, the 3’-terminal thymidine base formed stacking interactions with the neighboring thymidine nucleotide. Furthermore, in the S1–15 Fab structure in complex with the 5(dT)p (lacking 5’P), density is only observable for the 3’-terminal phosphate with the residual oligonucleotides being disordered. This suggests that the 5’P-binding site plays a crucial role in stabilizing the second oligonucleotide fragment via stacking interactions on the 3’P-binding site.

Finally, density for the thymidine base on the 5’ end was poor in the p5(dT)p liganded structure due to high thermal motion, consistent with the lack of stabilizing stacking interactions.

Structural Comparison with Single-stranded Nucleotide-specific Antibodies—Published anti-ssDNA antibody structures show that the backbone phosphates are usually oriented away from the combining site, with the CDRs interacting directly through stacking interactions with the hydrophobic bases.

Autoantibody BV04-01 does recognize the backbone phosphate groups in its complex with the trinucleotide p5(dT) (lacking the 3’P), with the central thymidine forming stacking interactions with a Tyr and a Trp residue on either side of the thymidine (56). Additionally, the 5’P and central phosphate groups of the trinucleotide contribute to binding through hydrogen bonds, and interestingly, the 5’P-binding site of BV04-01 contains many interactions that correspond to the 5’P (and the 4’P lipid A)-binding site of S1–15 (Fig. 3G), marking its importance for dual recognition of ssDNA and lipid A. Of the three phosphates of the trinucleotide bound to BV04-01, only one phosphate formed a salt bridge to the Fab.

Similarly the RNA-specific antibody Jel 103 Fab structure in complex with RNA nucleotide (Protein Data Bank code 1MRF) display base stacking interaction with a Tyr residue and two salt bridges to the phosphate moiety from Lys and Arg residues, respectively (47).

It is remarkable that S1–15 and A6 bind ssDNA at all, as both have undergone affinity maturation toward the lipid A carbohydrate antigen, and the corresponding germ line antibodies could exhibit alternative binding modes to ssDNA than observed here. Crucially, the higher avidity of decavalent IgM plays an important role in establishing polyspecificity toward single-stranded nucleic acids. Binding assays have shown significant binding to various ssDNA fragments for both antibodies (data not shown), and additional crystallization studies with these antigens are ongoing.

Conclusions—The structures of mAbs S1–15 and A6 provide a structural explanation of their inability to bind intact LPS.

The ability of antibodies to undergo somatic hypermutation implies that germ line antibodies in general must display a significant degree of cross-reactivity or polyspecificity to increase the number of antigens recognized. Although there has been significant progress toward structural characterization of antibody cross-reactivity, there is a dearth of structures demonstrating the polyspecific nature of antibodies. These structures show significant binding to terminal nucleotides and shorter ssDNA oligonucleotides via positively charged surface complementarity to the terminal phosphates. Structural insights into the molecular basis for polyspecificity against ssDNA is of clinical interest and may provide crucial clues into how these antibodies arise as implicated in autoimmune diseases such as systemic lupus erythematosus, thyroiditis, and rheumatic autoimmune diseases.
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Author Contributions—O. H. G. performed the experiments shown in Figs. 2 and 3, conceived some of the experiments, analyzed the data, and wrote a major part of the paper. S. M. L. performed the ELISA experiments shown in Fig. 1, conceived some of the experiments, analyzed the data, and contributed to the paper. T. R. performed some of the experiments. L. B. performed some of the experiments, analyzed the data, and contributed to the paper. P. K. provided reagents and contributed to the paper. H. B. provided reagents, conceived many of the experiments, and contributed to the paper. S. V. E. conceived many of the experiments and wrote a major part the paper. All authors reviewed the results and approved the final version of the manuscript.

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