Structure and specificity of lamprey monoclonal antibodies

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Adaptive immunity in jawless vertebrates (lamprey and hagfish) is mediated by lymphocytes that undergo combinatorial assembly of leucine-rich repeat (LRR) gene segments to create a diverse repertoire of variable lymphocyte receptor (VLR) genes. Immunization with particulate antigens induces VLR-B-bearing lymphocytes to secrete antigen-specific VLR-B antibodies. Here, we describe the production of recombinant VLR-B antibodies specific for BcLA, a major coat protein of Bacillus anthracis spores. The recombinant VLR-B antibodies possess 8–10 uniform subunits that collectively bind antigen with high avidity. Sequence analysis, mutagenesis, and modeling studies show that antigen binding involves residues in the β-sheets lining the VLR-B concave surface. EM visualization reveals tetrameric and pentameric molecules having a central core and highly flexible pairs of stalk-region “arms” with antigen-binding “hands.” Remarkable antigen-binding specificity, avidity, and stability predict that these unusual LRR-based monoclonal antibodies will find many biomedical uses.

The adaptive immune system in jawless vertebrates (agnathans) is comprised of clonally diverse lymphocytes that express variable lymphocyte receptors (VLRs) created by the combinatorial assembly of leucine-rich repeat (LRR) gene segments rather than the Ig V, D, and J gene segments used by jawed vertebrates (gnathostomes) (1, 2). Two VLR genes, VLR-A and VLR-B, have been identified in both lamprey and hagfish (1–3). The germline VLR genes are incomplete in that they have coding regions only for the invariant N-terminal and C-terminal sequences, which are separated by intervening sequences, lacking canonical splice sites (1, 2). During development of lymphocyte lineage cells, components of the flanking LRR modular units are sequentially inserted into the incomplete VLR gene with a concomitant deletion of the intervening sequences (4, 5). The stepwise assembly of a mature VLR gene is mediated via a gene conversion mechanism in which AID-APOBEC family members with lymphocyte-restricted expression may serve a catalytic role (3, 5). This somatic gene rearrangement process has been estimated to produce a potential repertoire of >1014 distinct VLR-B receptors (4).

VLR-B proteins constitute a major component of the humoral arm of the lamprey adaptive immune system. Earlier studies, before the discovery of VLR genes, established that lampreys produce serum antibody-like proteins with agglutination and neutralization activity in response to immunization with particulate antigens, such as bacteriophage, Brucella abortus, and human red blood cells (6–10). More recently, we demonstrated that immunization with Bacillus anthracis exosporum induces production of soluble, antigen-specific VLR-B proteins, much like the antibody responses of jawed vertebrates (4). The secreted VLR-B proteins may function analogously to antibodies in jawed vertebrates, whereby microbe-induced VLR-B antibodies promote clearance of the infectious agent, presumably by neutralization, opsonization, and other mechanisms.

Monoclonal antibodies are valuable research and therapeutic tools that take advantage of the remarkable ability of the jawed vertebrate adaptive immune system to recognize almost any foreign molecule. In theory, it should also be possible to capitalize on the tremendous repertoire diversity of the agnathan adaptive immune system to produce cloned VLR-B antibodies of known specificity, with similar properties to monoclonal antibodies. However, there is no long-term in vitro culture system for lamprey lymphocytes, nor are there means to immortalize them presently, and the lack of fusion partner cell lines precludes the use of hybridoma fusion technology. Here, we describe a method of producing soluble, recombinant monoclonal VLR-B antibodies of defined antigen specificity and use them to investigate the quaternary structure and antigen binding site of secreted VLR-B antibodies.

Results

Production of Recombinant, Antigen-Specific VLR-B Antibody Clones. To generate VLR-B antibody-producing cells, we developed a heterologous expression system in which HEK-293T cells were transfected with full-length VLR-B cDNAs derived from lymphocytes of lamprey larvae immunized with the exosporum (i.e., the outermost layer) of B. anthracis spores [supporting information (SI) Fig. 5]. Clones that secreted antigen-specific VLR-B antibodies into the culture supernatant were then identified by ELISA and immunofluorescence-based flow cytometry assays. The secreted recombinant VLR-B antibodies are large molecules similar in molecular weight to primary VLR-B antibodies in plasma samples (SI Fig. 6). Fourteen of 212 VLR-B transfectants (6.6%) were found to secrete VLR-B antibodies against the C-terminal domain of the major exosporium protein BcLA (BcLA-CTD) (11, 12), a major epitope recognized by primary VLR-B antibodies made in the lamprey response. We selected the eight recombinant antibodies that recognized BcLA-CTD at the highest levels above background and one weakly binding clone, VLR5, for more comprehensive analysis (Fig. L4). Seven of the eight BcLA-CTD-reactive clones were also reactive with wild-type B. anthracis spores, but not BcLA-deficient B. anthracis spores (∆BcLA) or strains of two closely related Bacillus species, Bacillus cereus T and Bacillus thuringiensis (subsp. Kurstaki) in ELISA (Fig. 1B) and flow cytometry (Fig. 1C) assays. B. anthracis BcLA-CTD differs from B. cereus T BcLA-CTD at 14 of 134 amino acid positions, only 9 of which are solvent exposed (SI Fig. 7) (13). These results indicate that monoclonal VLR-B antibodies can discriminate between closely related protein antigens on the basis of limited amino acid variation.


The authors declare no conflict of interest.

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. EU325533–EU325540).

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The recombinant VLR-B antibodies that reacted strongly with both recombinant BclA-CTD and B. anthracis spores were all different by sequence analysis (SI Fig. 8). However, most shared the same number of LRR units and displayed notable sequence similarity, even in hypervariable amino acid positions. To evaluate how the shared residues might contribute to BclA-CTD binding, we constructed a homology-based model of the VLR4 structure by using the crystal structure of hagfish VLR-B (14) as a template (Fig. 2). The amino acids in hypervariable positions of neighboring LRR units were located near each other in the potential antigen binding site on the concave surface of the VLR-B antibody. A deep pocket contributed by residues of the LRRV, LRRVe, and LRR-CP units in the center of the concave surface may form a complementary surface for BclA-CTD binding. The LRR-CT sequences of the BclA-CTD-specific clones were identical except for a small variable region consisting of two to three residues (Fig. 2B). Our model predicts that the variable residues are located at the tip of the loop formed by the LRR-CT that projects toward the concave surface. This suggests that the variable amino acids of the LRR-CT may contribute to antigen binding.

Secreted VLR-B Antibodies Are Composed of Disulfide-Linked Pentamers and Tetramers of Dimers. The BclA-CTD-specific recombinant VLR4 antibody was purified by antigen affinity chromatography to examine its quaternary structure. Although this antibody could not be eluted from a BclA-CTD affinity column by high salt concentrations or extreme acidic conditions (pH < 1.5), it could be dissociated by very basic conditions (pH > 11) (SI Fig. 9A). Despite these harsh conditions, the eluted VLR4 antibody retained full antigen binding ability even after storage for >12 months at 4°C, 1 month at room temperature, or 36 h at 56°C (data not shown). Two forms of the recombinant VLR4 antibody, both larger than 225-kDa molecular mass standards, were eluted from the BclA-CTD affinity column (SI Fig. 9B). To obtain an accurate estimate of the molecular mass of VLR4, Ferguson plots were constructed by comparing the relative mobility of VLR4 and molecular mass standards in native gels of 5–12% acrylamide. The data indicated a molecular mass of 400 kDa for the larger VLR4 band (Fig. 3A), whereas a molecular mass of 40 kDa was estimated for individual VLR4 monomeric units. Therefore, the largest VLR4 oligomer may have 10 subunits, and the lower molecular mass VLR4 oligomer may contain 8 VLR subunits. Notably, a partially reduced band of 80 kDa, indicative of dimeric subunits, was observed on Western blots after exposure to relatively low concentrations of reducing agents (Fig. 3B). To visualize this structure, purified VLR4 antibody was examined by negative stain transmission electron microscopy (TEM). Pentameric VLR4 molecules with thin, highly flexible arms were observed, wherein the basally connected dimeric subunits form a central consolidation (Fig. 3C). Structurally similar VLR4 tetramers were also seen (data not shown). The composite results suggest that oligomeric VLR-B antibodies are composed of...
The Cysteine-Rich C Terminus Is Required for Assembly of Monomeric VLR-B Peptides into Multivalent Antibodies. The VLR-B antibody multimeric structure raises the question of how these molecules are assembled and released, especially given that cell surface VLR-B molecules are tethered by GPI linkage (1). If VLR-B antibodies were released by cleavage of the GPI linkage, the cysteines used for oligomerization of solubilized VLR-B antibodies would have to be N-terminal to the GPI cleavage site, because amino acids C-terminal to the cleavage site would be removed by GPI addition in the endoplasmic reticulum. To examine this possibility, trypsinized peptides of the purified VLR4 antibody were sequenced by tandem mass spectrometry. This analysis revealed that the entire cysteine-rich peptide sequence of the C terminus is present in the secreted VLR4 antibody (VLR4WT) molecules produced by transfectants were separated by nonreducing SDS/PAGE, and their molecular weights were estimated by Western blotting. As predicted, the VLR4GPI-stop molecule migrated as a \( \approx 40 \text{-kDa} \) monomer (SI Fig. 11A). ELISA evaluation of antigen binding by the oligomeric VLR4WT versus the monomeric VLR4GPI-stop antibodies indicated strong BclA-CTD binding by the oligomeric VLR4 antibody and weak antigen binding by the monomeric VLR4 antibody (SI Fig. 11B). These results imply that, even though VLR-B monomeric units have a relatively low antigen binding affinity, the antigen binding avidity of a multimeric VLR-B antibody can be relatively high.

The \( \beta \)-Sheets on the Concave Surface of VLR-8 Antibodies Constitute the Primary Antigen Binding Site. Because of their high sequence variability, the parallel \( \beta \)-strands that form the concave surface of VLR-B are presumed to be the antigen binding site (4, 14). However, this assumption has not been experimentally tested. The amino acid sequences of four BclA-CTD-specific VLR-B antibodies (Fig. 11A) are similar in the predicted antigen binding region, and three of these (VLR4, Ba41, and Ba191) bind BclA-CTD with relatively high avidity. The other, VLR5, exhibits weak antigen binding, and its sequence differs from the consensus sequence of the high-avidity BclA-CTD VLR-B antibodies at 6 of 20 possible hypervariable amino acid positions on the concave surface (H34, Y55, T58, Q101, S103, W127) (Fig. 4A and B). These findings suggest that one or more of these six residues causes the relatively low antigen avidity of the VLR5 antibody. To test this hypothesis, we used site-directed mutagenesis to change the variant VLR5 residues to the corresponding amino acids in VLR-B antibodies with higher antigen binding avidity. By using surface plasmon resonance to evaluate antigen binding by VLR4WT, VLR5WT, and VLR5 mutants with modifications of the posited antigen binding site (VLR5H34N, VLR5Y55R, VLR5Q101H, VLR5S103A, and VLR5W127Y), we observed much higher binding responses to BclA-CTD for VLR5Y55R and VLR5Q101H relative to the VLR5WT antibody. The response curves were indicative of an increase in the
mutants (VLR5H34N, VLR5Q101H, VLR5S103A) displayed equivalent antigen binding residues of VLR5 that differ from the corresponding residues of the high-avidity clones at that position. Response curves are color-coded with respect to amino acid positions depicted in the high-avidity clones at that position. Response curves are color-coded with respect to amino acid positions depicted in A and B.

Fig. 4. The primary antigen binding site of VLR-B is located on the concave surface. (A) Sequence alignment of high-avidity (Ba41, Ba191, VLR4) and low-avidity (VLR5) VLR-B antibodies specific for BclA-CTD. Hypervariable positions are shaded black. Amino acids in VLR5 that differ from the high-avidity clones are shaded in color if they are in hypervariable positions or gray if located elsewhere, except V97/N98 (yellow). (B) Homology-based model of VLR5 structure. Amino acids in hypervariable positions are color-coded as in A. (C) Surface plasmon resonance measurement of the interaction between VLR4WT, VLR5WT, or VLR5 mutant antibodies with BclA-CTD. VLR5 residues in hypervariable positions that differ from the high-avidity clones were mutated to the consensus amino acid of the high-avidity clones at that position. Response curves are color-coded with respect to amino acid positions depicted in A and B.

Association rate of these VLR5 mutants with BclA-CTD; however, the VLR-BcA complexes formed by these VLR5 mutants dissociated more rapidly than VLR4 (Fig. 3C). The other VLR5 mutants (VLR5H34N, VLR5Q101H, VLR5S103A) displayed equivalent or slightly weaker binding avidity when compared with the VLR5WT antibody (Fig. 4C).

Because mutation of the Y55, T58, or W127 residues on the concave surface increased VLR5 antigen binding avidity, we made double mutants of VLR5 (Y55R/W127Y and T58I/W127Y) to test whether these residues could function in a cooperative manner for antigen binding (Fig. 4C). Both the VLR5Y55R/W127Y and VLR5T58I/W127Y double mutants exceeded the antigen binding response of their single mutation counterparts and their rate of dissociation was more comparable with that of the VLR4 antibody, indicative of enhanced binding avidity. In contrast, when we mutated two residues of VLR5 that differ from the corresponding residues of the high-avidity clones, but that are not located on the concave surface (V97T/N98K), BclA-CTD antigen binding response of this mutant was similar to that of VLR5WT. These results suggest that the hypervariable amino acids on the concave surface are the primary antigen binding residues.

Discussion

We have developed a method to exploit the vast repertoire diversity of the lamprey adaptive immune system to produce recombinant monoclonal VLR-B antibodies with high avidity and specificity for antigen. VLR-B antibody clones discriminated B. anthracis from B. cereus spores with 100% accuracy (seven of seven clones tested), despite 89.5% identity (120 of 134 aa) in peptide sequences of BclA-CTD from B. anthracis and B. cereus. Monoclonal VLR-B antibodies also agglutinated B. anthracis spores at a 1,000-fold higher dilution than an IgG2b monoclonal antibody, demonstrating the high avidity of the multivalent VLR-B antibodies for antigens with repetitive epitopes. The high avidity of VLR-B antibodies facilitated their purification from crude supernatant by antigen affinity chromatography. Surprisingly, VLR-B antibodies could not be eluted by high salt concentrations or extreme acidic conditions (pH <2.0). Although highly basic solutions (pH >11) were required to elute VLR-B antibodies, the structure and antigen binding activity of the antibodies was not compromised by the harsh conditions. Recombinant VLR-B antibodies also retained their antigen binding activity after prolonged exposure to high temperatures, including 1 month at room temperature and 36 h at 56°C. These studies indicate that the LRR subunits form a very stable structure that should be resistant to denaturation under most storage and assay conditions.

Lampreys produce multivalent serum antibodies with high antigen binding avidity even though the constituent VLR units exhibit relatively low avidity for antigen. Recombinant VLR-B antibodies are assembled into disulfide-linked tetramers and pentamers of dimers with approximate molecular weights of 320 and 400 kDa, respectively. The molecular weight estimates are remarkably similar to previous estimates of lamprey agglutinins, although not all of the biochemical properties ascribed to the agglutinins are consistent with VLR-B antibodies (i.e., lack of interchain disulfide bonds) (9). The pentameric/tetrameric arrangement of VLR-B subunits is similar to the subunit organization of IgM antibodies in higher vertebrates. Notably, however, VLR-B antibodies use monomeric subunits for antigen binding rather than the heterodimeric pairing of heavy and light chain peptides that form the antigen binding surface of most jawed vertebrate antibodies. The single peptide composition of VLR-B antibodies makes them more amenable to molecular engineering, including manipulation of the antigen binding site by mutagenesis and fusions to the coding sequences of other peptides, such as enzymes, toxins, and epitope tags to extend their functional capabilities. Furthermore, it may be possible to use random mutagenesis technologies to create libraries of VLR-B antibodies with higher avidity for antigen than can be produced in vivo to promote monomeric VLR-B antigen binding.

In conclusion, our studies reveal several unique and potentially advantageous characteristics of monoclonal VLR-B antibodies. Multivalency contributed by 8–10 identical antigen binding subunits assures relatively high-avidity binding to antigens with repetitive epitopes. In contrast with typical IgM antibodies, the VLR-B antibodies that we have examined are relatively stable and appear to have limited cross-reactivity. This difference in specificity may be because VLR-B antibodies use β-sheets as the primary antigen binding surface, whereas Ig-based antibodies use extended loops for antigen binding. The ease of mutational modification of the antigen binding site of VLR-B antibodies should allow their binding characteristics to be tailored to the requirements of a particular assay. Finally, VLR-B antibodies may be produced against mammalian antigen epitopes that would be forbidden for Ig-based antibodies because of self-tolerance.
VLR-B cDNA Library Cloning. Blood samples collected from immunized, lethally anesthetized lamprey were centrifuged at 50 × g for 5 min to pellet red blood cells, and then buffy coat leukocytes overlying the red blood cell pellet were collected by pipetting. VLR-B-1 lymphocytes labeled with anti-VLR-B mAb (4C4) (specific for the invariant stalk region) (4) were purified by using a MoFlo cell sorter (DakoCytomation). RNA isolated from VLR-B-1 lymphocytes with TRIzol (Invitrogen) was reverse transcribed into cDNA by using SuperScript III reverse transcriptase (Invitrogen) and random hexamer priming. VLR-B transcripts were amplified from VLR-B-1 lymphocyte cDNA by nested PCR using KOD high-fidelity DNA polymerase (Novagen). The first round of PCR used primers from the 5′- and 3′-untranslated region, LRR_N.F1 (CTCCGCTACTCGCGCTGCA) and LRR_C.R2 (C CGCCATCCCCGACCTTGG), respectively. PCR conditions were 25 cycles at 95°C, 30 s; 60°C, 30 s; and 70°C, 1.5 min. The second round of PCR used a 5′-phosphorylated primer, pIRES-koz-fwd (CCACTAGTGATCGAACGTTGCGGCCGCGGCCACCTTGG), and a 3′ primer containing a NheI site, pIRES-Nhel-rev (GGAGATCTGATCAAGACGGTGAGGCG). PCR conditions were 30 cycles at 95°C, 30 s; 62°C, 30 s; and 70°C, 1.5 min. PCR products were purified and digested with NheI (New England Biolabs). More than 500 VLR-B sequences from GenBank were examined for restriction enzyme cut sites; Nhel sites were not found in any of the sequences. The Nhel-digested PCR products were ligated into the EcoRV and Nhel sites of pIRES-puro-2 (Clontech) and transformed into NovaBlue competent cells (Novagen). Individual library colonies were picked and purified with the 96-well format QIAprep 96 Turbo miniprep kit (Qiagen) (clones Ba1-A1-Ba12). In a pilot screen, plasmids were purified from 30 colonies by using QIAprep spin miniprep kit (Qiagen) (clones VR1-VR30).

Recombinant VLR-B Expression and Purification. For library screening, purified library plasmids were transfected into HEK-293T cells cultured in RPMI medium 1640 containing 5% FBS using linear polyethylenimine (PEI), molecular weight (MW) 25,000 (Polysciences) at a 3:1 PEI-to-DNA ratio. Supernatants harvested 48 h posttransfection were cleared by centrifugation for 15 min at 20,000 × g. Recombinant VLR-B production was verified by Western blotting transfectant supernatants with anti-VLR-B mAb (4C4) and then with R-phycoerythrin (RPE)-conjugated goat anti-mouse antibodies. This work was supported by National Institutes of Health Grants AI057699 (to K.H.R. and C.L.T.) and AI072435 (to M.D.C.) and by the Howard Hughes Medical Institute (M.D.C.).

Methods

Animals and Immunization. Two- to 4-year-old lamprey larvae were maintained and immunized as previously described (4). All experiments were approved by Institutional Animal Care and Use Committee at the University of Alabama at Birmingham.

VLR-B Sequence Analysis and Structural Modeling. VLR-B sequence analysis and multiple sequence alignments were prepared by using VectorNTI (Invitrogen) and GeneDoc (http://www.psc.edu/biomed/genedoc). Homology-based models of VLR4 and VLR5 structure were constructed with MODELLER 9v2 (www.salilab.org/modeller) using the crystal structures of hagfish VLR-B as the template (Protein Data Bank (PDB) ID codes 206R and 206S).

Ferguson Plots. Purified recombinant VLR4 and high MW standards (thioglycol- lulin, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa; lactate dehydrogenase, 140 kDa; and BSA, 67 kDa) (GE Healthcare) were separated by electrophoresis using native gels containing 5%, 6%, 8%, 10%, and 12% acrylamide. After electrophoresis, proteins were visualized by Gelcode blue staining (Pierce). The relative mobility (R) of VLR4 and each of the MW standard proteins in each percent acrylamide gel was measured. A graph of log(100R) versus percent acrylamide was constructed for each protein and the retardation coefficient (K) of each protein was calculated from the negative slope of the line. The K value of each MW standard protein was plotted versus its MW to construct a standard curve, from which the MW of VLR4 was calculated.

Electron Microscopy. Negative-stain electron microscopy was performed as previously described (16). Briefly, VLR4 was diluted to 1.25 μg/ml BSA, 0.025 M Na2B4O7, 0.075 M NaCl, pH 8.3, affixed to carbon support membrane. The membrane was stained with 1% uranyl formate and mounted on 600 mesh carbon-coated grids for analysis. Electron micrographs were digitally photographed > 100,000 magnification at 100 kV on a JEOL JEM 1200 electron microscope.

Mass Spectrometry. For peptide sequencing by tandem mass spectrometry, recombinant VLR4 was separated on a reducing SDS/PAGE gel and stained with Gelcode blue (Pierce). VLR4 was excised from the gel, alkylated with iodoacetamide, and digested with trypsin. The tryptic peptides were separated by reverse-phase high-performance liquid chromatography and sequenced by electrospray ionization MS/MS.

Agglutination Assay. Stock solutions of the VLR4 antibody and anti-BclA-CTD mouse monoclonal antibody (EA2-1) (IgG2b) (11) at 0.5 μg/ml were prepared in PBS, and then serially diluted in 10-fold increments from 1/10 to 1/1010. EA2-1 was provided by John F. Kearney (University of Alabama at Birmingham). A total of 100 μl of each antibody dilution was added to 1 × 106 B. anthracis spores in 96-well flat-bottom plates and incubated at room temperature overnight before visual inspection for spore agglutination by light microscopy. Spore agglutination was scored on an arbitrary scale from 0 to 4 (0, single spores; 1, clusters of 2–3 spores; 2, clusters of 4–6 spores; 3, clusters of 7–10 spores; 4, large clusters of >10 spores).

Surface Plasmon Resonance Measurements of Antigen Binding Site Mutants. VLR5 antigen binding site mutants were constructed by using QuikChange Site-Directed Mutagenesis (Stratagene). Tissue culture supernatants of HEK-293T cells transfected with VLR4, VLR5WT, and VLR5 mutants were collected 48 h after transfection. VLR expression in the supernatants was detected Western blotting with anti-VLR-B mAb (4C4) followed by alkaline phosphatase-conjugated goat anti-mouse antibodies. This work was supported by National Institutes of Health Grants AI057699 (to K.H.R. and C.L.T.) and AI072435 (to M.D.C.) and by the Howard Hughes Medical Institute (M.D.C.).
B. anthracis  LGLPAGLYAFNSGCISLDLCNEPVPFNTVGSQFG 35
B. cereus T  LGLPAGLYAFNSGCISLDLCNEPVPFNTVGSQFG 35

B. anthracis  TAISQLDADTFVISETGFKITVITANATASVLGG 70
B. cereus T  TAISQLDADTFVISETGFKITVITANATASVLGG 70

B. anthracis  LTQVNVPGPTGSLISLGAPIVQAIITQITTT 105
B. cereus T  LTQVNVPGPTGSLISLGAPIVQAIITQITTT 105

B. anthracis  PSLVEVIVTGLGLSLALGTNASIIIEKVA 134
B. cereus T  PSLVEVIVTGLGLSLALGTNASIIIEKVA 134