THE ADAPTIVE IMMUNE SYSTEM OF SEA LAMPREY

by

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A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham, in partial fulfillment of the requirements for the degree of Doctor of Philosophy

BIRMINGHAM, ALABAMA

2008
Jawed vertebrates rearrange their immunoglobulin V, D, and J gene segments to generate a large repertoire of antibody receptors. Jawless vertebrates instead have evolved an adaptive immune system that does not use immunoglobulin genes. We show that lamprey and hagfish lymphocytes generate a large repertoire of variable lymphocyte receptors (VLR) by randomly assembling leucine-rich-repeat (LRR) gene segments. The assembly proceeds by insertion of flanking LRR cassettes into an incomplete germline VLR gene via a multi-step, piecewise gene conversion process. Monoallelic VLR assembly leads to the expression of unique cell surface VLRs by individual lymphocytes as the basis for a clonally diverse lamprey lymphocyte repertoire that is comparable to the antigen receptor repertoire of mammalian B lymphocytes (>10^14). We also characterize the cellular and humoral responses of lamprey to different immunogens. To identify the VLR-bearing cells and their soluble VLR products we used monoclonal antibodies specific for the invariant stalk region of VLRs. Immunization of lampreys with Bacillus anthracis exosporium induced lymphocyte transformation, differentiation, and secretion of multivalent VLR antibodies specific for the BclA spore surface protein. Lampreys immunized with blood group O erythrocytes produced VLR antibodies with specificity for the carbohydrate H antigen. The results of these experiments provide new insights into the evolution of adaptive immune systems.
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INTRODUCTION

Even the most elementary forms of life utilize barriers, such as cell walls and plasma membranes, to compartmentalize and protect their biochemical machinery from the chaotic extracellular milieu. As life evolved into multicellular organisms, more elaborate barriers and defense mechanisms were selected for in order to prevent invasion by microorganisms, including fungi, bacteria, and viruses. Evolution led to increasingly sophisticated methods of immunity, first innate and later adaptive, to defend against pathogens (Fig. 1). Innate immunity is present throughout the metazoan lineage and utilizes invariant germline encoded molecules that detect pathogens and activate effector mechanisms to prevent establishment of infection. Examples include toll-like receptors (TLR), antimicrobial peptides, and the complement cascade. This type of immunity has proven extremely effective in that the vast majority of species on earth, including many long-lived and complex species, rely on innate immunity alone to defend themselves from pathogens.

Beginning with vertebrates a more complex and flexible adaptive immune system arose. Central to the adaptive immune system is the somatic rearrangement of incomplete germline gene segments to generate a vast repertoire of receptors. Gene rearrangement occurs only in specialized cells called lymphocytes. The lymphocytes are long lived, quiescent cells each expressing a single antigen receptor that, when activated, induces proliferation and differentiation to effector cells with the same receptor specificity. In jawed vertebrates two major lineages of lymphocytes rearrange
Figure 1  Phylogenetic tree showing the relationship of major metazoan lineages and the distribution of innate and adaptive immunity within these lineages. Molecules that participate in adaptive immunity are in green: variable lymphocyte receptor (VLR) and immunoglobulin domain based T cell receptor (TCR) and immunoglobulins (Ig). Other examples of innate immune receptors that use immunoglobulin domains are shown in blue: fibrinogen-related proteins (FREPs) and V type Ig domains and a chitin binding domain containing proteins (VCBP). Not shown is the toll-like receptors that utilize LRR domains and are present in all major metazoan lineages.

antigen receptor gene segments to maintain different arms, humoral and cellular, of the adaptive immune system. Humoral immunity is mediated by B cells that first express their antigen receptor on the surface and following activation secrete their receptor. T cells express surface T cell receptors (TCR) that recognize foreign peptides in the presence of the major histocompatibility complex (MHC) to initiate cellular immunity. This model of adaptive immunity seems to hold true for all vertebrates with the notable exception of the most primitive jawless vertebrates, lamprey and hagfish. These jawless fish have cells that are morphologically similar to lymphocytes and exhibit several characteristic adaptive immune responses, such as more rapid rejection of second set skin allografts, delayed type hypersensitivity and lymphoblastoid transformation of their lymphocytes in response to immunization with a cocktail of antigens. In addition, lamprey immunized with heterologous erythrocytes produce high titer specific
agglutinins. However, definitive isolation of immunoglobulin based antibodies has not been achieved\textsuperscript{6,7}. Extensive searches for genes essential for T and B cell-mediated adaptive immunity revealed that many genes expressed specifically in mammalian lymphocytes are also expressed by lamprey lymphocytes, such as \textit{IKAROS, SPIB, VPRESS}, and \textit{CD4}, but the cardinal genes required for adaptive immunity (\textit{MHC, RAG1} and 2, rearranging \textit{TCR} and \textit{BCR}) have not been identified\textsuperscript{8-13}. Further, analysis of transcripts derived from lamprey lymphocytes revealed a significant number of highly variable transcripts containing leucine-rich repeats (LRR) motifs which were later called variable lymphocyte receptors (VLR)\textsuperscript{5}.

The large number of diverse VLR sequences isolated from lamprey lymphocytes suggested that these proteins may be participating in lamprey immune responses. LRR domains from other proteins have been associated with protein-protein interactions and implicated in innate pathogen recognition\textsuperscript{14}. TLRs and plant disease resistance genes are composed of LRRs which bind conserved epitopes on microbes\textsuperscript{15,16}. All LRR containing protein domains for which structural data is available form a horseshoe shaped solenoid. Most of these, including ribo-nuclease inhibitor and internalin, bind their ligand through interactions on the inner concave surface of the solenoid\textsuperscript{14}. When aligned, the VLR sequences exhibited great diversity, that when modeled on other LRR proteins structures, is located primarily on the inner concave surface. Each VLR sequence contains an N-terminal LRR (LRRNT), an 18 amino acid LRR1, a variable number of sequence diverse 24 amino acid LRRs (called LRRV), a 13 amino acid connecting peptide (CP), a C-terminal LRR (LRRCT), and a conserved threonine-proline rich stalk that is attached to the cell surface of lymphocytes by a glycosyl-phosphatidyl-inositol (GPI) linkage\textsuperscript{5} (Fig
2a). The VLR sequence diversity arises from differences in sequence of each LRR module, as well as in the number of modules in each transcript.

![Genetic layout of VLR transcripts and germline gene.](image)

**Figure 2** Genetic layout of VLR transcripts and germline gene. (a) Each VLR segment is shown: signal peptide (SP), LRRNT, first LRR (LRR1), 1 to 9 variable LRR (only one is depicted in this figure but up to 9 have been isolated with the average being between 2 and 3 segments; LRRV), connecting peptide (CP), LRRCT, and the invariant stalk region. (b) Organization of unrearranged VLR germline locus.

Southern blots with probes specific for the conserved threonine-proline rich stalk regions of the VLR transcripts and sequencing of the locus led to the conclusion that VLRs were encoded by a single copy gene\(^5\). The locus contains three coding regions, the first codes for the signal peptide and a small part of LRRNT, the second codes for most of LRRCT and the third codes for the latter portion of LRRCT and the stalk region. Roughly 20 Kb upstream from the transcription start site LRR1, LRRV, and LRRNT cassettes can be found. These cassettes code for portions of one, two or three joined LRR motifs. 5 Kb downstream from the transcription termination site more cassettes can be found containing portions of the LRRCT (Fig 2b). Large scale sequencing of the lamprey genome catalogued over 450 different cassettes distributed over a ~2 Mb locus\(^17\). Single cell PCR revealed that each lymphocyte only expressed a single VLR transcript\(^5,18\). These findings led to the hypothesis that the lamprey VLR locus undergoes molecular rearrangement of the LRR modules in order to generate an anticipatory
repertoire of antigen receptors, in a similar fashion to our own lymphocytes that undergo V(D)J rearrangement to generate their diverse repertoire of antigen receptors\textsuperscript{5} (Fig. 3).

\textbf{Figure 3} Comparison of jawed vertebrate (gnathostome) immunoglobulin based adaptive immune system and the model for the jawless vertebrates (agnathan) VLR based adaptive immune system.

The experiments described in this dissertation were designed to test this model of the lamprey adaptive immune system. The germline sequence of the VLR locus suggested some method of rearrangement would be required to generate a complete open reading frame that coded for a viable VLR protein, but through what mechanism? The first 100 VLR sequences were almost all different, suggesting the VLR diversity was quite large. But was the repertoire sufficient to mediate adaptive immunity? Could examples of VLRs binding antigens be demonstrated? These questions are addressed in the first chapter. The second chapter focuses on the lamprey lymphocyte and its similarities to our own lymphocytes. It also addresses the different types of antigens that initiate immune responses in lamprey. Finally, we show that stimulated lamprey lymphocytes undergo blast transformation, proliferation, and differentiation into VLR secreting plasmacytoid cells. During the time these experiments were carried out a
second VLR molecule from lamprey was discovered\textsuperscript{17}. The VLR molecule described above and throughout this dissertation is now referred to as VLR-B, while the newly discovered VLR-A is only mentioned in chapter 2 and in the conclusion because little has been learned about its potential role in lamprey immunity.
DIVERSITY AND FUNCTION OF ADAPTIVE IMMUNE RECEPTORS IN JAWLESS VERTEBRATES

by

MATTHEW N. ALDER, IGOR B. ROGOZIN, LAKSHMINARAYAN M. IYER, GALINA V. GLAZKO, MAX D. COOPER AND ZEEV PANCER


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ABSTRACT

Instead of immunoglobulin-type antigen receptors of jawed vertebrates, jawless fish possess variable lymphocyte receptors (VLRs), which consist of leucine-rich repeat (LRR) modules. Somatic diversification of the VLR gene is shown here to occur through a multistep assembly of LRR modules randomly selected from a large bank of flanking cassettes. The predicted concave surface of the VLR is lined with hypervariable positively selected residues, and computational analysis suggests a repertoire of about $10^{14}$ unique receptors. Lamprey immunized with anthrax spores responded with the production of soluble antigen-specific VLR. These findings reveal that two strikingly different modes of antigen recognition through rearranged lymphocyte receptors have evolved in the jawless and jawed vertebrates.
An adaptive immune system based on lymphocytes bearing clonally diverse antigen-specific receptors first appeared at the dawn of vertebrate evolution ~500 million years ago. Within less than 40 million years in the Cambrian, both jawless and jawed vertebrates evolved mechanisms of lymphocyte receptor diversification that were radically different. Thus, jawed vertebrates rearrange immunoglobulin and T-cell receptor (TCR) variable, diverse, and joining gene segments (VDJ) to generate highly diverse repertoires of T and B lymphocyte antigen receptors (1, 2). In contrast, lamprey and hagfish, jawless fish representatives of the oldest vertebrate taxon, assemble their VLRs from modular LRR units (3, 4). In the lamprey, a single incomplete germline VLR gene generates a diverse repertoire of cell surface receptors through somatic rearrangement of LRR cassettes that flank the gene. Each lymphocyte thus assembles a VLR gene of unique sequence. Hagfish have two germline VLR genes, called VLR-A and VLR-B, that can generate equivalently diverse receptor repertoires (4). On the basis of the existence of a sizeable repertoire of diverse lymphocyte receptors, we hypothesized that VLRs may serve as jawless fish equivalent of the anticipatory antigen receptors of jawed vertebrates.

The potential diversity of lamprey VLRs was estimated by analysis of 517 unique VLR sequences, including 129 previously reported sequences (3) and 388 new sequences derived mostly from animals immunized with the Bacillus anthracis spore coat (5). Analysis of the aligned VLR diversity regions revealed mixed clusters of sequences, with no exclusive clustering of VLRs from animals immunostimulated with particular antigens. The alignment was then converted into a matrix consisting of the individual types of constituent LRR modules (Fig. 1A). This included the 30 to 38 residue N-
terminal LRR (LRRNT), 18-residue first LRR (LRR1), 24-residue LRRs (LRRV), 13-residue connecting peptide (CP), and 48 to 65 residue C-terminal LRR (LRRCT). Noting that the terminal 24-residue LRR module adjacent to the CP had a distinct sequence signature in 98% of the cases (Fig. S1, 5), we designated this as the LRRV-end (LRRVe).

The dataset was screened for repetitive occurrence of each type of LRR module, singly or as recurring pairs (Tables 1 and 2). Most pairs of adjoining LRRVs or LRRVe’s were only observed once, but in some cases, repetitious pairs of LRRNT-LRR1 and CP-LRRCT were identified. These may represent VLRs that were assembled from multi-
Table 1. Distribution of unique and repeated adjoining pairs of LRR modules among 517 unique VLR sequences (the modules are shown in Fig. 1A).

<table>
<thead>
<tr>
<th>Number of pairs</th>
<th>LRRNT</th>
<th>LRR1</th>
<th>LRRV</th>
<th>LRRVe</th>
<th>CP</th>
<th>LRRCT</th>
</tr>
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<tr>
<td>1</td>
<td>287</td>
<td>390</td>
<td>270</td>
<td>388</td>
<td>449</td>
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<td>2</td>
<td>22</td>
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<td>3</td>
<td>16</td>
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</tr>
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<td>7</td>
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<td>3</td>
<td>8</td>
<td></td>
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<td>1</td>
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<td></td>
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<td>1</td>
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</tbody>
</table>

module genomic cassettes, such as one LRR1-LRRV-LRRV triplet previously identified in the VLR locus (3), or VLR selected for certain structural conformations. However, 94% of the LRRNT-LRR1 and CP-LRRCT pairs are either unique or consist of the same pair of adjoining modules occurring three times or less in the VLR dataset, and the pairing occurrence follows a random Poisson distribution (6). Most hagfish VLR-A modules were also found in random combinations (n=139; Tables S1 and S2) whereas the VLR-B sample (n=70) was too small for reliable analysis. The potential diversity of the VLR repertoire was therefore calculated by considering individual LRR modules as independent recombination units. For the lamprey we predict a potential repertoire of up to $10^{14}$ unique VLRs and up to $10^{17}$ for the hagfish VLR-A (5).

The number of LRR cassettes flanking the germline VLR gene is unknown. Thus far, 32 unique germline LRR modules have been identified in the partially sequenced lamprey VLR locus (3), and only 15 of these were identical to one of the 1,568 modules
Table 2. Different LRR modules and those found only once in adjoining pairs among 517 unique VLR sequences. The distribution of LRRV modules per transcript is shown separately.

<table>
<thead>
<tr>
<th>Module</th>
<th>Different (% total)</th>
<th>Uniquely paired</th>
<th>LRRV modules</th>
<th>Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>LRRNT</td>
<td>235 (45)</td>
<td>196</td>
<td>0</td>
<td>109</td>
</tr>
<tr>
<td>LRR1</td>
<td>191 (37)</td>
<td>148</td>
<td>1</td>
<td>228</td>
</tr>
<tr>
<td>LRRV</td>
<td>530 (78)</td>
<td>518</td>
<td>2</td>
<td>119</td>
</tr>
<tr>
<td>LRRVe</td>
<td>353 (68)</td>
<td>335</td>
<td>3</td>
<td>45</td>
</tr>
<tr>
<td>CP</td>
<td>71 (14)</td>
<td>54</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>LRRCT</td>
<td>188 (36)</td>
<td>160</td>
<td>5</td>
<td>8</td>
</tr>
</tbody>
</table>

Average \( (k) \) 1.31

*VLR sequences with LRRVe modules but no LRRV.

from the VLR dataset. To estimate the number of LRRV modules in flanking cassettes at the VLR locus, we used Monte Carlo simulations to predict at 95% confidence level an upper bound estimate of \( \sim 1500 \) lamprey LRRVs and \( \sim 2400 \) LRRVs for the hagfish VLR-\( A \) (5). These data suggest that the rearrangement process yielding mature VLR genes occurs by random selection of each module type from a large pool of genomic LRR modules.

The lamprey germline VLR gene of \( \sim 13 \) kb consists of three coding regions separated by two intervening sequences: (i) the signal peptide and 5` portion of LRRNT, (ii) the 5` portion of LRRCT, and (iii) the 3` portion of LRRCT and stalk region (Fig. 1B) (3). Previously, we identified germline VLR transcripts from lamprey and hagfish lymphocytes, which indicated VLR gene transcription before or during the rearrangement process (4). We therefore preferentially cloned those rare cDNA amplicons that retained portions of the intervening sequences. For this we used polymerase chain reaction (PCR) primer combinations, wherein one annealed within an intervening region and the other in a coding portion, followed by selection for amplicons shorter than the length expected for
germline VLR transcripts. Amplicons generated from genomic DNA (gDNA) were also analyzed. Among 37 unique rearrangement intermediates, we identified clones with large DNA deletions at different locations in the intervening regions (nine cDNA clones, two gDNA). In addition to deletions, some clones revealed modular LRR insertions within the germline VLR (24 cDNA clones, 2 gDNA). Deletion of coding portions of the germline gene were observed in five cDNA clones, as in no. 36 where the germline 5`LRRCT is missing. In other cases, the germ line-encoded 5`LRRCT was replaced with unique 5`LRRCT from the flanking cassettes (four cDNA clones, two gDNA). The insertions in two clones included non-coding DNA, as shown for the 78 nucleotide insertion flanking the terminal LRR module in no. 36. All of the LRR modules were inserted in-frame with germ line-encoded elements, but in most cases, the insertions terminated with an incomplete LRR module (92%). Insertion of the LRRV modules into the germline VLR occurs through multiple independent events as indicated by (i) the variable numbers of LRRVs in the rearrangement intermediates and as many as eight in some of the VLR transcripts, whereas only singlet or doublet LRRV cassettes have been identified in the VLR locus; (ii) the rarity of repetitive adjoining LRRV modules (Tables 1 and 2); and (iii) the random Poisson distribution of the number of LRRV modules per transcript (table S5). Among the 32 LRR modules identified in the intermediate clones, only 4 matched any of the 32 known germline modules in the VLR locus. Consensus sequences that could mediate rearrangement of the LRR cassettes via recombinase activity were not found. The mechanism for the stepwise VLR rearrangement process remains unknown, but the final maturation step into functional VLR genes may involve recombination between the ends of the partially rearranged germline gene, thereby eliminating the remaining
intervening sequences and any incomplete modules.

A hallmark of genes undergoing positive Darwinian selection is the prevalence of codons with nonsynonymous nucleotide substitutions (Ka), which alter the encoded residue, over codons with synonymous substitutions (Ks). For instance, multiple alleles of the polymorphic major histocompatibility complex antigen-presenting molecules differ by only a few positively selected residues located in the diverse antigen-presentation sites (7). In B lymphocytes however, somatic hypermutation of immunoglobulin genes followed by a selection stage can also result in prevalence of non-synonymous mutations. We therefore analyzed the distribution of nucleotide substitutions in all the related VLR sequences of identical length that differ by 1-21 nucleotides (n=20; two triplets and seven pairs). In most cases, the substitutions clustered discretely in one or more of the LRR modules in a non-random distribution (P<0.01) (8). Only in one case were "mutations" randomly scattered throughout the VLR diversity region (P=0.37). Hence, the presence of one or more unique LRR modules distinguish most of the VLR sequences, indicating that somatic hypermutation is not a significant contributing factor in VLR diversification. This conclusion is supported by the finding of recurring identical LRR modules among VLR collected from different animals (Table 2) and by the observation that scaffold residues in the LRR modules are highly conserved, for example, 10 out of 24 residues are invariant in 90 to 100% of the LRRVe modules (fig. S1).

To identify regions in the VLR that may be undergoing positive selection, we used a three-dimensional (3D) model of the lamprey VLR (Fig. 1C) to predict the position of solvent-exposed and buried residues in the VLR. The residues in each VLR were then divided into three categories: (i) solvent-exposed residues on the concave VLR
surface; (ii) solvent-exposed residues elsewhere; and (iii) buried residues. Analysis of nucleotide substitution revealed a rate higher significantly for non-synonymous substitutions only in the concave VLR surface. A concentration of non-synonymous substitutions was also found on the concave surface of hagfish VLR-A and VLR-B (Table 3; Fig. S2). The invariant scaffold residues within each LRR module are interspersed with hypervariable sites (fig. S1), indicating that some of these sites may be under positive selection (7, 9, 10). Positive selection can be distinguished by the ratio of $K_a$ to $K_s$ substitutions: a ratio $>1$ indicates positive selection, a ratio $<1$ indicates purifying selection and a near 1 ratio indicates neutral selection (9).

Using both maximum parsimony (11) and maximum likelihood (12, 13) for independent calculations, we identified one to six sites that could be confidently considered as having been under positive selection in all six module types, with the exception of the hagfish VLR-A LRRCT and VLR-B CP (tables S3 and S4). The positively selected sites predicted by both methods were mapped onto lamprey and hagfish VLR models (Fig. 1C; fig. S2). In each LRR module type, except for the CP, one to three of the positively selected residues are solvent-exposed on strands of the central $\beta$ sheet that forms the concave surface of the VLR model, for example, codons 7 and 9 in lamprey LRRV (table S4). Another set of positively selected sites localize at one or both ends of the LRRNT and LRRCT. A conservative estimate of the combinatorial diversity that can be generated by the positively selected solvent-exposed residues on the concave VLR surface is $5 \times 10^7$ for the lamprey, $7.1 \times 10^{13}$ for the hagfish VLR-A and $1.5 \times 10^6$ for VLR-B. Notably, in many LRR-containing proteins, the concave surface is the ligand-binding interface (14-19).
Table 3. Average Ks and Ka among solvent-exposed and buried residues of the lamprey VLR (n=517), hagfish VLR-A (n=139) and hagfish VLR-B (n=70). A ratio of Ka/Ks>1 indicates positive selection; Ka/Ks<1 indicates purifying selection; and Ka/Ks≈1 indicates neutral selection. For Ks and Ka, standard error in parentheses.

<table>
<thead>
<tr>
<th>Site class</th>
<th>Ks</th>
<th>Ka</th>
<th>Mode of selection</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lamprey VLR</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exposed residues on concave VLR surface</td>
<td>0.28 (0.03)*</td>
<td>0.44 (0.05)</td>
<td>Positive selection (Z=2.61, P=0.004)†</td>
</tr>
<tr>
<td>Exposed residues elsewhere on VLR surface</td>
<td>0.25 (0.02)</td>
<td>0.21 (0.03)</td>
<td>Neutral evolution (Z=1.55, P=0.12)</td>
</tr>
<tr>
<td>Buried residues</td>
<td>0.21 (0.02)</td>
<td>0.12 (0.02)</td>
<td>Purifying selection (Z=3.43, P=0.001)</td>
</tr>
<tr>
<td><strong>Hagfish VLR-A</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exposed residues on concave VLR surface</td>
<td>0.37 (0.05)</td>
<td>0.53 (0.05)</td>
<td>Positive selection (Z=3.63, P&lt;0.001)</td>
</tr>
<tr>
<td>Exposed residues elsewhere on VLR surface</td>
<td>0.26 (0.03)</td>
<td>0.29 (0.03)</td>
<td>Neutral evolution (Z=0.37, P=0.90)</td>
</tr>
<tr>
<td>Buried residues</td>
<td>0.25 (0.02)</td>
<td>0.10 (0.02)</td>
<td>Purifying selection (Z=4.77, P&lt;0.001)</td>
</tr>
<tr>
<td><strong>Hagfish VLR-B</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exposed residues on concave VLR surface</td>
<td>0.35 (0.04)</td>
<td>0.65 (0.02)</td>
<td>Positive selection (Z=8.37, P&lt;0.001)</td>
</tr>
<tr>
<td>Exposed residues elsewhere on VLR surface</td>
<td>0.30 (0.03)</td>
<td>0.17 (0.03)</td>
<td>Purifying selection (Z=3.75, P&lt;0.001)</td>
</tr>
<tr>
<td>Buried residues</td>
<td>0.32 (0.02)</td>
<td>0.09 (0.02)</td>
<td>Purifying selection (Z=8.72, P&lt;0.001)</td>
</tr>
</tbody>
</table>

The remarkable diversity of the VLR repertoire suggested that these may serve as lymphocyte antigen receptors in lamprey immunity. To assess the VLR’s role in antigen recognition, we injected animals with anthrax spore coat (exosporium) as a particulate immunogen bearing an immunodominant antigen for mice, the collagen-like BclA glycoprotein (20). We then examined cellular and humoral responses after exosporia injections at weekly intervals. Flow cytometric analysis, using a VLR-specific antibody against the conserved stalk, indicated a dramatic increase in large lymphocytes among the VLR-positive cells. Compared to unstimulated animals, the fraction of large VLR-positive lymphocytes increased during the 8-week stimulation period from 4% to 93% in the blood, from 11% to 90% in the kidney and from 7% to 76% in the typhlosole, the
major hematopoietic tissue in larvae. Mitogenic activity of the exosporium may have induced the dramatic activation of VLR-bearing lymphocytes, as in lamprey stimulated with a mixture of antigens and mitogens (3). Plasma VLR concentrations in 8-week immunized animals were increased by 8- to 10-fold over preimmunization levels (5). An ELISA assay, used to measure levels of soluble anthrax-reactive VLR, revealed a progressive increase in spore recognition over the immunization period (Fig. 2A). VLR specificity was indicated by selective reactivity with \(B.\ anthracis\) versus \(B.\ subtilis\) spores, a related bacterium used as a control. BclA antigen-specific VLR also increased in plasma samples from immunized animals (Fig. 2B), and longer immunization periods led to progressively higher levels of anti-BclA VLRs. These data indicate that lampreys are capable of humoral responses to anthrax exosporium by producing increasing levels of soluble BclA-specific VLRs.

![Antigen recognition by lamprey VLR.](image)

In summary, our data indicate that jawless fish generate a very large repertoire of VLRs, comparable to the predicted diversity of \(\sim10^{14}\) mammalian antibody repertoire (21, 22). These repertoires would clearly be sufficient to recognize a wide range of antigenic determinants, yet this remarkable extent of receptor diversity in both jawless and jawed vertebrates is intriguing given that the available repertoire is limited by the presence of
less than 10 million lymphocytes in lamprey larvae and in jawed vertebrate representatives like the zebrafish (23). Apart from antibodies, TCRs and VLRs, such a spectacularly complex repertoire has only been reported for the \( \sim 10^{13} \) C-type lectin fold variants in the receptor of the *Bordetella* bacteriophage (24).

Analysis of intermediates in the *VLR* gene assembly process indicates a multistep mechanism for insertion of various LRR modules from flanking cassettes into the framework germline gene. These are incorporated precisely in-frame with the coding regions in the incomplete *VLR* and in tandem with previously inserted LRR modules. The molecular machinery used in assembly of mature *VLR* genes is clearly an interesting arena for future investigation, and our prediction that an array of 1500 to 2400 diverse LRR modules in agnathan genomes provides the primary source of VLR diversity will be tested when the sea lamprey genome sequencing project is completed.

Most importantly, the present studies indicate that lamprey can use their VLR for specific recognition of particulate and soluble protein antigens in a humoral response. Within 4 weeks of anthrax immunization, soluble anthrax-specific VLRs were abundant in the circulation, and these included VLRs that recognize the exosporium BclA protein. Our data thus strongly suggest convergent evolution of remarkably different strategies for generating anticipatory lymphocyte receptors in jawless and jawed vertebrates.
References and Notes


5. Methods and materials are available as supporting material on *Science* Online.


25. Reported sequences were deposited in GenBank: DQ150997-DQ151421.

26. We thank C.L. Turnbough and J.F. Kearney labs for advice and anthrax reagents; L. Gartland for the anti-VLR, G.L. Gartland for help with the FACS; L. Aravind, M.F. Flajnik, A.N. Haines, M. Criscitiello for critical comments. I.B.R. and L.M.I. were supported by the NLM/NIH/DHHS Intramural Research Program; M.D.C. is a HHMI Investigator; Z.P. was funded by NSF-MCB-0317460; Contribution #05-122 from COMB.
Methods and Materials

Animals and immune stimulation. Anthrax spores, exosporia, recombinant BclA protein and B. subtilis spores were generous gifts from Charles L. Turnbough Jr., (S1). Sea lamprey larva, 10-13 cm long, were maintained at 16°C. For immune stimulation the larvae were sedated for 10 minutes in 100 mg/l MS222 (Sigma, St. Louis, MO). Animals were given 4, 6 and 8-weekly intraperitoneal injections of 2 µg B. anthracis exosporia diluted in 75 µl 0.67x PBS (1x PBS per liter: 8 g NaCl, 0.2 g KCl, 1.44 g Na2HPO4, 0.24 g KH2PO4, pH=7.4). Blood from euthanized tail-severed animals was collected in 0.57x PBS and 30 mM EDTA 5-7 days after final injection. Buffy coat leukocytes were isolated by 5 minutes centrifugation at 50x g. Intestines and kidneys were ground between two glass slides, sedimented 5 minutes at 50x g and then the supernatant leukocytes were collected. Plasma was separated from blood cells by 5 minutes centrifugation at 300g.

Anti-VLR monoclonal antibody. The conserved C-terminal VLR fragment, from the end of LRRCT to the beginning of the hydrophobic tail (82 residues, nucleotides 624-873 in clone 2913; accession AY578059), was PCR amplified using Expand High Fidelity PCR (Roche Applied Science, Indianapolis, IN) with the primers TAT_LRR.F1+TAT_LRR.R1 that include BamHI and HindIII overhangs (see Table S6 for all primers). Digested product was cloned into the corresponding sites in pET-24b (Novagen, Madison, WI). Following expression in bacteria, recombinant protein was purified under denaturing conditions via the 6 Histidine tag using Ni-NTA Agarose (QIAGEN, Valencia, CA). Recombinant protein was then used to immunize mice.
Lymph node cells were fused with the Ag8.653 myeloma variant (S2). The hybridoma 4C4 mAb stained a subpopulation of lamprey blood, typhlosole, and kidney lymphocytes. The antibody isotype is IgG2b kappa as determined by indirect capture ELISA (Zymed, San Francisco, CA).

**Anti-VLR staining.** Buffy coat leukocytes were pelleted at 300x g for five minutes. Cells were resuspended in 0.67x PBS with 4 µg/ml anti-VLR 4C4 mAb for ten minutes on ice followed by two washes with 0.67x PBS. RPE-conjugated goat anti-mouse antibodies (Southern Biotechnology Associates, Birmingham, AL) diluted 1:100 were then applied for ten minutes on ice followed by two washes. Cells were analyzed using a MoFlo cytometer (Cytomation, Fort Collins, CO).

**ELISA of lamprey plasma VLR.** For spores, 96 well plates (#3590; Corning, Corning, NY) were coated one hour with poly-L lysine (Sigma) at 50 µg/ml, washed with water, seeded with 10⁶ spores per well and allowed to dry over night. For protein ELISA, plates were coated with 2.5 µg/ml soluble BclA or recombinant protein from the empty pET-24b vector as control, for 2 hours at 37°C. To quantify the plasma VLR, plates were coated with lamprey plasma samples for 2 hours at 37°C. All following incubations were for one hour at 37°C. Plates were blocked with 1% BSA (Sigma) in PBS and then the plasma was diluted serially in 1% BSA starting at 1:50 and up to 1:109,350. After washing, 1 µg/ml of the 4C4 mAb in 1% BSA was added and after two washes, we added alkaline phosphatase conjugated goat anti-mouse mAb (Southern Biotechnology) diluted 1:500 in 1% BSA. Following two washes the phosphatase substrate (Sigma) was added
and the plates were read at 405 nm (Versamax microplate reader; Molecular Devices, Sunnyvale, CA). All plasma samples were normalized to the same protein concentration as measured by a spectrophotometer at 280 nm (Beckman DU 640B; Beckman, Fullerton, CA).

**VLR clones and rearrangement intermediates.** We cloned and sequenced 110 new VLR sequences from four individual larva injected four times with exosporia at weekly intervals: from buffy coat blood leukocytes of larvae numbers 14 (n = 21) and 15 (n = 22), and from 4C4 mAb VLR-positive blood lymphocytes sorted from animals 16 (n = 23) and 17 (n = 44/47 unique sequences). In addition, 270 VLR were cloned from pooled samples of three animals that were immunostimulated by 8 weekly injections of exosporia: from buffy coat blood leukocytes (n = 76/84 unique sequences), kidney leukocytes (n = 91/94 unique sequences) and typhlosole leukocytes (n = 82/92 unique sequences). Out of 270 sequences 249 were unique.

Total RNA was extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA) and Poly(A)$^+$ RNA was selected with Dynabeads mRNA purification Kit (Dynal Biotech, Lake Success, NY). First strand cDNA synthesis (SuperScript III First Strand cDNA Synthesis kit; Invitrogen) was primed with 20 pmol of the primer LRR_C.R2 (Table S6), and the products were purified (QIAquick PCR purification; Qiagen). PCR amplification of VLR was with Expand High Fidelity PCR (Roche). Reactions of 50 µl contained: 10 pmole of each primer LRR_N.F1, LRR_C.R2, 1 µl dNTP at 10 mM, 5 µl 10x buffer, 36.25 µl water, 5 µl cDNA and 0.75 µl Expand enzyme. Reactions were amplified using one cycle of 94°C 1 min, then 35 cycles of 94°C 30 s, 59°C 30 s and 72°C 1 min, and a final 7 min at 72°C. Products were cloned in pCRII-TOPO (Invitrogen).
Clones of rearrangement intermediates belong to three groups. For the F.1+R.1 amplicons, cDNA was primed with IntSeq1.R3 and then amplified using Slit.F+IntSeq1.R4 followed by nested PCR with LRR_NF.2+IntSeq1.R5. The F.1+R.2 amplicons were primed with IntSeq2.R6 and amplified using Slit.F+IntSeq2.R7. The F.2+R.3 amplicons were primed with LRR_CR.2, amplified using LRR_CR.2+Intseq1.F1, and then with the nested pair LRR_CR.1+IntSeq1.F2. The primary PCR was performed with Expand Long Template (Roche) in 50 µl reactions containing: 15 pmole of each primer, 2.5 µl dNTP at 10 mM, 5 µl 10x buffer 2, 36.25 µl water, 2.5 µl cDNA and 0.75 µl Expand enzyme. Reactions were amplified using one cycle of 93°C 20 s, then 35 cycles of 93°C 15 s, 57°C 30 s and 68°C 7 min, and a final 7 min at 68°C. Nested PCR was performed using Expand High Fidelity (Roche) in 50 µl reactions containing: 15 pmole of each primer, 1 µl dNTPs at 10 mM, 5 µl 10x buffer, 37.25 µl water, 2 µl of 1:500 dilution of primary PCR, and 0.75 µl of the Expand enzyme. Reactions were cycled as follows: one cycle of 94°C 2 min, then 30 cycles of 94°C 20 sec, 57°C 30 sec and 68°C 4 min, and a final 7 min at 68°C. PCR amplicons of specific molecular weights were gel-excised (QIAquick gel extraction Kit, QIAGEN) and cloned in pCR-XL-TOPO or pCRII-TOPO (Invitrogen). DNA harvested from whole lamprey larval carcass served as template for the genomic clones.

**VLR data analysis.** The dataset of lamprey VLR was assembled from 129 previously reported sequences (accessions: AY577943-AY578057, CK988616, CK988617, CK988619, CK988620, CK988622, CK988625-CK988627, CK988629-CK988633, CK988635, CK988637-CK988639, CK988646, CK988648-CK988651) and 388 new
sequences: 351 from the four individuals and from the pooled samples mentioned above, and 37 additional sequences were assorted from other experiments. Pacific hagfish (Eptatretus stoutii) VLR sequences were reported previously (S3): 139 VLR-A (AY964719-AY964931) and 70 VLR-B (AY965520-AY965612).

Multiple alignments of VLR amino acid sequences were constructed using the T-Coffee program (S4). Nucleotide sequence alignments were constructed with the protein alignments as guides. Using Perl scripts, individual LRR modules were converted into a matrix of numeric codes for the analysis of LRR adjoining pairs.

The amino acid sequence alignments were used to calculate consensus motifs for the LRRV and LRRVe modules. The LRRV consensus is \( xLxxLxxLxxNqLxxlPxGvFd \), and the LRRVe consensus is \( xLxxLxxLxxNQLkSiPRGAFD \), with 90-100% consensus in capitals, 75-89% consensus in small letters and \( x \) indicates any residue.

Analysis of adjoining LRRNT-LRR1 and CP-LRRCT pairs was done with the ClusterP program (S5). The distribution of LRRNT-LRR1 and CP-LRRCT pairs was separated into three classes, the first of which included unique pairs and those repeated less than four times. Classes 2 and 3 represent a small fraction (<6%) of non-randomly distributed pairs that were repeated four times or more.

The equation below was used to calculate the potential diversity \( (V) \) of the lamprey VLR repertoire with the assumption that each unique module occurs once per genome. \( N \) is the number of each type of the LRR modules that were found only in unique neighboring combinations amongst the 517 VLR dataset, and \( k=1.31 \) is the average number of LRRV module per sequence (Table 2).
\[ V = N(LRRNT=169) \times N(LRR1=148) \times N(LRRV=518)^{k=1.31} \times N(LRRVe=335) \times N(CP=54) \times N(LRRCT=160) \]

For hagfish VLR-A the calculation was as follows:

\[ V = N(LRRNT=51) \times N(LRR1=86) \times N(LRRV=361)^{k=3.1} \times N(LRRVe=119) \times N(CP=59) \times N(LRRCT=45) \]

To estimate the number of LRRV modules in the genome we used a Monte Carlo approach with the assumption that these are independent recombination units. Amongst the 517 unique VLR sequences we identified 675 LRRV modules, 530 of which were unique (Table 2). An ad hoc simulation program was developed to generate sets of numbers from 1 to \( N_G \) (range 700-10,000, incremented by 100 per set; 10,000 repeats per \( N_G \)). For each set of 1 to \( N_G \) that was generated, 675 numbers were randomly chosen allowing repeated selection of numbers. 'Success' was scored only for samples that included at least 530 unique numbers.

Maximum likelihood (ML) analysis of positively selected codons was done using the PAML program (S6). The likelihood ratio test was used to implement a comparison of the M1 (neutral) vs. M2 (selection) models for all categories of repeats as described (S7), and reconfirmed the M1 vs. M2 conclusions using the M7 \( 0<\omega<1 \) vs. M8 models \( \omega>1 \) (S6) for several arbitrary selected repeats. For all LRR modules the M1 vs. M2 model and the M7 vs. M8 model predicted the same codons to be under positive selection.

Maximum parsimony (MP) analysis for codon substitution was as described (S8). Only those codons predicted independently by both MP and ML methods were considered in our calculations as positively selected positions. The modified Nei-Gojobori method (S9) was used to estimate the number synonymous (Ks) and non-synonymous (Ka) nucleotide
substitutions per site. Individual LRR modules were divided into clusters using neighbor-joining trees constructed with synonymous substitutions (S9).

Diversity regions of lamprey VLR and hagfish VLR-A and VLR-B, LRRNT to LRRCT, were three dimensionally modeled via the META server (http://cubic.bioc.columbia.edu/predictprotein/submit_meta.html). The lamprey VLR was modeled based on residues 22-319 from clone AY577974, comprised of eight LRRV and one LRRVe. For the hagfish VLR-A we modeled residues 40-299 from clone AY964913 and for hagfish VLR-B residues 32-279 from clone AY965603, both of which consist of five LRRV and one LRRVe module. The three dimensional models were displayed using the Pymol program (http://www.pymol.org).

The equation below was used to estimate the combinatorial diversity that can be generated by positively selected solvent exposed residues on the lamprey concave VLR surface. N is the number of different amino acids observed at particular sites (Table S4) assuming equal likelihood of occurrence, and an average $k=1.31$ of LRRV per VLR.

$$V = N(LRRNT_{23} = 13) \times N(LRR_{13} = 13) \times [N(LRRV_{7} = 15) \times N(LRRV_{9} = 16)]^{k = 1.31} \times N(LRRVe_{7} = 15) \times N(LRRVe_{9} = 15)$$

For hagfish VLR-A the calculation was as follows:

$$V = [N(LRRV_{9} = 13) \times N(LRRV_{11} = 14) \times N(LRRV_{12} = 15)]^{k = 3.1} \times N(LRRVe_{9} = 11) \times N(LRRVe_{11} = 12) \times N(CP_{12} = 12)$$

For hagfish VLR-B the calculation was as follows:

$$V = N(LRRNT_{13} = 10) \times N(LRR_{13} = 10) \times [N(LRRV_{9} = 13)]^{k = 1.8} \times N(LRRVe_{9} = 15) \times N(LRRCT_{26} = 10)$$

The distribution of positively selected residues in the VLR models resembles that found in several of the LRR-containing plant disease resistance genes (S10–S12).
Figure S1. Amino acid variability plots for the lamprey VLR modules LRR1, LRRV, LRRVe and CP. The mean of all pairwise differences in residues per site was calculated for the aligned constituent modules from 517 unique VLR sequences. In each column the most common residue is indicated and the variability values can range from 0 for a single residue in that position and up to 1 when all 20 residues are represented; *Positively selected residue; **Positively selected residue predicted on the concave VLR surface. The variability in length of LRRNT and LRRCT modules prevents such calculations.
Figure S2. Three dimensional models of hagfish VLR-A and VLR-B diversity regions. Colored spheres represent Cα atoms of positively selected residues whose side chains lie in the solvent exposed concave surface of the VLR (site predictions are according to Table S4): red, LRRNT; yellow, LRR1; blue, LRRV; white, LRRVe; orange, CP, pink, LRRCT; green, β-strands; magenta, α-helices.
**Supplementary Tables**

Table S1. Distribution of unique and identical pairs of adjoining LRR modules among 139 unique hagfish VLR-A sequences (the modules are shown in Fig. 1A).

<table>
<thead>
<tr>
<th>Number of pairs</th>
<th>LRRNT</th>
<th>LRR1</th>
<th>LRRV</th>
<th>LRRV</th>
<th>LRRVe</th>
<th>CP</th>
<th>LRRCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>117</td>
<td>125</td>
<td>266</td>
<td>125</td>
<td>124</td>
<td>108</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>5</td>
<td>8</td>
<td>5</td>
<td>6</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

Table S2. Unique LRR modules and those found only once in adjoining pairs among 139 unique hagfish VLR-A sequences. The distribution of LRRV modules per transcript is shown separately.

<table>
<thead>
<tr>
<th>Module</th>
<th>Unique ( % total)</th>
<th>Uniquely paired</th>
<th>Distribution of LRRV modules per transcript</th>
</tr>
</thead>
<tbody>
<tr>
<td>LRRNT</td>
<td>60 (43)</td>
<td>51</td>
<td>0 LRRV cases</td>
</tr>
<tr>
<td>LRR1</td>
<td>96 (69)</td>
<td>86</td>
<td>1 LRRV cases</td>
</tr>
<tr>
<td>LRRV</td>
<td>384 (90)</td>
<td>365</td>
<td>2 LRRV cases</td>
</tr>
<tr>
<td>LRRVe</td>
<td>126 (91)</td>
<td>119</td>
<td>3 LRRV cases</td>
</tr>
<tr>
<td>CP</td>
<td>74 (53)</td>
<td>59</td>
<td>4 LRRV cases</td>
</tr>
<tr>
<td>LRRCT</td>
<td>53 (39)</td>
<td>45</td>
<td>5 LRRV cases</td>
</tr>
</tbody>
</table>

*VLR transcripts with one LRRVe module but no LRRV.*
Table S3. Positively selected codons in lamprey VLR (n = 517), hagfish VLR-A (n = 139) and hagfish VLR-B (n = 70) predicted by maximum parsimony (MP) and maximum likelihood (ML) methods. For each LRR module, the number of residues is shown in parentheses. Numbers in bold represent codon positions that were predicted independently both by maximum parsimony and maximum likelihood methods. Only some of these residues are located on the concave surface (bold residues in Table S4).

<table>
<thead>
<tr>
<th>LRR module Cluster number</th>
<th>Positively selected positions (MP)</th>
<th>Positively selected positions (ML)</th>
<th>Negatively selected positions (MP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamprey VLR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LRRNT (30-38)</td>
<td>2</td>
<td>12, 23, 36, 38</td>
<td>12, 21, 23, 24, 33, 36, 38, 39</td>
</tr>
<tr>
<td>LRR1 (18)</td>
<td>1</td>
<td>3</td>
<td>1, 3, 5, 6</td>
</tr>
<tr>
<td>LRRV (24)</td>
<td>5</td>
<td>7, 9, 16, 22</td>
<td>1, 4, 7, 9, 11, 12, 16, 20</td>
</tr>
<tr>
<td>LRRVe (24)</td>
<td>3</td>
<td>1, 3, 7, 9, 12, 18, 20</td>
<td>1, 3, 4, 6, 7, 9, 11, 12, 17, 18, 22</td>
</tr>
<tr>
<td>CP (13)</td>
<td>1</td>
<td>4, 7</td>
<td>7, 9, 11, 12</td>
</tr>
<tr>
<td>LRRCT (48-65)</td>
<td>2</td>
<td>44</td>
<td>5, 7, 30, 31, 32, 33, 44, 46, 49</td>
</tr>
<tr>
<td>Hagfish VLR-A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LRRNT (37)</td>
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<td>10, 30</td>
<td>10, 20, 30</td>
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<td>LRR1 (18)</td>
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<td>14, 15</td>
<td>3, 6, 14</td>
</tr>
<tr>
<td>LRRV (24)</td>
<td>3</td>
<td>4, 9, 11, 12, 17, 20</td>
<td>9, 11, 12, 20</td>
</tr>
<tr>
<td>LRRVe (24)</td>
<td>3</td>
<td>9, 11, 12, 20</td>
<td>9, 11</td>
</tr>
<tr>
<td>CP (13)</td>
<td>1</td>
<td>12</td>
<td>7, 11, 12</td>
</tr>
<tr>
<td>LRRCT (46-52)</td>
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<td>7, 34, 42</td>
</tr>
<tr>
<td>Hagfish VLR-B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LRRNT (31)</td>
<td>1</td>
<td>13</td>
<td>11, 13, 15, 16</td>
</tr>
<tr>
<td>LRR1 (18)</td>
<td>1</td>
<td>1, 3</td>
<td>3, 5, 6</td>
</tr>
<tr>
<td>LRRV (24)</td>
<td>1</td>
<td>9</td>
<td>7, 9, 11, 12</td>
</tr>
<tr>
<td>LRRVe (24)</td>
<td>1</td>
<td>9</td>
<td>7, 9, 11, 12</td>
</tr>
<tr>
<td>CP (13)</td>
<td>1</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>LRRCT (36-50)</td>
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<td>26, 34</td>
<td>24, 25, 26, 27, 31, 32</td>
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</table>
Table S4. Position of positively selected codons in LRR modules from unique VLR sequences of lamprey (n = 517)*, hagfish VLR-A (n = 139) and VLR-B (n = 70). Only sites predicted by both methods of maximum parsimony and maximum likelihood were included (Table S3). Numbers in bold represent residues predicted on the concave VLR surface.

<table>
<thead>
<tr>
<th>LRR module</th>
<th>Lamprey VLR</th>
<th>Hagfish VLR-A</th>
<th>Hagfish VLR-B</th>
</tr>
</thead>
<tbody>
<tr>
<td>LRRNT</td>
<td>12, 23, 36, 38</td>
<td>10, 30</td>
<td>13</td>
</tr>
<tr>
<td>LRR1</td>
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<td>14</td>
<td>3</td>
</tr>
<tr>
<td>LRRV</td>
<td>7, 9, 16</td>
<td>9, 11, 12, 20</td>
<td>9</td>
</tr>
<tr>
<td>LRRVe</td>
<td>1, 3, 7, 9, 12, 18</td>
<td>9, 11</td>
<td>9</td>
</tr>
<tr>
<td>CP</td>
<td>7</td>
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<tr>
<td>LRRCT</td>
<td>44</td>
<td></td>
<td>26</td>
</tr>
</tbody>
</table>

* Numbers in bold represent residues predicted on the concave VLR surface.

Table S5. Poisson distribution of LRRV modules per transcript in 517 unique lamprey VLR sequences.

<table>
<thead>
<tr>
<th>Observed distribution</th>
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Chi-square statistic 13.4 (P > 0.05)

Table S6. VLR PCR primers. GenBank accession numbers are for gVLR Contig, AY577941; for cDNA 2913, AY578059

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Supplementary References


VLR ANTIBODY RESPONSES IN THE LAMPREY

by


Submitted to *Nature Immunology*

Format adapted for dissertation
ABSTRACT

Living representatives of jawless vertebrates, lamprey and hagfish, use genomic leucine-rich-repeat cassettes for the combinatorial assembly of diverse antigen receptor genes encoding variable lymphocyte receptors of two types, VLR-A and VLR-B. We describe here the VLR-B bearing lineage of lymphocytes in the sea lamprey. They respond to repetitive carbohydrate or protein determinants on bacteria and mammalian cells with lymphoblastoid transformation, proliferation and differentiation into plasmacytes that secrete multimeric antigen-specific VLR-B antibodies. Lacking a thymus and the ability to respond to soluble protein antigens, the lampreys appear to have evolved a B cell-like system for adaptive humoral responses.
Jawless vertebrates have recently been shown to have an adaptive immune system that rivals our own in the extent of clonal diversity, wherein each lymphocyte expresses a unique anticipatory receptor for antigen\(^1\)\(^-\)\(^3\). However, the antigen receptors in lamprey and hagfish, the only two living representatives of the jawless vertebrates (agnathans), are constructed with building blocks that differ from the immunoglobulin \(V\), \(D\), and \(J\) gene segments used in construction of our T cell receptors (TCR) and B cell receptors (BCR) for antigen. The variable lymphocyte receptors (VLR) expressed by lamprey and hagfish lymphocytes are composed of leucine-rich repeat (LRR) protein segments and an invariant stalk region that is tethered to the lymphocyte surface via glycosyl-phosphatidyl-inositol (GPI) linkage. The LRR segments in VLRs vary in numbers and amino acid sequences. The sequence variations are concentrated in the \(\beta\) sheets that form the inner concave surface of the crescent shaped LRR proteins, thereby suggesting this to be the antigen binding surface\(^2\)\(^-\)\(^4\).

Two germ line VLR genes, \(VLR-A\) and \(VLR-B\), have been identified in lamprey and hagfish\(^1\)\(^,\)\(^3\)\(^,\)\(^5\). Each is incomplete by virtue of having coding sequences only for the invariant 5’ and 3’ ends of the VLR molecules. Multiple LRR cassettes flank these germ line \(VLR\) genes, and they encode all or a portion of the LRR modular units needed for \(VLR\) gene completion. During lymphocyte differentiation, pieces of the flanking LRR sequences are stitched into the germ line \(VLR\) gene in sequential fashion\(^1\)\(^,\)\(^2\)\(^,\)\(^5\)\(^,\)\(^6\). This process is achieved via a gene conversion mechanism in which short stretches of nucleotide sequence homology between donor and recipient \(VLR\) gene elements serve as anchorage sites for step-wise extension, culminating in the completion of a mature \(VLR\) gene. The gene conversion process begins on either the 5’ end or 3’ end of the germ line.
\textit{VLR} gene, is confined to one allele, and may involve an AID-APOBEC family member. The dozens of flanking LRR cassette sequences appear to be randomly selected for use in \textit{VLR} gene assembly, except that short stretches of homology are required for donor cassette anchorage to the acceptor region of the \textit{VLR} gene being constructed. These gene assembly features assure the generation of an extensive repertoire of lymphocytes, the potential diversity of which is estimated to be $>10^{14}$ for the agnathan VLRs$^{2,5}$. Long before discovery of the VLRs, lamprey and hagfish were shown to produce ill defined agglutinins against particulate antigens$^{7-14}$. In an earlier study, we found that lamprey produce soluble antigen-specific VLRs in response to immunization with \textit{Bacillus anthracis} exosporium$^{2}$, the outmost layer of spores of the bacterium that causes anthrax$^{15}$. In the present studies, we have revisited the lamprey humoral response to define requirements for the antigen induction of VLR-B antibody responses, the molecular composition of VLR-B antibodies, and their potential protein and carbohydrate binding specificities. We have also explored the cellular basis for the humoral response by using mouse monoclonal antibodies against the invariant stalk region of the VLR-B molecules to characterize the morphology, distribution, proliferation, and differentiation of VLR-B producing lymphocytes in the lamprey.
RESULTS

VLR-B antibody production against heterologous erythrocytes

The VLR-B portion of the humoral response was the focus of this analysis because it is the prevalent component of the VLR-based immune system in the sea lamprey\(^1,5\).

Humoral agglutinin, hemolysin, and bactericidal responses following immunization of lamprey and hagfish with heterologous erythrocytes and bacteria have been reported previously\(^7-14,16,17\). However, descriptions of the responsible humoral factors have either been inconsistent or inconclusive with regard to their molecular size, antigen specificity, relative heat stability, immunoglobulin versus non-immunoglobulin nature, and other physical characteristics\(^7-12,17\). For our analysis of the potential role of VLR antibodies in the erythrocyte agglutinin response, two to four-year-old sea lamprey larvae approximately 13 cm in length\(^18\) were immunized intraperitoneally with either mouse or human erythrocytes. Two VLR-B stalk region specific monoclonal antibodies, 4C4 (IgG2b isotype) and 6C3 (IgM isotype) were used to measure VLR-B antibody responses. Following an intraperitoneal injection of \(10^7\) human erythrocytes, hemagglutinin responses were found to reach peak levels around 19 days later. Booster immunization with the same immunogen dosage on day 14 resulted in a ~ 20-fold increase in the VLR-B response when compared to animals immunized with a single dose (Fig. 1a). In other experiments, the hemagglutinin response to erythrocytes was shown to be antigen dose dependent (Fig. 1b) and to be specific for donor mouse or human erythrocyte antigens (Fig. 1c).
Figure 1. Analysis of VLR-B antibodies produced after immunization with human blood group O erythrocytes. (a) Time course of plasma hemagglutinin titers following a single immunization (filled circles) or second immunization (arrow, open triangles) at day 14 with 1X10^7 erythrocytes. (b) Hemagglutinin responses of animals immunized with variable numbers of human O erythrocytes (n=3 per antigen dose). (c) Specificity of the agglutinin response to mouse (open circles) and human erythrocytes (closed circles). Blood samples for (b) and (c) were obtained on day 28, after immunizations on days 0 and 14. (d) Hemagglutinin titers before and after plasma adsorption beads coated with a monoclonal anti-VLR-B or isotype control antibody. Error bars indicate one standard error of the mean (S.E.M., n=7). (e) Flow immunocytometric analysis comparing H antigen reactivity of plasma from immunized lamprey with an anti-H mouse monoclonal antibody; staining is shown for α1,2-fucosyltransferase CHO cell transfectants expressing the H antigen. No reactivity was observed for non-transfected CHO cells (data not shown). (f) Depletion of H antigen-specific VLR-B antibodies by adsorption with H antigen-bearing CHO cells.

To determine whether or not the erythrocyte agglutination was mediated by VLR-B antibodies, sepharose beads coated with an anti-VLR-B antibody were used to remove VLR-B antibodies from immune plasma samples. Adsorption with the anti-VLR-B coated beads resulted in almost complete removal of the hemagglutinin activity, whereas plasma adsorption with beads coated with a control antibody of irrelevant specificity had no demonstrable effect (Fig. 1d). These findings indicate that the erythrocyte-specific
agglutinins which are made by immunized lamprey are predominately VLR-B antibodies.

**Erythrocyte carbohydrate specificity of VLR-B antibodies**

Earlier studies in lampreys suggested that agglutinins made against blood group O erythrocytes were specific for the H trisaccharide cell surface antigen that defines this blood type in humans\(^8,11\). To test for H antigen specificity of the VLR-B antibodies, we employed Chinese hamster ovary (CHO) cells that were stably transfected with the \(\alpha 1,2\)-fucosyltransferase enzyme that generates the H trisaccharide antigenic determinant\(^19\). In these studies, animals immunized with human blood group O erythrocytes were shown to produce VLR-B antibodies that recognize CHO cells expressing the H trisaccharide antigen (Fig. 1e), while they did not produce VLR-B antibodies against control CHO cells transfected with the vector alone. Adsorption of the immune plasma samples with H antigen-positive cells removed the agglutinating VLR antibodies without noticeably affecting the level of VLR-B antibodies in the blood stream (Fig. 1f). These findings confirm that the H trisaccharide is a dominant antigenic determinant in the lamprey humoral response to blood group O erythrocytes, indicate the response is primarily attributable to the production of VLR-B antibodies, and demonstrate that these antigen specific VLR-B antibodies constitute a minor fraction of the total pool of circulating VLR-B antibodies.

**VLR-B antibodies are disulfide-linked multimers**

The ability of lamprey VLR-B antibodies to agglutinate erythrocytes inferred that they were multivalent. Western blot analysis of plasma samples to test this possibility revealed that VLR antibodies in the circulation are large proteins of >250 kDa (Fig. 2),
Figure 2. Analysis of VLR-B antibody composition. Western blot analysis of lamprey plasma treated with increasing concentrations of the reducing agent, 2-mercaptoethanol (2-ME).

whereas the molecular weights predicted for the VLR-B proteins on the basis of amino acid composition vary on average between 22 kDa and 30 kDa. Use of 2-mercaptoethanol in sufficient concentration to reduce disulfide bonds resolved the large VLR-B antibodies into individual protein components that migrated slightly higher in sizing gels than expected for the predicted molecular weights for VLR-B monomers. Multiple potential O-linked glycosylation sites in the stalk region of the VLR-B proteins could account for the difference in predicted protein size versus that estimated by gel migration. Additionally, a dimeric subcomponent in the form of ~70 kDa protein bands was observed following treatment with intermediate concentrations of the reducing agent. This analysis suggests that the VLR-B antibodies are composed of multiple VLR-B monomers linked by disulfide bonds to form large oligomeric molecules comprised by dimeric subunits.

VLR-B antibody response to *B. anthracis*

Our previous studies indicated that after immunization with *B. anthracis* exosporium lamprey produce VLR antibodies against the spore surface glycoprotein BclA. We reexamined this response to define antigen dosage requirements, kinetics, and epitope specificity of the VLR-B antibody response. Following a single intraperitoneal
immunization with 10 µg of *B. anthracis* exosporium, anti-spore VLR-B antibodies were detectable around seven days later and peak levels were reached at ~26 days. A significant increase in specific VLR-B antibodies was observed when animals were given a second immunization on day 14 and plasma samples were analyzed on day 28 (Fig. 3a). As was observed for the erythrocyte response, increasing the exosporium dosage resulted in the production of higher levels of VLR-B antibodies to BclA (Fig. 3b). A major portion of the VLR-B antibody response appears to be directed against the BclA protein,

**Figure 3.** VLR-B antibody response to immunization with *B. anthracis* exosporium. (a) Plasma samples were collected over a 7 week period after a single immunization with exosporium (10 µg) to examine for reactivity with *B. anthracis* spores by ELISA. VLR-B response to a booster immunization on day 14 (arrow) was examined on day 28 (closed bar). The numbers of animals for each data point (except day 49) were 3-11; error bars indicate S.E.M. except for day 49 where the range of values for two animals is indicated. (b) Evaluation of antigen dose requirement. VLR-B antibodies made against BclA before [x] and after intraperitoneal injections of 0.05 [a], 0.5 [o] and 5 [e] µg of exosporium on days 0 and 14. BclA reactive VLR-B antibodies in plasma samples were detected by ELISA at day 28 (n=3 per antigen dose). (c) Specificity of VLR-B antibodies for *B. anthracis* after two immunizations with exosporium (5 µg; n=4). BclA Δ represents BclA deficient *B. anthracis* spores. *, P < 0.05; **, P < 0.01 (d) VLR-B antibody response to the C-terminal domain of the spore coat protein BclA (BclA-CTD) and glutathione S-transferase (GST) control protein. Plasma samples from immunized (black bars) and unimmunized (white bars) lampreys were assayed by ELISA. Error bars indicate S.E.M. for four animals. *, P < 0.01
in that only minimal VLR-B reactivity was detected for *B. anthracis* spores deficient in the surface protein BclA (ΔBclA) (Fig. 3c).

Since mice make antibodies predominantly against the C-terminal domain (CTD) of BclA after *B. anthracis* immunization\(^{15,20}\), we evaluated the lamprey response to this determinant. The results of this experiment showed that immunized lamprey also make VLR-B antibodies against BclA-CTD (Fig. 3d). Adsorption of immune plasma with BclA-CTD-coated beads removed most of the VLR-antibody reactivity with *B. anthracis* spores (data not shown). Moreover, the VLR-B antibody response was directed primarily against the *B. anthracis* strain; much lower levels of VLR-B antibody reactivity were observed for the closely related *B. thuringiensis* and *B. cereus* spores (Fig. 3c). Notably, the BclA-CTD of *B. cereus* differs from the BclA-CTD of *B. anthracis* by approximately 10% of constituent amino acids. These observations indicate that the lamprey VLR-B response to *B. anthracis* exosporium is dose-dependent and highly antigen-specific. They also suggest that the CTD of the BclA surface protein is a major antigenic determinant for this humoral response.

**Non-responsiveness to a soluble protein antigen**

To examine whether sea lamprey larvae make antigen specific VLR-B antibodies when immunized with a protein antigen, we immunized lamprey with 10 µg of bovine serum albumin (BSA) that was either unmodified, alum-precipitated or combined with commercially available adjuvants, Ribi and Titermax, which contain bacterial products in a water-in-oil emulsion. In other immunizations, BSA was conjugated to the surface of polystyrene beads, 1X10⁸ of which were injected either alone or together with 1 µg each of lipopolysaccharide, lipoteichoic acid, or peptidoglycan. For these experiments, in
which we used an immunization protocol that resulted in a strong VLR-B humoral response to *B. anthracis* exosporium proteins, primary immunization followed by booster immunization two weeks later and plasma collection for antibody assessment by ELISA at four weeks but, none of these methods of BSA immunization resulted in the production of ELISA-detectable VLR-B antibodies by immunized lamprey (n = 30, 4-5 per immunization group, data not shown). When other groups of animals were immunized with keyhole limpet hemacyanin (50 µg, KLH, n = 10), ELISA-detectable VLR-B antibodies were not detected against this soluble protein antigen over a 56 day period. Moreover, the BSA and KLH immunized lamprey did not respond with the lymphoblastoid transformation of circulating lymphocytes that was observed following hyperimmunization of lamprey with exosporium (see below). These model protein immunogens thus failed to elicit a VLR-B antibody response, even when given with adjuvants, in aggregated form or coated onto the surface of a solid matrix in the case of BSA.

**Tissue distribution of VLR-B⁺ lymphocytes**

To determine the cellular basis for the lamprey humoral response to a particulate antigen, we examined the tissue distribution of VLR-B⁺ cells by immunohistochemical staining using the two monoclonal antibodies against the invariant VLR-B stalk region. In pilot studies, the 6C3 anti-VLR-B antibody yielded better definition of the VLR-B⁺ lymphocytes due to a lower level of background staining for paraffin embedded sections. This antibody therefore was used for tissue analysis, which revealed the presence of VLR-B⁺ cells in the kidney and typhlosole, as well as within blood vessels throughout the lamprey larvae. Notably, VLR-B⁺ lymphocytes were not detected within or beneath the
epithelium of the intestine, which in the filter feeding larval stage is essentially an unvariegated tube extending from the last gill slit region to the cloaca. Over most of its length, the intestine is folded like an elongated horseshoe over the typhlosole, which is filled with hematopoietic lineage cells surrounding blood filled sinuses. VLR-B⁺ lymphocytes were dispersed throughout the typhlosole, wherein they exhibited greater morphological diversity and variability in VLR staining intensity than VLR-B⁺ lymphocytes elsewhere (Fig. 4a). Small VLR-B⁺ cells were intermixed with other hematopoietic lineage cells in ventral interstitial regions of the kidneys surrounding the renal tubules. The blood vessels in the gill regions and elsewhere contained many VLR-B⁺ lymphocytes. In accordance with the abundance of VLR-B antibodies in plasma samples, prominent extracellular VLR-B staining was evident within the blood vessels and typhlosole sinuses. Conversely, minimal VLR-B staining was seen in extravascular compartments throughout the lamprey larvae.

Immunofluorescence analysis of VLR-B bearing cells was also conducted for cell suspensions freshly prepared from blood, kidney, and typhlosole. Among cells with the light scatter characteristics of lymphocytes, 15-35% of blood cells were surface VLR-B positive versus ~50% in kidney cell suspensions and 15-30% of the typhlosole cells (Fig. 4b). The VLR-B bearing cells from blood and kidney expressed significantly higher VLR-B levels than those for VLR-B⁺ cells from typhlosole, which also exhibited greater variability in cell surface VLR-B expression (Supplementary Fig. 1 online). Interestingly, we observed a temporary loss of VLR-B⁺ cells in the typhlosole, but not VLR-B⁺ cells in kidney or blood, immediately after the shipment of lamprey larvae by air freight, and this phenomenon was recapitulated by treatment with exogenous
Figure 4. Tissue distribution of VLR-B⁺ lymphocytes. (a) Immunohistochemical analysis of VLR-B⁺ cells in different organs. Paraffin sections stained with hematoxylin and eosin (top) or anti-VLR mAb using DAB as a chromogen marker (bottom). (b) Immunofluorescence analysis of VLR-B surface expression by lymphocytes from blood, kidney, and typhlosole. Histograms represent results for live analysis of cells in the light scatter ‘lymphocyte gate’. (c) Transmission EM of VLR-B⁺ and VLR-B⁻ cells sorted from ‘lymphocyte gate’ of blood sample: photomicrographs of a resting VLR-B⁺ lymphocyte (top) and a thrombocyte with characteristic nuclear cleft (bottom). (d) Quantitative PCR analysis of VLR transcripts in VLR-B⁺ and VLR-B⁻ cells isolated by fluorescence activated cell sorting of cells with lymphocyte-like light scatter characteristics. Error bars represent S.E.M. for three separate experiments. Note: the VLR-B⁻ cells contain a mixture of thrombocytes and lymphocytes.

Corticosteroid treatment (Supplementary Fig. 2 online). In both instances, recovery of the VLR-B bearing population in the typhlosole occurred over the ensuing two to three weeks. These observations indicate that mature VLR-B⁺ cells and their soluble VLR-B products are confined primarily within the vascular compartment except in the typhlosole and the kidney, wherein interstitial VLR-B⁺ lymphocytes are abundant around the tubules. They also suggested that, together with other blood cell types²¹, the VLR-B producing cells may be generated in the larval typhlosole.

VLR-B⁺ lymphocyte morphology and differential VLR gene expression
When the VLR-B⁺ and VLR-B⁻ cells in the ‘lymphocyte gate’ were isolated by fluorescence activated cell sorting, the VLR-B⁺ cells in non-immunized lamprey were found by transmission electron microscopy to resemble the small lymphocytes of jawed vertebrates (Fig. 4c). The VLR-B⁺ lymphocytes typically have a relatively large nucleus with peripheral concentration of the chromatin. The narrow rim of cytoplasm surrounding the nucleus contains relatively few distinguishable organelles, such as mitochondria. Many of the VLR-B⁻ cells in the ‘lymphocyte gate’ were identifiable as thrombocytes with a characteristic deep nuclear cleft and relatively abundant cytoplasm (Fig. 4c, lower panel), whereas cells with lymphocyte morphology were in the minority (~15 %).

Analysis of VLR transcripts in these isolated VLR-B⁺ and VLR-B⁻ populations of cells indicated that the purified VLR-B⁺ cells exclusively expressed VLR-B transcripts, whereas cells within the VLR-B⁻ population expressed VLR-A, but did not express VLR-B transcripts (Fig. 4d). We conclude from these results that VLR-B⁺ and VLR-A⁺ cells belong to separate lymphocyte populations.

VLR-B⁺ lymphocyte response to immunization

Intraperitoneal injection of lamprey with a cocktail of antigens and phytomitogens had been shown to induce a lymphoblastoid response¹, and a similar response was observed following the injection of a large dose of *B. anthracis* exosporium (>25 µg) (Supplementary Fig 3a online). Most of these responding lymphoblastoid cells expressed cell surface VLR-B at reduced levels (Supplementary Fig 3b online). These observations suggested that, when given in sufficient dose, the *B. anthracis* exosporium serves as a lymphocyte mitogen in the lamprey. They also raised the question of whether
a global mitogen response is required for the VLR antibody response to the BclA antigen. When lamprey larvae were immunized with lower doses of exosporium, however, antigen specific VLR-B antibodies were produced in the absence of a lymphoblastoid response that could discerned by flow cytometric analysis (data not shown). To examine the response of antigen-specific VLR-B-bearing cells, we determined the frequency of *B. anthracis* spore-binding VLR-B\(^+\) cells before and after two immunizations with exosporium (10 µg). When blood samples were examined on day 28, a four-fold increase was observed in the percentage of VLR-B\(^+\) cells that bound fluorescence labeled *B. anthracis* spores, whereas background levels of *B. cereus* spore binding cells were unchanged (Fig. 5a and b).

Since the increase in numbers of antigen-binding VLR-B\(^+\) cells could reflect binding by cytophilic VLR-B antibodies, we incubated blood cells from naïve lamprey in immune plasma samples containing high levels of anthrax-specific VLR-B antibodies. When the preincubated cells were examined for antigen binding, no increase was observed in the numbers of anthrax spore-binding cells (Supplementary Fig. 4 online). As another test of specificity of the antigen-binding VLR-B\(^+\) lymphocytes, we immunized larvae simultaneously with two immunogens, *B. anthracis* exosporium and heat-killed *S. typhimurium*, before examining whether or not this dual immunization resulted in the appearance of lymphocytes that could bind both of these antigens. Discrete subpopulations of cells were later found to bind one or the other immunogen, thereby suggesting that VLR-B\(^+\) cells bind antigen via their own endogenous antigen receptor and not by cytophilic antigen receptors (Fig. 5c). When the percentage of VLR-B\(^+\) cells that bound to fluorescence labeled spores was measured over a seven week
Figure 5. Immunization with *B. anthracis* results in enhanced spore binding by VLR-B⁺ cells. (a) Percentage of *B. anthracis* spore-binding VLR-B⁺-bearing cells before and after (25 days) immunization with *B. anthracis* exosporium (10 µg on days 0 and 14). Error bars indicate S.E.M. for three animals per group, *p* < 0.05. (b) Flow cytometric analysis of antigen-binding VLR-B⁺ cells in blood samples from naïve and immunized animals by costaining with 4C4 anti-VLR-B monoclonal antibody and fluorescent-tagged spores. (c) VLR-B⁺ lymphocytes from animals immunized at the same time with two separate antigens, *B. anthracis* exosporium and *Salmonella typhimurium*, only bind fluorescently labeled *B. anthracis* spores or *S. typhimurium* and not both. (d) Kinetics of antigen-binding cells after a single immunization of *B. anthracis* exosporium (10 µg). Flow cytometric analysis of antigen-binding VLR-B⁺ cells in blood samples by costaining with 4C4 anti-VLR-B monoclonal antibody and fluorescent-tagged spores. Error bars indicate S.E.M. for 3-9 animals for each data point, except for 49 days when the mean of two animals is shown.

interval following a single injection of exosporium (10 µg), the highest percentage of spore-binding VLR-B⁺ cells occurred on ~day 26 (Fig. 5d). In view of these results, the increase in numbers of antigen-binding VLR-B⁺ cells after *B. anthracis* exosporium immunization is most easily explained by antigen induced cellular proliferation. To test this interpretation, animals immunized with exosporium (25 µg) were pulsed with before looking for VLR-B⁺ cells undergoing proliferation. When the lymphoid tissues were examined later by immunohistology (Fig. 6), we found significant increases in the numbers of BrdU-containing VLR-B⁺ cells in immunized animals compared to naïve
Figure 6. Assessment of BrdU incorporation by VLR-B+ cells before (a, c) and after (b, c) immunization with *B. anthracis* exosporium. Animals were given a single injection of exosporium (25 µg) and five days later pulsed with BrdU for seven hours before processing for staining. Sections of gill regions (a, b) and ventral kidney regions (c, d) were stained for the nuclear dye DAPI (blue), BrdU (red), and VLR-B (green). Note the increased numbers of BrdU-incorporating VLR-B+ cells (white arrows) in the gills (b) and kidney (d) of immunized animals; * indicates representative plasmacytoid cell. (a-d, 100x magnification) (e) Comparisons of the numbers of BrdU+VLR-B+ cells in the different tissues before (white bars) and after (black bars) immunization. Error bars indicate S.E.M. for three animals per group. * p < 0.05.

BrdU-treated animals. The increase numbers of BrdU-containing VLR-B+ cells was much greater in the gills and kidney than in the typhlosole (Fig. 6e). VLR-B containing cells with lymphoblastoid and plasmacytoid features were also seen more frequently in the gill region and kidneys than in the typhlosole of immunized larvae (Fig. 6).

**Plasmacytoid cells secrete VLR-B antibodies**

To characterize the cells that secreted the VLR-B antibodies following antigenic stimulation, VLR-B+ and VLR-B- subpopulations of cells from immunized lamprey were isolated on the basis of their relative cell size and analyzed for their ability to secrete
VLR-B antibodies to the BclA-CTD antigen using an ELISPOT assay. The different subpopulations of cells derived from blood, kidney and typhlosome tissue were placed in culture for 18 hours before analysis of VLR-B antibody secretion. In these experiments, cells that secreted BclA-CTD specific antibodies were found exclusively among the relatively large VLR-B bearing cells (Fig. 7a). When these VLR-B producing cells were isolated for morphological evaluation by transmission electron microscopy, they were found to be large plasmacytoid cells with an extensive amount of cytoplasm that contained multiple organelles and a prominent network of rough endoplasmic reticulum.

**Figure 7.** Characterization of VLR-B secreting cells induced by immunization with *B. anthracis* exosporium. Cells were pooled from the blood, kidney and typhlosome six lamprey larvae 14 days after a booster immunization with 5 µg of exosporium. Representative data from the kidney is shown. (a) VLR-B⁺ and VLR-B⁻ cells were sorted into the three populations (indicated by circles) on the basis of light scatter characteristics. Secretion of antigen-specific VLR-B antibodies was detected by ELISPOT. (b) Transmission EM analysis of large VLR-B⁺ producing cells indicating plasmacyte morphology with expanded rough endoplasmic reticulum, a representative portion of which is enlarged in the right panel.
(Fig. 7b). The VLR-B antibody secreting cells were more abundant in blood and kidney samples than in the typhlosole four weeks after immunization with *B. anthracis* exosporium. The mean frequency of cells that secreted antigen specific VLR-B antibodies per million leukocytes was 215 (±101 one standard error of the mean) for blood, 272 (±100 S.E.M.) for the kidney, and 93 (±25 S.E.M.) in the typhlosole. The above observations indicate that immunization of lamprey with an effective immunogen induces antigen-specific lymphocytes to undergo lymphoblastoid transformation, proliferation, and differentiation into plasmacytes that secrete antigen-specific VLR-B antibodies, while continuing to express cell surface VLR-B antibody.

**DISCUSSION**

This analysis indicates that the VLR-B bearing lymphocytes belong to a distinct lineage of cells which play a dominant role in the humoral response of lamprey to antigenic stimulation. The prototypic small VLR-B+ cells constitute a major lymphocyte subpopulation in the hematopoietic typhlosole, circulation, and ventral regions of the kidney in healthy animals. The lamprey VLR-B lymphocytes, unlike T and B lymphocytes in jawed vertebrates, are not found in organized lymphoid organs, such as lymph nodes, spleen, or intestinal lymphoid tissues. Follicular accumulations of VLR-B+ lymphocytes were not demonstrable in three-year-old lamprey even after hyperimmunization and, more surprisingly, VLR-B bearing lymphocytes were not seen within or beneath the intestinal epithelium. A convincing thymus-like structure also has not been found in lamprey\textsuperscript{22,23}, and we could not find VLR-B+ lymphocyte accumulations in the gill regions. With regard to VLR-A producing lymphocytes, it is notable that *VLR-
A transcripts were found only in the mixed VLR-B population. These findings suggest that the VLR-A and VLR-B loci, which in hagfish are a great distance apart on the same chromosome, are under different regulation. When VLR-A specific antibodies become available to allow direct identification of the VLR-A expressing cells, it will be interesting to determine their contribution to the immune response and their relationship to the VLR-B lymphocytes.

Our analysis of the requirements for induction of humoral responses in the lamprey indicates that particulate antigens initiate strong VLR-B antibody responses, whereas soluble antigens do not. We failed to detect VLR-B antibodies production against model protein antigens, bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH), over an eight week period after immunization irrespective of the mode of immunization. Concordant results were obtained in earlier studies by other investigators, who could not demonstrate agglutinin responses using antigen-coated erythrocytes after immunization with BSA, KLH, and bovine gamma globulin. In contrast, VLR-B antibodies were consistently induced against the repetitive protein and carbohydrate epitopes on the surface of the representative particulate antigens, *B. anthracis* exosporium and mammalian erythrocytes. Others have observed agglutinin production following immunization with *Brucella abortus* and, in our unpublished studies, we found that lamprey larvae make VLR-B antibodies against other particulate immunogens with repetitive cell surface antigens, including *E. coli*, *S. typhimurium*, influenza virus, and human lymphocytes. This pattern of antigen responsiveness is reminiscent of mammalian antibody responses to T independent antigens and accords
with the apparent absence of a thymus dependent T lymphocyte population in the lamprey.

The mechanism(s) by which particulate antigens initiate specific VLR-B responses is presently unknown. VLR-B molecules are attached to the lymphocyte surface via GPI linkage\(^1\) and therefore lack a cytosolic portion that could trigger currently recognized intracellular signaling pathways of lymphocyte activation. Although ligation of some GPI linked molecules, such as Thy-1, CD16b, and CD14, may trigger cellular activation, the mechanisms for signal induction are either unclear or they involve interaction with transmembrane adaptor proteins that have signaling capacity\(^{25-27}\). The latter mode could be operative in VLR-B lymphocytes or the GPI-linked VLR-B molecules could serve merely as antigen focusing receptors to facilitate immunogen contact with a secondary mitogenic receptor, such as TLRs or other pattern recognition molecules with signaling capacity. In this context, two TLR genes have been identified recently in lamprey\(^{28}\). However, we found that immunization with polystyrene beads coated with BSA failed to elicit an immune response, even when given together with candidate TLR ligands. Although the cellular activation mechanism is still unknown, our studies clearly indicate that cells bearing antigen specific VLR-B receptors are activated by immunization to undergo lymphoblastoid transformation, proliferation and differentiation into mature VLR-B antibody secreting cells. Moreover, a \(>20\)-fold increase in VLR-B antibodies was induced following a booster immunization with human blood group O erythrocytes and this response was shown to be directed specifically against the trisaccharide H antigen. The features of this response are most easily
explained by clonal expansion of the antigen specific VLR-B lymphocytes, although formal proof of this interpretation is needed.

VLR-B bearing lymphocytes are thus remarkably similar to mammalian B lymphocytes in their response to immune stimulation, in keeping with the idea that cells with lymphocyte-like characteristics evolved in a common ancestor of lamprey and jawed vertebrates\textsuperscript{29,30}. This leads us to speculate that the immunoglobulin based V(D)J rearranging system may have evolved in an early ancestor for jawed vertebrates, perhaps one of the ostracoderms, to generate B lymphocytes before the evolution of thymus derivative T lymphocytes and MHC genes for antigen presentation. However, this hypothesis will very likely remain untested, because key representatives in the vertebrate evolutionary pathway are now extinct\textsuperscript{31}.

It should be noted that the results of our analysis of the antigen induced responses of VLR-B\textsuperscript{+} lymphocytes are remarkably consistent with observations made in earlier studies of the immune response in agnathans. In both lamprey and hagfish, immunizations with bacteria or heterologous erythrocytes were shown to elicit a humoral response that could be measured by antigen agglutination assays\textsuperscript{7-14}. Moreover, the hemagglutinins made against human O\textsuperscript{+} erythrocytes appeared to be specific for the polysaccharide H antigen\textsuperscript{8,11}. Erythrocyte rosetting cells were found to increase in frequency within days following erythrocyte immunization\textsuperscript{32}, and cells with the morphological features of plasma cells were also reported\textsuperscript{33-36}. All of these observations may now be explained by the antigen recognition and responsiveness of a diverse repertoire of VLR-B expressing lymphocytes that differentiate into plasmacytes that secrete multimeric VLR-B antibodies into the circulation. Our analysis suggests these
antibodies are large multimeric molecules composed of disulfide-linked pairs of identical VLR-B chains, a structural configuration that is supported by an analysis of recombinant VLR-B antibodies. The multivalency of VLR-B antibodies accounts for their ability to agglutinate particulate immunogens with repetitive antigenic epitopes. Future studies are needed to understand the molecular mechanisms that allow lamprey plasmacytes to simultaneously express cell surface VLR-B and secrete multimeric VLR antibodies.

Finally, our observations offer clues regarding the primary generation site for VLR-B lymphocytes in lamprey larvae. We observed relatively low and more variable levels of VLR-B expression before and after immunization for the population of lymphocytes in the typhlosole relative to the lymphocyte populations in blood and kidney. Remarkably, animal stress and treatment with exogenous corticosteroids led to the loss of VLR-bearing cells exclusively in the typhlosole. These findings are reminiscent of the corticosteroid susceptibility of immature B lineage cells in the avian bursa of Fabricius and the mammalian bone marrow and of immature mammalian thymocytes. In addition to their implied immaturity, relatively few typhlosole based VLR-B lymphocytes responded to immunization with proliferation and differentiation into antibody secreting plasmacytes. These findings collectively infer that VLR-B lymphocytes are generated along with other types of blood cells in the typhlosole.
METHODS

Animal maintenance and immunization

Sea lamprey larvae (11-15 cm) supplied by Lamprey Services (Ludington, MI) were from the Great Lakes of North America. They are less diverse that lamprey from the sea, presumably because of founder influence\(^4\). Lamprey were maintained in sand-lined aquariums at 16-18 °C and fed brewer’s yeast. Purified Sterne strain of *Bacillus anthracis* exosporium\(^1\), erythrocytes, or recombinant proteins were injected intraperitoneally into lamprey anesthetized by immersion in 0.1 g/L MS222 (Sigma, St. Louis, MO).

Monoclonal anti-VLR antibodies and recombinant VLR antibody

Two mouse monoclonal antibodies were produced by hyper-immunization of mice with a recombinant VLR-B invariant stalk region protein produced in *E. coli* and subsequent fusion of regional lymph node cells with the non-productive Ag8.653 myeloma variant\(^4\). Two hybridoma clones that produced antibodies with VLR-B specificity, 6C3 (IgM) and 4C4 (IgG2b), were identified by ELISA and flow cytometric screening. By immunofluorescence staining of viable cells and by immunohistochemical staining of fixed sections, the 6C3 and 4C4 antibodies were shown to recognize the same lymphocyte populations in lamprey blood and tissues. The 4C4 antibody was also reactive with VLR-B protein by Western blotting.

Immunohistochemistry, immunofluorescence and electron microscopy

Lamprey were sacrificed by emersion in 1 g/L MS222 to obtain tissue and blood samples. For immunohistology, 1 cm corpse transections were fixed in 10% neutral buffered formalin and embedded in paraffin. Cut sections were deparaffinized and rehydrated
through sequential emersion in 100%, 95%, and 70% ethanol before antigen retrieval by heating the sections for 10 minutes at 15 psi in 0.01 M citric acid (pH 6) for the 6C3 anti-VLR antibody or in 0.01 M EDTA (pH 8) for 4C4 antibody staining\textsuperscript{43}. The sections were then treated with 3% hydrogen peroxide for five minutes before blocking with 3% goat serum for 30 minutes. Processed tissue sections were incubated with one of the primary antibodies at room temperature for 1 hour before washing with Tris buffered saline and addition of a biotinylated secondary antibody and streptavidin-HRP (Signet Laboratories, Dedham, MA), 20 minutes each, followed by addition of the diaminobenzidine substrate (BioGenex, San Ramon, CA) for chromogenic labeling. Labeled slides were immersed briefly in Mayer's hematoxylin for counterstaining, then dehydrated through sequential baths of ethanol and xylene before application of cover slips. The same protocol was used for immunofluorescence, except that cover slips were placed after addition of the secondary antibody with Prolong Gold with DAPI mounting media (Invitrogen, Carlsbad, CA,). For electron microscopy, sorted blood cells were resuspended in sodium cacodylate or Sorsenson’s buffer with 2.5% glutaraldehyde for four hours at 4° C. Cells were then post-fixed in 1% osmium tetroxide for 1 hour, dehydrated in a series of graded acetone, and embedded in epoxy resin. For quantitation of lymphocytes in the VLR-B\textsuperscript{+} and VLR-B\textsuperscript{-} populations of cells with lymphocyte-like light scatter characteristics, cytospins of sorted cells were fixed in methanol for 30 seconds and then stained with the Wright-Geimsa stain (EM Seeince, Gibbstown, NJ) for 2 minutes. Stained slides were washed in water and coverslips added before analysis of the cells by light microscopy.

\textbf{BrdU\textsuperscript{+} VLR-B\textsuperscript{+} cell detection by immunohistochemistry}
Lamprey were immunized with 25 µg of *B. anthracis* exosporium and immediately returned to their water tanks. Five days later 200 µg BrdU (Beckton Dickinson, Franklin Lakes, NJ) was injected in 60 µl 2/3 PBS and the animals were returned to their tanks for seven hours before animal sacrificing, tissue processing and paraffin embedding of tissues. Fluorescence staining was done with anti-VLR-B mAb 6C3 (IgM) and anti-BrdU clone Bu20a (IgG1; Dako, Glostrup, Denmark) with isotype specific fluorescently labeled secondary antibodies (Southern Biotech) as described above.

**Antigens and VLR antibody assays**

BSA immunizations were injections of 10 µg of BSA in 50 µl of one of the following vehicles: sterile 0.66% PBS, 200 µg Al(OH)₃ absorbed with protein for four hours before injection, or emulsions with Ribi and Titermax Gold (Sigma) adjuvants prepared according to manufacturer’s protocol. For BSA coated beads, BSA was conjugated to 1 micron carboxylate polystyrene beads with the carbodiimide kit according to manufacturer’s protocol (Polysciences, Warrington, PA) and lipopolysaccharide, lipoteichoic acid, and peptidoglycan (Invivogen, San Diego, CA) was added before injection. Erythrocytes from B6 mice or human blood group O donors were washed three times prior to injection. For antibody assays, washed erythrocytes (5X10⁶) mixed with lamprey plasma at varying dilutions and allowed to settle in conical bottom microwell plates for 1 hour before visual assessment of agglutination after tilting the plate at 80° for two minutes. ELISA assays were performed as previously described² using BSA, KLH, and BclA-CTD at 5µg/ml or 1X10⁶/well *B. anthracis, ΔBclA B. anthracis, B. cereus T,* and *B. thuringiensis* spores to coat the plates²⁰. VLR reactivity with H antigen was determined by incubating CHO cells that were stably transfected with constructs for α1,2-
fucosyltransferase or vector alone\textsuperscript{19} with test plasma samples. The CHO cells were then stained by incubation with 4C4 VLR mAb and goat anti mouse Ig (H+L)-RPE (Southern Biotech, Birmingham, AL) for 10 minutes each before analysis of immunofluorescence using a Cyan flow cytometer (Cytomation, Fort Collins, CO). For plasma VLR adsorption, test samples were mixed with the 4C4 anti-VLR mAb or BclA-CTD recombinant protein conjugated to sepharose or CHO cells (3X10\textsuperscript{6}) fixed by paraformaldehyde for one hour at 4° C. Beads or cells were spun down and the supernatant transferred to a new test tube before repeating the adsorption process prior to the analysis of antigen reactivity by agglutination, ELISA, or western blot assay. For staining of lamprey lymphocytes with fluorescent spores or bacteria, 1X10\textsuperscript{6} leukocytes were mixed with 1X10\textsuperscript{6} spores and/or 5X10\textsuperscript{7} \textit{S. typhimurium} labeled\textsuperscript{44} with Alexa 488 or 647 (Invitrogen) on ice for 20 minutes and then 4C4 anti-VLR monoclonal antibody for an additional 10 minutes. Cells were then washed and incubated with a goat anti-mouse Ig (H+L)-RPE (Southern Biotech) for 10 minutes on ice before two washes and flow cytometric analysis. For the cytophilic VLR antibody binding assays, leukocytes from naïve animals were incubated in plasma from hyperimmunized animals for 30 minutes at room temperature before washing and incubating with fluorochrome-labeled spores.

**Dexamethasone treatment of lamprey larvae**

Lamprey were injected intraperitoneally with 3-30 µg of dexamethasone (Sigma) or maintained in tanks with 250 µg/ml dexamethasone for three days. Animals were sacrificed on serially collect leukocytes and organs for flow cytometric analysis.

**ELISPOT analysis of VLR secreting cells**
Microwells in 96 well plates (Millipore, Billerica, MA) were coated overnight at 4°C with 100 µl of recombinant BclA C-terminal domain protein \(^{20}\), 50 µg/ml, then blocked with 1% BSA in PBS for 2 hours at 37°C before adding test cell suspensions in IDMEM (Mediatech, Herndon, VA) supplemented with 10% FBS, L-glutamine, penicillin, streptomycin, insulin, and transferrin for an 18 hour incubation at 25°C in 5% CO₂. The cells were then washed away with PBS before adding 1 µg/ml VLR antibody in 1% BSA for one hour at 37°C. After washing the wells with PBS-0.5% tween, goat anti-mouse Ig conjugated with horseradish peroxidase (Southern Biotech) was added for one hour at 37°C before washing the wells with PBS-tween and PBS. AEC peroxidase substrate (Moss Inc, Pasadena, MD) was then added for one hour before washing with deionized water and counting of VLR antibody spots using Immunospot 2.0 software (Cellular Technology Ltd., Cleveland, OH).

**Western blots**

Plasma samples (1 µl) were electrophoresed in a 10% SDS page gel either without or with 0.25%, 0.5%, or 5% 2-mercaptoethanol before transfer onto a nitrocellulose membrane which was blocked with 3% milk before incubation with the 4C4 anti-VLR mAb for one hour. The membranes were then washed 5 times with PBS-0.5% tween before adding goat anti-mouse HRP (Southern Biotech) and a final wash one hour later. A SuperSignal chemiluminescent kit (Pierce, Rockford, IL) was used to detect VLR-antibody conjugates.

**Quantitative PCR**

RNA was extracted from VLR-B⁺ and VLR-B⁻ sorted cells using Trizol (Invitrogen) and RNeasy with on-column DNA digestion (Qiagen, Valencia, CA) according to
manufacturer’s protocol. First strand cDNA was generated using random hexamer primers with Superscript III (Invitrogen). Quantitative PCR was carried out with primers designed at splice sites, when known, using SYBR Green on a 7900HT ABI Prism (Applied Biosystems).

**Statistical analysis**

Statistical analyses were performed with Statdisk software (version 10.0.0) using the two-sample Student’s t-test.
ACKNOWLEDGEMENTS

We thank Louise Stansell for providing human erythrocytes, Leigh Millican and Elizabeth Weeks for assistance with electron microscopy, and Marsha Flurry for help with preparation of figures. This work was supported by NIH grants AI72435 and AI57699.

AUTHOR CONTRIBUTIONS

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44. Oliva, C. et al. The integrin Mac-1 (CR3) mediates internalization and directs *Bacillus anthracis* spores into professional phagocytes. *Proc Natl Acad Sci U S A*. In press
Supplementary Figure 1
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Supplemental figure 1 Comparison of VLR-B cell surface levels on lymphocytes from blood, kidney and typhlosole. (a) Note the significantly lower mean fluorescence intensity for VLR-B-bearing lymphocytes from typhlosole versus blood and kidney (n=21-24 per organ; bars indicate S.E.M.; *, p < 0.01). (b) Higher variation in VLR-B staining levels for typhlosole VLR-B bearing lymphocytes. (n = 21-24; bars indicate S.E.M.)
Supplemental figure 2  Assessment of cell surface of VLR-B in blood and typhlosole four days after corticosteroid treatment (250 μg/ml dexamethasone treatment for three days). Note the selective loss of VLR-B bearing cells in typhlosole. Kidneys also retained their VLR-B⁺ cells after steroid treatment (data not shown).
Supplementary Figure 3
Alder et al.

Supplemental figure 3  Lymphoblastoid response of VLR-B+ lymphocytes in lamprey hyperimmunized with B. anthracis exosporium. (a) Flow cytometric analysis of forward and side light scatter characteristics of all blood leukocytes versus the VLR-B bearing population. Blood samples were from animals 14 days after booster injection of a large dose of exosporium (25 µg). (b) Cell surface expression of VLR-B before and after hyperimmunization with exosporium.
Supplementary Figure 4
Alder et al.

Supplemental figure 4 In vitro assessment of VLR-B antibodies in B. anthracis immune plasma to facilitate spore binding by cytophilic adherence to lymphoid cells. These results can be compared with those in text figure 5.
CONCLUSION

The majority of the work in this dissertation supported what was hypothesized following the initial discovery of the lamprey VLR. Lamprey lymphocytes maintain a VLR repertoire, which is comparable to that of jawed vertebrates and sufficient to mediate adaptive immune responses. Rearrangement of the VLR locus occurs exclusively in lymphocytes by addition of LRR cassettes into the incomplete germline VLR gene via multi-step, piecewise gene conversion process that relies on short stretches of homology between donor and acceptor sequences to align incoming sequences. The assembly process begins at either of the incomplete ends of the germline VLR gene. VLR antigen receptors can recognize a broad range of epitopes including protein and carbohydrate antigens. Lamprey lymphocytes undergo lymphoblastoid transformation and proliferation following immunization with some lymphocytes taking on plasmacytoid characteristics to secrete VLR antibodies.

The characteristics defined for VLR antibodies explain many of the earlier experimental results from studies on the lamprey immune system. Like earlier investigators, we were unable to induce immune responses to soluble proteins\(^1,6\). In the initial work from the 1960’s, the production of agglutinins after immunization with erythrocytes had been attributed to either primitive immunoglobulins or uncharacterized non-immunoglobulin proteins\(^1,6,7,19\). We now show that the multivalent nature of the VLR antibodies accounts for their ability to agglutinate erythrocytes and bacteria. While the attempts to identify the agglutinins were unsuccessful, molecular weight estimates of
~320 kDa and subunits of ~75 kDa were remarkably close to those predicted for VLR multimers and their dimeric subunits\textsuperscript{7}. Previous work had suggested an H antigen specificity of the agglutinating proteins made in response to immunization with human blood group O erythrocytes\textsuperscript{19}, a finding that was confirmed for the VLR antibodies in this dissertation.

The phylogenetic relationships between lamprey, hagfish, and jawed vertebrates are controversial\textsuperscript{20,21} (Fig. 1). Paleontological evidence suggests that lamprey and hagfish are paraphyletic, with lamprey being closer to jawed vertebrates than to hagfish (Fig. 1a), while molecular data suggests lamprey and hagfish are monophyletic and more related to each other than to jawed vertebrates (Fig. 1b). If lamprey and hagfish are paraphyletic (Fig. 1a), this implies the VLR immune mechanism would have been present in the common ancestor of all three groups and that it was subsequently lost in the jawed vertebrates. This scenario would favor the likelihood that remnants of the rearranging VLR locus will be found in early jawed vertebrate genomes, a question that has not yet been addressed. An analysis of LRR protein structure revealed that lamprey and hagfish VLR contain an insertion of several amino acids in the LRRCT between the helix portion and first beta strand. A similar insertion could only be found in the GPIb\(\alpha\) receptor.
suggesting these two genes may have arisen from a single ancestral gene\textsuperscript{17}. The presence of this insertion could be used to search for remnants of the VLR system when the genomes of more basal vertebrates become available. Alternatively, if lamprey and hagfish are monophyletic (Fig 1b), it is difficult to ascertain which of the two immune systems is more ancient. The acquisition of the VLR recombinatorial system could have arisen from a duplication of the GPIb\textalpha receptor gene in a common ancestor of lamprey and hagfish (Fig 3b, arrow). This would also imply that the immunoglobulin based adaptive immune system evolved independently of the VLR based immune system, potentially in a now extinct group of jawless vertebrates known as the ostracoderms (Fig 3b, asterisk), which eventually gave rise to the jawed vertebrates. It is possible that the evolution of these two different adaptive immune systems may account for the selective survival of lamprey, hagfish, and jawed vertebrates while other jawless vertebrates perished. The apparent convergent evolution of the two immune systems supports the idea that at some point in evolution it proved advantageous [or necessary] to have an immune system that was capable of anticipatory adaptive immunity.

Any evolutionary scenario still leaves at least 500 million years of history since the last common ancestor. Yet the similarities between the two adaptive immune systems are striking. The genes for both types of antigen receptors are assembled only in lymphocytes and, in each case, only one allele is expressed. Immunoglobulins and VLR molecules are both initially expressed as antigen receptors on lymphocytes but can also be secreted, although the mechanism for simultaneous surface expression and secretion of VLRs is still not fully understood. The lymphocytes from lamprey respond to immunization in a similar fashion as mammalian lymphocytes, with lymphoblastoid
transformation followed by, proliferation and plasmacytoid differentiation. In jawless vertebrates VLR-B antibodies participate in at least a humoral immune response, but testing of protective immunity, as is seen with immunoglobulins in jawed vertebrates, awaits the studies of a model pathogen for lampreys.

Through the work presented here and the related work of others, much has been learned about the VLR adaptive immune system. For the first time since the discovery of our own adaptive immune system, we now know of another adaptive immune system for comparison. While the progress toward understanding the lamprey adaptive immune system has been rapid, many questions still remain unanswered, just a few include: 1) the mechanism for signaling through GPI linked VLR surface receptors, 2) how lamprey express cell surface and secreted VLR molecules, 3) the mechanism for allo-recognition that has been demonstrated in lamprey and hagfish, 4) preliminary evidence for memory type responses have been demonstrated, definitive proof is still needed, 5) the role of VLR-A in lamprey adaptive immune responses, 6) the mechanism for avoiding VLR antibodies with self specificity. This is just a short list of future investigations that might lead to interesting new discoveries about the VLR based adaptive immune system of jawless vertebrates.
GENERAL LIST OF REFERENCES


APPENDIX

IACUC APPROVAL FORM

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: November 2, 2007
TO: Max Cooper, M.D.,
SHL 431 2182
FAX: 975-7218

FROM: Judith A. Kasp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Characterization of the Lamprey Adaptive Immune System
Sponsor: NIH
Animal Project Number: 071107964

On October 31, 2007, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

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Animal use is scheduled for review one year from November 2007. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 071107964 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.