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Analysis of the Diversity of a Sheep Antibody Repertoire as Revealed from a Bacteriophage Display Library

Keith A. Charlton,* Sarah Moyle,† Andrew J. R. Porter,* and William J. Harris2*

We have applied bacteriophage display technology to construct and analyze the diversity of an IgG library of $>1 \times 10^8$ clones from an adult sheep immunized against the hapten atrazine. We have identified eight new $V_H$ gene families ($V_{H}2$–$V_{H}9$) and five new $V_{\kappa}$ gene families ($V_{\kappa}5$–$V_{\kappa}10$). The heavy and $\kappa$ light chain variable region gene loci were found to be far more diverse than previously thought. The Journal of Immunology, 2000, 164: 6221–6229.

The ability to display the entire functionally active Ab repertoire of a suitable host on the surface of filamentous bacteriophage has been successfully applied to the generation of mAbs without the need for B cell immortalization (1–4). The majority of research has been conducted using libraries of human origin, but libraries have also been produced from rabbit, chicken, mouse, and cattle (5–10), often with a view to producing Abs against Ags conserved in humans and/or mice or in mammals as a whole. The use of libraries circumvents the difficulties associated with generating stable heterohybridoma cell lines and also enables studies to be made of the immunology of the selected host species.

The diversity and organization of the variable region $V_{H}$–$D$–$J_{H}$ heavy chain and $V_{\kappa}$–$J_{\kappa}$ light chain genes of humans and mice are well understood, as are the mechanisms involved in generating the enormous primary Ab repertoire necessary to fulfil the immune system’s protective role (11, 12). In recent years much research has also been directed toward the study of domesticated animals of economic importance such as chickens and large farm animals, revealing variations in both the site of primary repertoire generation and the mechanisms used.

In humans and mice, B cell lymphopoiesis occurs in the bone marrow (13) and continues throughout life. In the chicken the bursa of Fabricius has been recognized as the site of B cell development for some time (14), and in rabbits the appendix functions as a bursal equivalent (15). More recently it has been shown that the bursa of Fabricius has been recognized as the site of B cell development and the mechanisms used.

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In this study, we demonstrate for the first time that phage display technology can be used to study diversity, that sheep possess and utilize a more diverse Ig germline gene pool than was previously thought, and that sequences derived from pseudogenes may contribute to an ongoing process of IgG diversification.

Materials and Methods

Bacterial strains

Phagemid vector was transformed into Escherichia coli TG1 (supE thi-1 Δlac-proAB Δ(mcrB-hsdSM)Str^R mcrB Δ(mcrB-hsdSM)lacIq Δtrd30 proAB lacFΔ15ΔM15). Soluble expression was conducted in E. coli XL1-Blue (supE44, hsdR17, recA1, endA1, gyrA96, thi-1, relA1, lacFΔ1 proAB, lacFΔ15ΔM15, th10 tetR).

Isolation of mRNA and production of cDNA

Total mRNA was isolated with the Quick-prep-mRNA purification kit (Pharmacia, Milton Keynes, U.K.) from a total of 400 mg spleen removed from a 10-year-old Welsh breed/Suffolk sheep that had been hyperimmunized against a hapten target (atrazine) conjugated to bovine-thyroglobulin (Guildhay, Surrey, U.K.). To prepare cDNA, 200 ng mRNA was used up to 25 µl with RNase-free water, and 25 pmol of FOR primer specific for sheep heavy, A, or $\kappa$ light chain constant regions was added. The mixture was heated to 70°C for 10 min and cooled to 42°C before adding 8 µl of 5× concentration first-strand buffer (Life Technologies, Paisley, U.K.), 4 µl 0.1 M DTT, and 1 µl dNTP mix (10 mM each). The mixture was incubated at 42°C for 2 min before adding 1 µl (200 U) SuperScript II (Life Technologies) reverse transcriptase, and incubation continued at 42°C for 50 min and then for 15 min at 70°C.

PCR rescue and linking of variable heavy and light chain genes

PCR reactions comprising 25 pmol each OvVHBACK and OvVHFOR primers, 1 µl dNTP mix (25 mM each), 5 µl 10× concentration Bioline reaction buffer (160 mM NH₄SO₄, 670 mM Tris-HCl (pH 8.8 at 25°C), 0.1% Tween 20), 2 µl 50 mM MgCl₂, 1 µl heavy chain cDNA, and sterile water to 50 µl were prepared. The reactions were heated to 94°C for 5 min and held while 0.5 µl Bioline Taq DNA polymerase (5 U/µl) was added. They were then incubated for 30 temperature cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and then a final incubation of 72°C for 7 min. Separate sets of 10 PCR reactions were performed using each combination of OvVHBACK and OvVHFOR primers (Fig. 1). Both $\lambda$ and $\kappa$ thought to express relatively few $V_H$ genes belonging to a single $V_H$ family, that of the former two being homologous to human $V_H4$ and the latter to human $V_H3$ (19–23). The sheep Ig light chain primary repertoire is diversified by extensive somatic hypermutation and is independent of Ag (16, 24), whereas cattle and possibly swine also use templated gene conversion by nonreciprocal recombination (17, 25, 26). Chicken and rabbit have more extensive $V_H$ loci in that each possess ~100 genes, related to human $V_{\kappa}3$. However, $V_H$ gene usage is limited by restricted functionality or preferential expression, and gene conversion plays a significant role in Ab diversity (27, 28).

In this study, we demonstrate for the first time that phage display technology can be used to study diversity, that sheep possess and utilize a more diverse Ig germline gene pool than was previously thought, and that sequences derived from pseudogenes may contribute to an ongoing process of IgG diversification.

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light chains were amplified as above using OvVABACK/OvJAFOR and OvVeBACK/OvJeFOR primer combinations with λ and κ cDNA templates, respectively. PCR products were purified by electrophoresis through a 1% TAE agarose gel, and bands of the correct size were excised. DNA was recovered using QIAquick columns (Qiagen, Surrey, U.K.) and eluted a 1% TAE agarose gel, and bands of the correct size were excised. DNA was recovered from gel slices with DNA extraction and ethanol precipitation.

Construction of scFv-phage library

Purified V<sub>H</sub> and V<sub>L</sub> DNA was digested with T<sub>aqI</sub> and T<sub>aqII</sub> (Roche, Basel, Switzerland) and then with BstEII (New England Biolabs, Beverly, MA). DNA was ligated with vector (2 μg each) using 10 U T4 DNA ligase. After incubation at 16°C overnight, the ligation mixture was transformed into E. coli DH5α (Invitrogen, Carlsbad, CA). Transformants were plated onto ampicillin-containing LB agar plates, and colonies were selected and amplified as described above. The DNA was purified using QIAquick columns (Qiagen) and eluted into sterile water.

Sequence analysis

The nucleotide sequences of the V<sub>H</sub> and V<sub>L</sub> genes were determined using the ABI 377 automated DNA sequencer (Applied Biosystems, Foster City, CA) in both directions. Sequences were compared and edited using the GAP and PILEUP programs (Daresbury, U.K.). Comparisons were restricted to those parts of the rearranged genes encoded by the V<sub>µ</sub> or V<sub>k</sub> gene segments.

Results

Library construction

The library was constructed from a sheep immunized with atrazine conjugated to BSA for the isolation high-affinity anti-atrazine Abs

Ab gene sequence analysis

Ab V<sub>H</sub> and V<sub>L</sub> genes from clones selected at random from the original library glycerol stocks were PCR amplified using the AH-1 and Fd seq1 primers to determine the proportion of clones containing a scFv fragment of the correct size (~800 bp). After incubation overnight at 30°C, the colonies were scraped off into 2 ml of 2X YT-Amp-15% glycerol per plate and pooled. Aliquots were prepared and stored at ~80°C. To reseque, 100 μl of glycerol stock (~3 × 10<sup>8</sup> cells) was inoculated into 500 ml of 2X YT-Amp-Glu and incubated with shaking at 37°C to an OD<sub>600</sub> of 0.6 (1–2 h). M13KO7 helper phage (Pharmacia) was added at 200 multiplicity to 50 ml of the culture that was incubated at 37°C without shaking for 30 min. Infected cells were pelleted, resuspended in 500 μl 2X YT-Amp-Kan-Glu, and incubated overnight with shaking at 30°C. Phage particles were concentrated from the culture supernatant by two successive precipitations with 1/5 volume PEG (20% polyethylene glycol weight to volume ratio, 2.5 M NaCl) as described by Griffiths et al. (30).

FIGURE 1. Primers were designed using published sheep antibody gene sequences (16, 19, 24, 33–36) except OvJAFOR, which was derived from cDNAs amplified and sequenced as part of this study (data not shown), and OvH4aBACKSfi, which was derived from a sheep immunized with atrazine conjugated to BSA for the isolation high-affinity anti-atrazine Abs.

scFv fragments were ligated with vector (2 μg each) using 10 U T4 DNA ligase. After incubation at 16°C overnight, the ligation mixture was transformed into E. coli DH5α (Invitrogen, Carlsbad, CA). Transformants were selected and amplified as described above. The DNA was purified using QIAquick columns (Qiagen) and eluted into sterile water.

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Sequence analysis

Variations in the nomenclature used by researchers when discussing the organization and ancestry of heavy chain variable region genes can lead to confusion. In the context of this article and in reference to others works, the following system will be used: heavy chain gene families, where applicable, will be numbered using Arabic numerals; and the three major homologous groups will be referred to as “clans” and will be identified by Roman numerals using the classification of Chaudhary et al. (31), enables all heavy and light chains to be joined without bias via the center 15 bases.

Sheep heavy chains

A large number of clones were selected at random and the VH and Vl chain genes were amplified by PCR with the AH-1 and Fd seq1 primers (Fig. 1). The sequences of 45 rearranged heavy chain genes are compared with the V5a germline sequence (19) and JH 1. As with the 5' end of FR1, the terminal eight codons of FR4 are encoded by primers used in PCR, and so sequence variability should be ignored in this region. Of the sequences belonging to VH1, 3 of 20 have used the JH2 gene segment as determined by the presence of G in position 2 of codon 105 (Kabat numbering) encoding an arginine (CAG). The remaining 17 have a proline here (CCA). This preferential use of JH2 is in agreement with previous findings (38).

Examination of the JH-encoded regions of the remaining sequences belonging to families other than VH1 reveals some interesting features. All such sequences except H261 have either AG (19 clones) or AA (five clones) in positions 2 and 3 of codon 105, giving rise to a glutamine residue. The frequency with which this occurs is too great to be a result of convergent somatic mutation. Analysis of the published JH segments reveals that the pseudogenes JHps2, JHps3, and JHps4 all encode codons 103–105 in this way (5'-TGGGCCCAG-3') (38). Moreover, clones H242, H225, H168, H9, and H297 include the motif 5'-TGCTTTTGA-3' (boxed sequence in Fig. 3). Once again this sequence is found in the correct position in the pseudogene JHps3. A second motif, 5'-ACGG-3' is found spanning the codons 2 and 3 aa upstream of

Heavy chain D-JH regions

The lengths of CDR3s we have observed in sheep heavy chains range from 23 aa in clone H257 down to 3 aa in clones H69 and H261. The JH locus has been characterized by Dufour and Nau (38), who identified two functional JH segments and four pseudogenes in a region 5 kb upstream of the Cmu gene and spanning 1.85 kb. Both functional genes have been included in Fig. 3, and the rearranged sequences are compared with I. As with the 5' end of FR1, the terminal eight codons of FR4 are encoded by primers used in PCR, and so sequence variability should be ignored in this region. Of the sequences belonging to VH1, 3 of 20 have used the JH2 gene segment as determined by the presence of G in position 2 of codon 105 (Kabat numbering) encoding an arginine (CAG). The remaining 17 have a proline here (CCA). This preferential use of JH2 is in agreement with previous findings (38).

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3 Abbreviations used in this paper: FR, framework region; CDR, complementarity-determining region.
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**Note:** The table represents a section of the sheep Ab repertoire from bacteriophage display, with amino acid sequences detailed in the rows and columns.
position 101 (Fig. 3) in clones H3, H204, H11, H264, H217, H257, H15, and H23. This motif is found in JH2, and so its presence in H3 is not unexpected. However, the region encoding positions 101–102 in six of the other seven clones suggests that these rearrangements involved the use of JH1 and not JH2.

κ Chains

Thirteen of the clones sequenced from the unselected phage library contained κ light chains (Fig. 5). Five new families can be identified (Table II), two of which include two genes and a further three each assigned from a single gene. The phylogenetic relationship of all of the sheep Vκ families is illustrated in the dendogram in Fig. 6. In a previous study (41), the amino acid at the 3′ end of CDR3 (Kabat position 97) was found to be either alanine or serine, corresponding to the use of the Jκ1 and Jκ2 gene segments, respectively. All of the clones we have identified have a threonine (ACT/ACG) in this position, the ACT codon being found in the pseudogene Jκ3. In three clones (K227, K13, and K321) the last two codons of CDR3 are 5′-TGG ACG-3′, which does not correspond with any of the three known Jκ gene segments.

λ Chains

Forty-seven rearranged VA sequences were aligned with the 5.1 germline gene described previously (Ref. 16 and Fig. 7). All were found to segregate with members of the VλI family described by Reynaud et al. (Ref. 16 and not shown). GAP analysis of λ light chains revealed a wide range of diversity between these sequences that belong to the same family (not shown). Twenty-five percent of the clones had less than 80% homology with the majority of other clones. Clones L123 and L21 have >80% identity with the 17 germline genes belonging to the closely related but distinct family IV described by Reynaud et al. (16). However, L123 has 91.6% identity with 16.1 and L21 has 94.9% identity with 4.1, and so these clones are clearly members of the VλI family.

Discussion

Sheep Ab sequences

By producing a phage display library, we potentially have access to the whole expressed IgG repertoire of the host animal. The primer sequence used to produce heavy chain cDNA (CH1FOR) is conserved in both IgG1 and IgG2 isotypes. In total, 45 heavy chains, 47 λ light chains, and 13 κ light chains were sequenced from clones selected at random. Sequences have been analyzed according to the established method of Kabat et al. (32) which assumes that sequences that diverge by greater than 80% are derived from different germline gene families. In some cases this
analysis has provided only a single family member sequence and may be less decisive. However, in these cases homology with all other sequences is less than 70% with no clustering of sequence variation, and therefore it is unlikely due to PCR errors or artifacts such as template jumping during library construction. Because we have sequenced only 47 clones, a germline family providing 2% of the repertoire would be represented by a single clone.

Heavy chain genes

All previously reported \( \text{V}_H \) genes from sheep belong to a single family (19) that shows greatest homology to the single family expressed in cattle (22). When compared with human \( \text{V}_H \) genes, both sheep and cattle are homologous to \( \text{V}_H 4 \), a member of clan II (38). The heavy chain genes of other species expressing a single family such as swine, rabbit, and chicken are more closely related to human \( \text{V}_H 3 \) (clan III) (23, 44, 45), which has been proposed as the ancestral \( \text{V}_H \) gene family (46). We have identified nine heavy chain gene families in sheep, eight of which have not been previously reported (Fig. 4). Of the new families, \( \text{V}_H 5 \), \( \text{V}_H 7 \), \( \text{V}_H 8 \), and \( \text{V}_H 9 \) are homologues of clan \( \text{V}_H II \), together with the \( \text{V}_H 1 \) family already described. \( \text{V}_H 3 \), \( \text{V}_H 4 \), and \( \text{V}_H 6 \) are homologues of clan \( \text{V}_H I \), and \( \text{V}_H 2 \) is a homologue of clan \( \text{V}_H III \). The isolation of sheep genes related to clan \( \text{V}_H III \) confirms the evidence obtained by Tutter and Riblet (46), who observed hybridization of probes derived from the murine S107 and 7183 gene families to sheep genomic DNA. The sheep families \( \text{V}_H 1 \), \( \text{V}_H 7 \), \( \text{V}_H 8 \), and \( \text{V}_H 9 \) are most similar to human \( \text{V}_H 4 \), and the sheep \( \text{V}_H 5 \) is most similar to human \( \text{V}_H 6 \) (not shown). Saini et al. (47) reported detecting homologues of murine \( \text{V}_H 11 \) in bovine genomic DNA by Southern blot. In view of the close homology between ovine and bovine Ig genes, the sequences reported in this study may prove valuable in a more extensive analysis of the bovine genome.

All of the sequences we have obtained were derived from cDNA and so were being expressed in the host animal. Greater than 55%
of the V_H genes belong to new sheep families. Previous detailed studies have not revealed the heavy chain diversity that we have seen. Selective breeding has not resulted in variations in the diversity of Ig light chain loci between sheep belonging to different breeds (35). A possible factor is the age of the donor sheep. Heavy chain genes sequenced by Patri and Nau (36), Dufour et al. (19), or gene conversion events. The clones with pseudogene sequences may represent further somatic mutation of these genes or gene conversion events.

**Light chain genes**

The κ genes represent six separate families. There is evidence that sheep preferentially express certain κ gene families at different stages of development (41), with VκIV dominating during the final stages of gestation. The single published gene isolated from adult tissue (35) belongs to this family. That this group is a major contributor to the adult Vκ repertoire is confirmed by our data in that six of the 13 genes we have identified are VκIV. However, phylogenetic comparison (Fig. 6) suggests that they form a separate subgroup to the Vκ4 gene. The Jκ-encoded region of our clones does not closely match the distinctive regions of any of the three known Jκ segments and may indicate that sheep possess a more extensive genomic Jκ region than that sequenced to date. The sheep Vα repertoire is known to be diverse, including at least six different families (16). Only genes from VαI, II, and VI have been identified from cDNA and so are known to be expressed (41). All such rearranged genes were isolated from fetal material.

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**FIGURE 6.** Dendogram of rearranged sheep Vκ genes. Sequences comprise five genes previously reported (43) labeled VκI, Vκ2.1, Vκ2.2, Vκ3, and Vκ4, together with 13 additional genes. Nine sheep Vκ families are identified based on >80% nucleotide homology and are labeled VκI–VκIX. = Families described previously.
The 47 unselected sequences described in this study belong to the VA I family, which leads us to suggest that in contrast to the V\(_{\text{H}}\) and V\(_{\text{K}}\) repertoires, VA gene usage is restricted in adult sheep. We have found that expressed VA I genes are frequently highly divergent from known germline sequences. The extent of the observed divergence is such that two genes, L2 and L222, have >80% sequence identity only with each other, and a further two, L21 and L123, have >80% identity not only with VA I germline genes but also with the "17" germline gene belonging to the related but separate family VA II.

**FIGURE 7.** Sheep light chain VA-JA rearranged genes. The sequences were obtained by PCR from cDNAs and are compared with the 5.1 germline gene (16). Gaps in sequences have been introduced to maximize homology, and dashes indicate nucleotide identity. The region of FR4 encoded by J FOR PCR primers is indicated. Positions of CDRs and amino acid numbering are as Kabat et al. (32).
In conclusion, ruminants are thought to possess a smaller and less diverse gene pool than humans and mice and to utilize different mechanisms for generating their primary immune repertoire. Our studies indicate a greater level of functional diversity than previously described in sheep, though this does not necessarily imply a larger gene pool. Ab diversity and repertoire development are important components in animal health and understanding of disease processes. The studies we describe demonstrate the value of sheep Ab phage display libraries and provide a powerful new tool for such research.

Acknowledgments

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7. Yamanaka, H. I., T. Inoue, and O. Ikeda-Tanaka. 1996. Chicken monoclonal antibody diversity and repertoire development imply a larger gene pool. Ab diversity and repertoire development are important components in animal health and understanding of disease processes. The studies we describe demonstrate the value of sheep Ab phage display libraries and provide a powerful new tool for such research.

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