4
Antibodies from Other Species

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4.1
Introduction

Immunoglobulins are the molecular basis of humoral immunity. Across different species, these macromolecules maintain a common quaternary structure, which is typically comprised of two identical heavy chains with covalently attached oligosaccharide groups and two identical non-glycosylated, light chains. These glycoprotein molecules recognize and bind a particular antigen in a highly complex and exceedingly specific immune response. Antibodies are the primary protective molecules elicited by most vaccines, and recombinant antibodies are now a major class of therapeutics for multiple diseases. The earliest antibody therapeutics were derived from serum of nonhuman species. In particular, horse serum served as anti-venom yet had substantial toxicity (serum sickness) due to the immune response against the nonhuman antibody protein [1, 2]. Other antibody preparations such as anti-thymocyte globulin produced in rabbit had therapeutic benefit but also had significant toxicity. The use of alternative species for these therapeutic preparations was largely due to ease of production, as they were developed prior to the advent of modern molecular biology techniques, which have enabled rapid discovery and engineering of recombinant antibodies. Thus, most current approaches for producing recombinant antibodies rely on humanizing antibodies derived from other species, usually mice, or beginning with human scaffolds engineered into libraries or transgenic "humanized" mice.

Recently, however, novel features of antibodies derived from other species have sparked interest in developing antibodies that may have particular unique features in binding certain antigens or epitopes [3–7]. For example, several heavy chain only antibodies (HCAbs) originally derived from camelids are now in

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clinical trials [8]. Other unique features of antibody paratopes have been found in cows, chickens, and shark. These paratopes may be a reflection of the fact that diseases, and their susceptibility, vary greatly among the various species. This reflects the staunch differences in genetic background, physiology, phylogeny, lifestyle, immune systems, and environment that exist in nature. These differences have clearly driven the evolution of the adaptive immune system. In this chapter we review the genetic and structural features of the antibody system of several diverse species, with an emphasis on those with social or economic importance to humans, but also including unique examples of novel antibody genetics or structure that have been identified in evolutionarily important organisms (Figure 4.1).

Although antibodies maintain a common structure, they exist in various isotypes, which differ in their biological function, structure, and tissue distribution. There are five major immunoglobulin isotypes in mammals: IgM, IgD, IgG, IgA, and IgE. IgM is widely conserved throughout vertebrates, with the potential unique exception of the coelacanth [9]. Additionally, IgM, IgD, and IgA, or its analog, IgX, have been described in nonmammalian tetrapods. Birds, reptiles, and amphibians express IgY, a likely evolutionary precursor to IgG and IgE. More extreme differences are present in some species. An example is the development of unique immunoglobulin isotypes, such as IgX in amphibians or IgW in sharks. Variability also exists in the type and usage frequencies of light chains. In certain species, the κ and λ light chains are utilized equally, while in others one or the other is preferred. The diversity of the immunoglobulin repertoire depends on several factors, and different species have evolved distinct mechanisms to generate antibody diversity. In particular, novel structures have evolved that may have unique functions in binding antigen in different species, such as antibodies devoid of light chains in camelids and sharks, ultralong complementarity-determining region 3 of the heavy chain (CDR H3) antibodies in cows, and unusual isotypes for both heavy and light chains in various organisms (Figures 4.1 and 4.2; Tables 4.1 and 4.2).

4.2 Mammals

4.2.1 Rat and Mouse

4.2.1.1 *Passive Transfer*

A unique feature of mammals is the passive transfer of immunoglobulins from mother to offspring. IgG is transferred from maternal serum into the offspring by the neonatal Fc receptor (FcRn) expressed in placenta and/or the infant intestine. In rats, this occurs both prenatally through the placenta as well as postnatally via the colostrum through the intestines. However, the majority of antibody transport occurs through the consumption of colostrum and milk after birth [10].

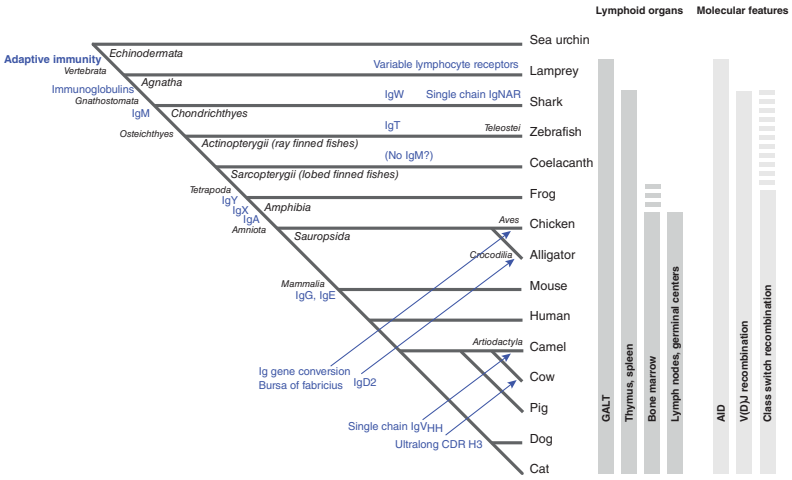

Figure 4.1 Phylogenetic tree of species of immunological interest. Appearance of unique features, isotypes, and binding structures are indicated in blue. Appearance of lymphoid organs and molecular mechanisms involved in antibody diversity are indicated in the bars on the right. AID stands for the activation-induced cytidine deaminase and GALT for gut-associated lymphoid tissue.

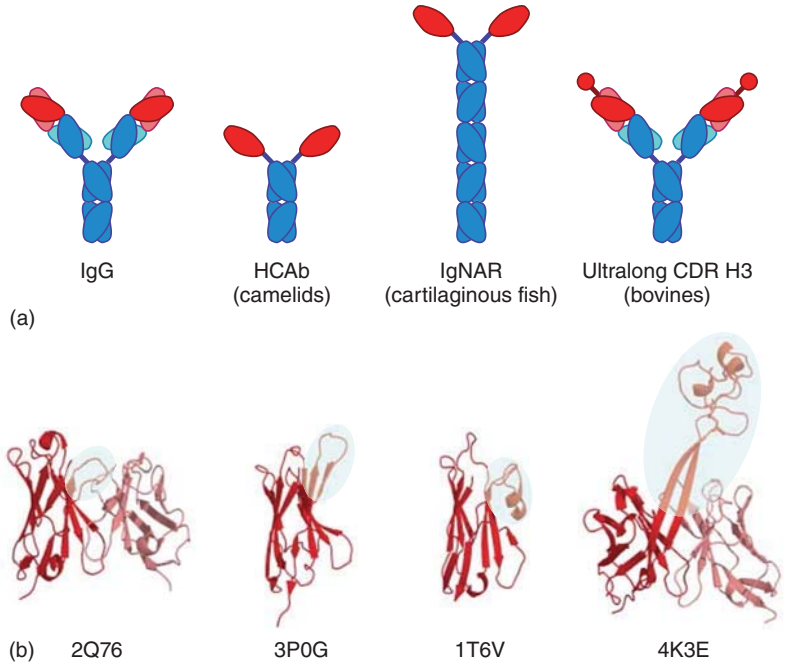

Figure 4.2 Comparison of different species' antibody structures. Common IgG antibody structure found in most species. The heavy chain only antibodies, or HCAbs, are found in camels, which only possess C_H2 and C_H3. The heavy chain only antibodies, IgNAR, are found in sharks, have 3–5 C_H (C_H1–5) regions. The stalk and knob structure found in ultralong cow antibodies. Cartoon (a) of the antibody and ribbon (b) diagrams of the variable region of each antibody type are shown. The CDR H3 regions are highlighted in each ribbon diagram. The PDB codes of the structures are indicated below each graphic.

Table 4.1 Different immunoglobulin isotypes found in each species.

	IgM	IgD	IgG	IgA	IgE	IgD2	IgNAR	IgT	IgW	IgX	IgY	HCAbs
Rat/mouse	X	X	X	X	X							
Cat/dog	X	X	X	X	X							
Pig	X	X	X	X	X							
Cow	X	X	X	X	X							
Camel	X	X	X	X	X							X
Chicken	X			X							X	
Sauropsida	X	X		X		X					X	
Xenopus	X									X	X	
Teleost	X	X						X				
Shark	X						X					

Table 4.2 Presence and use of light chains in various species.

	λ	κ	σ	σ-cart
Rat/mouse	X	X*		
Cat/dog	X*	X		
Pig	X	X		
Cow	X*	X		
Camel	X	X		
Chicken	X*			
Sauropsida	X	X		
Xenopus	X	X		X
Teleost	X	X		
Shark	X	X	X	X

Asterisks indicate known preferential use.

4.2.1.2 Lymphoid System

The immune system of rodents has been very well characterized, as these animals have been major model systems in immunology. As with most mammals, the lymphoid system can be organized into two regions: primary and secondary. The primary lymphoid organs, where lymphocytes are formed and mature, comprise the bone marrow and thymus. The secondary lymphoid organs are the spleen, lymph nodes, Peyer's patches, and mucosa-associated lymphoid tissue. Similar to other mammals, in rodents, the majority of B-cell development occurs in the bone marrow.

4.2.1.3 Antibody Organization

Like in other mammals, antibody diversity in the rat and mouse is created by the combination of heavy and light chain gene segments. The heavy chain locus is comprised of a variable (V_H), diversity (D_H), joining (J_H), and constant region genes. The light chain locus only contains a variable (V_L) and joining (J_L) region plus a constant region. Diversity is generated by the combination of different VDJ and VJ regions in the variable domain genes of the antibody. Addition of non-templated (N) or palindromic (P) nucleotides at the V–D, D–J, or V–J joints and exonuclease-mediated deletions greatly increase the diversity within CDR 3 regions. The constant region encodes the immunoglobulin isotype. The variable regions are encoded by a series of duplicated exons. In rodents, approximately 100 V_H, 4–30 D_H, and 4–6 J_H exons for the heavy chain region and 50 V_L and 4–5 J_L segments for light chains are present [11].

4.2.1.4 Antibody Isotypes

The rodents contain five immunoglobulin isotypes: IgM, IgD, IgG, IgA, and IgE, which are equivalent to their human homologs (Table 4.1) [12]. In the rat, the

IgG isotype contains four subisotypes: IgG1, IgG2a, IgG2b, and IgG2c, while IgG1, IgG2a, IgG2b, and IgG3 are present in the mouse [13]. There is significant homology between these subisotypes in the rat and mouse, but they are not direct homologs to their human counterparts IgG1, IgG2, IgG3, and IgG4. For example, human IgG1 appears to be a functional homolog of mouse IgG2a rather than mouse IgG1. Thus, it is important to remember that rodent IgG1 and human IgG1 are not interchangeable and therefore do not harbor the exact same functionality. Similar to other mammals, only one IgA subtype exists in mice and rats; however, two IgA subtypes, IgA1 and IgA2, exist in humans. In rodents, as well as rabbits, the light chain that is highly preferred is κ (Table 4.2) [14].

4.2.2 Cat/Dog

4.2.2.1 Antibody Organization

Because of the popularity of dogs and cats as companion animals and their excellent ability to serve as models for human diseases, some research on the immune systems of the dog and cat has been performed, yet detailed examination of the genetics and structural biology of their antibodies has not been published. Recent work has provided some information on the immunologenetics of the dog. Eighty variable (V_H) genes, six diversity (D_H) genes, and three joining (J_H) genes have been mapped to chromosome 8 in the dog [15]. The heavy chain locus in the dog appears to be made up of three variable gene families. However, only approximately half of the variable region genes are potentially functional. Additionally, the complementarity-determining region 3 of the heavy chain (CDR H3) in the dog is longer than in the mouse, which averages around 10 amino acids in length, but still shorter than their human counterpart, which averages 15 amino acids in length [16].

4.2.2.2 Antibody Isotypes

The five major isotypes (IgA, IgG, IgM, IgD, IgE) and two forms of light chain (κ and λ) are present in dogs and cats (Table 4.1). In both species, the λ light chain is more commonly utilized (Table 4.2) [11]. In the dog, four subisotypes for IgG have been identified, IgG1–4; however, only three have been recognized in the cat to this point (IgG1–3) [17]. IgA is typically found as a monomer in serum and in a dimeric form called secretory IgA in mucous secretions. Unlike in other species, in the cat, IgA is present as a dimer in serum and mucosa, while in most species IgA is only present as a dimer in its secreted form [18]. In both species, there is little transfer of immunoglobulins across the placenta. The majority of passive immunity occurs via the postnatal consumption of colostrum and milk [19]. Given that both species serve as important model systems in toxicology and infectious diseases, in addition to their role as companion animals, more research is warranted in the basic biology of their antibody systems.

4.2.3 Pig

4.2.3.1 Antibody Organization

Porcine antibody genes are arranged in the same way as in other mammals. Current characterizations of the porcine immunoglobulin heavy chain locus include 15 variable (V_H) genes, 2 functional diversity (D_H) genes, 1 functional joining (J_H) gene, and the constant genes [20–22]. Complementary DNA evidence indicates that additional variable genes may exist upstream from the 15 that are characterized [20]. Additionally, swine appear to only utilize a small number of V_H genes to form the majority of their antibody repertoire [22]. Thus with only 15–17 V_H genes, 2 D_H genes, and 5 J_H genes, the combinatorial potential of the heavy chain is relatively small compared to that of other mammals. A robust antibody repertoire is a result of somatic hypermutation within a small number of V_H genes [22].

Pigs use the κ and λ light chains in equal proportions (Table 4.2) [23, 24]. The porcine κ and λ light chain loci are located on chromosomes 3 and 14, respectively [23, 24]. The kappa locus contains at least 14 variable (V_L) genes, 5 joining (J_L) genes, and a single constant gene. Of the 14 κ V_L genes, 9 are proposed to be functional and can be divided into five families. The lambda locus contains 23 annotated V_L genes arranged in two clusters, while the constant and J_L genes are arranged in four sets of tandem cassettes. The 23 lambda V_L genes can be separated into seven families; IGLVl, IGLV2, IGLV3, IGLV5, IGVL7, IGLV8, and a poorly defined Group III [24, 25]. Ten of these genes appear functional and belong to either the IGLV8 or IGLV3 families. Deep sequencing indicates that there is considerable variation from one individual pig to another, including the absence of the λ V_L genes 3–6 in some animals and truncated versions in others [25]. Interestingly, it has been suggested that antibody gene variation at the population level may compensate for reduced diversity within an individual with regard to pathogen protection [26].

4.2.3.2 Antibody Isotypes

All five mammalian immunoglobulin isotypes are present in the pig (IgA, IgG, IgM, IgD, IgE) (Table 4.1). And, similar to many higher mammals, with the exception of humans and rodents, there appears to be no trans-placental transfer of maternal antibodies [27]. In humans and rodents, IgD cannot be expressed by class-switch recombination. Instead, alternative splicing occurs to produce either the IgD or IgM isotype. However, at the DNA level, class switching to IgD is possible in cattle and perhaps in porcine due to their unique IgD switch region [28].

4.2.4 Cow

An unusual paradigm for creating both genetic and structural diversity in antibodies is present in the cow. Antibodies in the cow can have an unusually

long complementarity-determining region 3 of the heavy chain (CDR H3), which can reach lengths of nearly 70 amino acids long [29–32]. Approximately 10–15% of the repertoire is comprised of these ultralong CDR H3s. On a structural level, these ultralong CDR H3 regions form β-ribbon "stalk" and disulfide-bonded "knob" mini domains (Figure 4.2). In addition to the ultralong antibodies, cows generate a shorter repertoire that is still significantly long compared to other species. The average cow CDR H3 sequence is between 20 and 40 amino acids in length, which, in comparison to the average length in rodents and humans (10–15 amino acids), are still considered unusually long.

4.2.4.1 Antibody Organization

As seen in several ungulate species, cows have a limited number of heavy chain variable (V_H) genes. Twelve V_H regions are thought to comprise one highly homologous heavy chain family [33–35]. There are nine diversity genes and six joining genes [36]. Cows perform V(D)J recombination similar to other species; however, the ultralong subset of cow antibodies appears to preferentially use a single V_H (termed V_HBUL) gene and an ultralong D_H2 gene [37]. Cows preferentially use the λ light chain (Table 4.2) [38]. The genomes of many species (human, mouse, rat, pig, dog, cat, etc.) including the cow have been published [39]. However, the difficulty in assembly and annotation of the heavy and light chain loci leaves open the possibility that additional highly homologous regions can still be discovered.

Unlike in rodents and humans, cows activate somatic hypermutation during development of the primary repertoire, compensating for the limited V(D)J combinatorial diversity [34, 40]. An unusual feature of cow germline D_H regions is that they encode multiple cysteines. In the D_H2 region, used in the formation of the ultralong CDR H3s, amino acids in repeats of Gly-Tyr-Gly or Gly-Tyr-Ser are encoded by the uncommon codons GGT (for Gly) and AGT (for Ser) and TAT (for Tyr). Each of these can be mutated to cysteine with one nucleotide change [37]. Frequent RGYW somatic hypermutation hot spots are present throughout the D_H, which may drive a high frequency of mutation. Thus this D_H region may be easily mutated to cysteine through somatic hypermutation. This was confirmed with deep sequencing, which identified mutations both to and from cysteine in antibody sequences undergoing somatic hypermutation [37]. These results indicate that cysteine mutations can alter the disulfide patterns in the ultralong CDR H3s of the cow, resulting in wholesale changes in loop structures and compositions, suggesting a novel mechanism for structural diversity generation unique to the cow [37].

Wang *et al.* solved the structures of two cow antibody Fab fragments, which contained ultralong CDR H3s [37]. Although the sequences of these two ultralong CDR H3s were highly dissimilar, their structures shared common features. Both structures were composed of a β-ribbon "stalk" and a distal disulfide bonded "knob" that rested upon the stalk. These structural features have never been seen in antibodies derived from other species. Each CDR H3 had six cysteines that were not conserved between their linear amino acid sequences. The structures clearly revealed different disulfide bonding patterns in the two knobs. Despite

the unique "stalk" and "knob" features of the two antibodies, all other attributes were very different. The sequences, disulfide bonding pattern, surface shape and charge, and loop lengths within the knob were dissimilar between the two antibody fragments. The remaining five CDRs and variable region framework regions were almost identical in structure and sequence, indicating that the diversity of these cow antibodies appears to reside solely within the ultralong CDR H3 region. Deep sequencing also revealed that most sequences contained an even number of cysteines at different positions, further strengthening the hypothesis that disulfide bonds, and their associated loops, are an important component of the structural repertoire. Significantly, all of the antigen binding was found to reside within the ultralong CDR H3 "knob" domain in a model antibody [37].

Three more ultralong CDR H3 antibody crystal structures were solved by Stanfield *et al.* [41]. Each contained the characteristic "stalk" and "knob" architecture; however, additional conserved features were identified. Despite having very little sequence conservation in the knob regions, each knob contained a very short three-stranded β sheet. This conserved β sheet is structurally similar to other small disulfide-bonded domains. The closest match was Kalata B1, which plays a role in pathogen protection in plants. A conserved disulfide in the antibody knobs was identified that utilized a germline-encoded cysteine, but the bonding partner cysteine was not conserved. Furthermore, the loops between the strands were different in length and amino acid content, suggesting that the short loops within CDR H3 may be functionally analogous to the CDRs of a traditional antibody.

4.2.4.2 **Antibody Isotypes**

The biological function of ultralong CDR H3 antibodies remains unknown. The heavy chain locus of the cow is unusual compared to those of other mammals, with a duplication that results in two IgM genes [42]. Interestingly, ultralong antibodies seem to be completely associated with rearrangement to the IgM2. The biological roles, if any, of the two IgM genes remain to be determined.

4.2.4.3 **Therapeutic Applications**

Serum-derived bovine immunoglobulin/protein isolate (SBI) is a protein powder composed of immunoglobulin and other serum proteins, similar to those found in colostrum and milk but do not contain any milk products such as lactose, casein, or whey. The use of SBI has been shown to be effective for the prevention and nutritional treatment of childhood malnutrition and gastrointestinal disease, including acute diarrhea and necrotizing enterocolitis. Well-established applications for the use of SBIs include HIV-associated enteropathy and diarrhea-predominant irritable bowel syndrome. The use of SBI could become important components of the treatment regimen for inflammatory bowel disease, conditions associated with the depletion of circulating and luminal immunoglobulins, and in critical care nutrition [43]. Currently, there are several ongoing clinical trials examining the effects of SBI on these diseases. In a similar vein, Avaxia Biologics is investigating polyclonal anti-TNF antisera as an oral formulation for inflammatory bowel disease [44]. Sevion Therapeutics (formerly Fabrus, Inc.)

is now developing recombinant monoclonal humanized cow antibodies with ultralong CDR H3s, with a lead molecule targeting the ion channel Kv1.3, which is a very challenging target for traditional antibodies.

4.2.5 Camel

4.2.5.1 Antibody Organization

Camels possess two populations of circulating antibodies. The first comprises 25% of total circulating antibodies and is composed of the conventional heterotetrameric antibodies with identical heavy chains paired with identical light chains (Table 4.2) [45]. In the second population, termed HCAbs, antibodies are similar to the conventional IgG molecule but have identical heavy chains that lack the C_H1 domain and do not pair with light chains (Figure 4.2). A simple point mutation from G to A that disrupts a consensus splicing sequence may be the cause for HCAbs lacking a C_H1 domain [46]. These HCAbs represent a significant fraction of the Igs in the serum, constituting up to 75%, and are significantly smaller than a conventional IgG molecule. The HCAbs have a molecular weight of about 90 kDa compared to the typical IgG molecular weight of about 150 kDa [47]. Different isoforms of these HCAbs have been identified and are classified by the length of the hinge region sequence between the variable domain and the C_H2 domain. Shorter hinge length isoforms are referred to as IgG3 and the longer hinge regions as IgG2 [47]. Alpacas and llamas also have HCAbs that are very similar to those found in camels [48].

HCAbs have a dedicated variable domain referred to as the V_{HH} domain, which is structurally and functionally similar to a typical IgG F_v fragment. The V_{HH} domains only have three CDR variable loops, which define the antigen binding surface. The CDR H3 of the V_{HH} contains long loops (Figure 4.2b), which may enable V_{HH} regions to interact with and inhibit unusual targets or epitopes not available to the flat binding surface of conventional antibodies, such as enzyme active sites or other recessed crevices [49].

Camel HCAbs share the same gene locus as their conventional IgG tetrameric counterpart [50]. In addition, both HCAbs and IgGs have dedicated variable region genes encoded in germline sequences and undergo classical V(D)J recombination [51]. HCAbs can be encoded by over 30 unique variable region (V_{HH}) sequences, possible unique splicing events of the mRNA, and promiscuous V genes that can produce either V_H (which will also pair with V_L molecules) or V_{HH} domains, each of which can undergo somatic hypermutation to produce further diversity [51].

4.2.5.2 Therapeutic Applications

The HCAbs found in camels are being researched for multiple pharmaceutical applications and have the potential for use in the treatment of acute coronary syndrome, cancer therapies, and Alzheimer's disease. A Belgium biopharmaceutical company, Ablynx, has spearheaded many of these technologies coining the term

"nanobody" when referring to the HCAbs found in camels, llamas, and alpacas. Because of their small size and conformational stability, these antibodies are able to access difficult epitopes, making them excellent targets for diagnostic and therapeutic applications. As an example of their diagnostic capabilities, HCAbs were immobilized on sensor surfaces sensing human prostate-specific antigen (hPSA) and tested. They outperformed the classical antibodies in detecting clinically significant concentrations of hPSA [52]. Therapeutically, HCAbs have been shown to suppress the replication of influenza A virus subtype H5N1 *in vivo* and neutralize cytopathic effects of toxin A and toxin B of *Clostridium difficile* in fibroblasts *in vitro* [53, 54]. Furthermore, several clinical trials have been completed using HCAbs as therapeutics for psoriasis (anti-IL-17A/F), acquired thrombotic thrombocytopenia purpura (anti-von Willebrand Factor), rheumatoid arthritis (anti-IL6R), and rotavirus diarrhea in infants. Additionally, the biopharmaceutical company arGEN-X uses llamas for human therapeutic antibody discovery. arGEN-X is developing a human anti-CD70 antibody and a human antibody against c-Met proto-oncogene, which have entered into clinical trials [55].

4.3 Reptiles

4.3.1 Chicken

4.3.1.1 Lymphoid System

Birds are often classified as a subgroup of reptiles [56]. The chicken is the most primitive vertebrate species with lymph nodes, true IgA, and pronounced affinity maturation. B lymphocytes mature in the bursa of Fabricius, and then migrate to other body tissues. The bursa is a blind sac that extends from the dorsal side of the cloaca, the common portal of the reproductive, urinary, and digestive systems, and then atrophies around the time of sexual maturation. The key role of the bursa of Fabricius in B-cell development was revealed when bursectomized chicks failed to produce antibodies [57, 58].

4.3.1.2 Antibody Organization

Chickens produce a diverse repertoire of antibodies that is unique compared to development of antibodies in humans or mice. Chicken antigen receptor genes undergo a single VDJ recombination event followed by gene conversion utilizing multiple upstream V-region pseudogenes [59]. Rearranged variants of the pseudogenes can further diversify the complementarity-determining region of the heavy chain 3 (CDR H3) by inserting sequence into the D_H region. Interestingly, the gene conversion process is dependent on the activation-induced cytidine deaminase (AID) enzyme [60], the same factor that is required for performing somatic hypermutation and class-switch recombination in several

other species [61]. Framework diversity is limited in chicken antibodies. This is most likely a result of the need for DNA homology, which allows for efficient gene conversion. The potential diversity of creating different CDR combinations and content through multiple theoretical gene conversion recombination events is enormous [62].

As is the case with many species, few detailed structural studies on chicken antibodies have been performed. In repertoire analysis by gene sequencing, Wu *et al.* analyzed the amino acid content of chicken heavy chains [62]. Interestingly, the cysteine content was substantially higher in chicken CDRs (9.4%) than in mice (0.25%) or humans (1.6%). Six families of putatively different disulfide patterns were identified, which may include disulfide bonds within CDR H3, or between CDR H3 and CDR H1 or CDR H2. Tyrosine is an important and abundant amino acid in antibody CDRs [63–65]. This amino acid is found less frequently in chicken CDR H3 (9.2%) compared to humans (16.8%). While, on average, chicken CDR H3s are not longer than most mammals, certain CDR H3s may form longer and unique disulfide-stabilized structures [62]. In structural studies of two chicken scFvs, Conroy *et al.* identified unique canonical classes of CDR L1 and a disulfide-bonded CDR H3 [66]. The data strongly suggests that chickens may have a novel repertoire of paratopes.

4.3.1.3 Antibody Isotypes

In chickens, B lymphocytes produce three classes of antibodies. Chickens have serum IgM, IgA, and IgY, the first two being homologs of their mammalian counterparts; however, they do not have IgE or IgD (Table 4.1) [67]. IgY appears to be related to both mammalian IgG and IgE [59] and may be an evolutionary common ancestor to both [68]. Similar to amphibians, immunoglobulins switch from IgM to IgY. Similar to mammals, IgA is found primarily in the mucus secretions of the eyes, gut, and respiratory tract. In chickens, the light chain repertoire is derived solely from one isotype, which is similar to the λ isotype in other vertebrates (Table 4.2) [69, 70].

4.3.1.4 Therapeutic Applications

Monoclonal antibodies are an essential tool in the treatment of many diseases. However, limitations exist with antigen recognition when mammalian hosts are used because of the evolutionary relationship to humans. The company Crystal Bioscience is working to develop transgenic chickens expressing human antibody genes. This could allow the access of human epitopes that have been difficult in mammalian hosts due to the tolerance to conserved proteins. By the expression of human immunoglobulin variable regions in a chicken DT40 B cell line and further diversification of these genes by gene conversion, Crystal Bioscience has demonstrated that a diverse pool of human antibody sequences in chicken B cells is produced, which suggests that a functional repertoire of chimeric antibodies will be expressed in transgenic chickens [71]. Additionally, others have performed clinical trials examining the effects of IgY on diseases of the alimentary track. Oral administration of IgY has been successfully used to prevent or treat specific diseases including dental caries (*Streptococcus mutans*), infant rotavirus diarrhea, gastritis (*Helicobacter pylori*), and periodontitis (*Porphyromonas gingivalis*) [72–75].

4.3.2 Sauropsida

4.3.2.1 Antibody Isotypes

Reptiles, including avian and non-avian species, evolved in parallel to mammals [76]. The Testudine species is one of the oldest reptile groups in existence, older then the crocodilians. This group of animals provides important insight into evolutionary immunology. A complex immunoglobulin heavy chain locus has been identified in two Testudine species [77]. The heavy chain locus of these species encodes an IgM gene similar to that found in all vertebrates, a gene coding for an IgD immunoglobulin with 11 exons, genes for 7 IgY isotypes, as well as 5 additional immunoglobulin D2 (IgD2) genes [78]. Similarities exist between the IgD2 heavy chain genes and avian IgA, but it does not appear that IgD2 is a true ortholog to IgA; rather, IgD2 and IgA may share a common ancestor similar to the amphibian IgX, further indicating the complexity in the evolution of the immunoglobulins [79].

By studying the full genome sequences, the structure and content of immunoglobulin heavy chain loci has been analyzed from two crocodilian species: *Alligator mississippiensis* and *Crocodylus porosus,* which originated from the evolutionary lineage that led to birds [80]. In the examined loci, IgD2 and IgA encoding genes were found in addition to IgM, IgD, and IgY genes (Table 4.1) [81]. Thus, an ancestral immunoglobulin may have given rise to IgX in amphibians, IgA in reptiles and mammals, and IgD2 in reptiles by recombination, leading to a chimeric IgD/IgA chain. Similar to most mammals, both κ and λ light chains are present in non-avian reptiles (Table 4.2).

Some of what is known about the immunology of crocodilians is that they do possess class switching, somatic hypermutation, and affinity maturation. Yet, their evolutionary history and unusual isotype repertoire begs for further investigation. Merchant *et al.* have presented some enticing data suggesting that there is a wide spectrum of antibacterial, antiparasitic, and antiviral properties of alligator serum [82–85]. Their data suggest that alligators have evolved a high-,affinity humoral immune response.

4.4 Amphibians

4.4.1 Xenopus

4.4.1.1 Lymphoid System, Antibody Organization, and Antibody Isotypes

The major lymphoid tissues in amphibians are the thymus and spleen [86]. As in mammals, the antibody diversity in this species is generated via somatic rearrangement and combinatorial joining of multiple V, D, and J elements within immunoglobulin heavy and light chain loci [87]. There are three isotypes of immunoglobulin heavy chain constant region genes: (i) IgM [88], (ii) IgY, a homolog of mammalian IgG [89], and (iii) IgX, which is preferentially expressed

in the gut and is orthologous and analogous to mammalian IgA (Table 4.1) [90]. Three light chains are present in Xenopus: κ [91], λ [92], and σ (Table 4.2) [93]. The σ light chain is quite different from the λ and κ light chains. The latter two have longer CDR L1s and shorter CDR L2s; whereas, the σ light chains have longer CDR L2s and shorter CDR L1s [94]. Somatic hypermutation and affinity maturation are present in Xenopus. Interestingly, compared to mammals, the antibody serum response is slower in Xenopus than in mammals, being weeks compared to days, which is consistent with other poikilotherms [95].

Because amphibians undergo a unique metamorphosis from the water-bound tadpole to a terrestrial adult, their immune systems undergo a distinctive maturation unlike other vertebrates. Major changes during metamorphosis occur, yet there is a lack of autoimmunity. All three immunoglobulin isotypes, IgM, IgY, and IgX, are present in both the tadpole and the adult. However, different antibody repertoires are present in pre- and post-metamorphosed Xenopus [11]. Sequential rearrangement of the heavy chain variable genes occurs in the developing tadpoles, and by day 13 all heavy chain variable families can be used [96]. In the early stages of tadpole development, all heavy chain diversity and joining genes are randomly expressed. However, by about day 40, certain genes are overexpressed. At approximately day 10, the Xenopus tadpoles are capable of producing specific antibodies. Antibody memory is transferred between the tadpole and adult [97]. Most of the larval and young post-metamorphic immunoglobulin gene samples have a complementarity-determining region of the heavy chain 3 (CDR H3), which is between 3 and 10 acids in length. This is 2 amino acids shorter than the adult CDR H3s, which are between 5 and 12 amino acids long [98]. Amphibians are the most primitive vertebrate with an immunoglobulin isotype switch [99]. There is a switch from IgM to IgY, which can be prevented by thymectomy of Xenopus tadpoles. The switch from IgM to IgX is not hindered in thymectomized tadpoles [100]. This process of class switching in Xenopus is also highly dependent on temperature, and appears to be less efficient at the larval stage. Class switching from IgM to IgY is more pronounced after metamorphosis is complete [101].

4.5 Fish

4.5.1 Teleost

4.5.1.1 Lymphoid System

Bony fish comprise ~96% of the world's fish population with over 20 000 species, while cartilaginous fish, such as the shark, only comprise 3.7%. Jawless fish make up the remaining percentage of fish species in the world [102]. The immunology of the bony fish, teleosts, is similar to that of higher vertebrates with some differences. The major lymphoid organs in this group are thymus, kidney, spleen, and gut-associated lymphoid tissues. The four main mucosal immune compartments

found in bony fish are (i) the gut-associated lymphoid tissue with the lamina propria and intraepithelial compartments, (ii) the skin-associated lymphoid tissue, (iii) the gill-associated lymphoid tissue, which includes the gills and the interbranchial immune tissue, and (iv) the nasopharynx-associated lymphoid tissue [103], which is composed of a diffuse network of immune cells. Similar to cartilaginous fish, they lack lymph nodes and germinal centers.

4.5.1.2 Antibody Isotypes

Immunoglobulins have been identified in several teleost species through the discovery of heavy and light chain genes [104–107]. Similar to cartilaginous fish, four light chains are present in bony fish: κ, λ, σ, and σ-cart (Table 4.2) [94]. The latter two are closely related and are early light chains that differ significantly from the λ and κ light chains, specifically in the variable region [94]. The immunoglobulin heavy chain isotypes in the teleost are IgM, IgD, and IgT (Table 4.1) [108–110]. IgT has been reported in gut mucosal immunity; however, IgT and IgA are phylogenetically distant, suggesting that their similar functions are a result of convergent evolution [111]. IgT (also referred to as IgZ) is found in some, but not all, teleosts. Unlike in mammals, where IgM forms a pentamer, in teleost IgM forms a tetramer by varying the degree of disulfide polymerization of monomer subunits [112]. The prevalent serum immunoglobulin in most teleosts is a high molecular weight (600–850 kDa) antibody corresponding to tetrameric IgM [112]. A low molecular weight immunoglobulin was identified over 40 years ago [113]; however, the molecular nature of this protein remains a mystery. Teleost antibodies are found in the skin [114], intestine [115], gill mucus [116], bile [117], and systemically in the plasma. No class switching at the DNA level occurs in this species, and they lack a secondary response. However, AID is present in both cartilaginous and bony fish, and they are able to undergo somatic hypermutation [118]. In fish, the catalytic domain and carboxy-terminal region in AID differ from those seen in the AID in other species [118]. Recombinational class switching is dependent on switch regions and multiple constant regions, which are lacking in fish. In cartilaginous and teleost fish, different isotype production is driven by the constant regions being dedicated to certain V, D, or J segments, which exist in various arrangements upstream of the constant region [119]. In some cases, the V–D, or D–J rearrangement event can be instructive of isotype lineage commitment [120]. Given the enormous diversity of teleost species, with relatively few studied immunologically at significant depth, it would not be surprising if further unusual characteristics are uncovered.

4.5.2 Shark

4.5.2.1 Lymphoid System

Nearly half a billion years ago, the adaptive immune system evolved in cartilaginous fish (reviewed in Ref. [121]). The major lymphoid tissues are the well-developed thymus and spleen, which in the shark are their earliest

phylogenetic appearance. Because sharks are cartilaginous, they lack bone marrow and lymph nodes; however, they possess unique lymphomyeloid tissues with similar immune functions. These include the epigonal organ [122] that surrounds the gonads, and the Leydig organ [123] that surrounds the esophagus. These organs participate in red blood cell production and immune function. Gut-associated lymphoid tissues are also present in the shark [124].

4.5.2.2 Antibody Organization

Antibody diversity in shark is created through V(D)J recombination of immunoglobulin genes like other jawed vertebrates, and it is one of the earliest vertebrates with this ability (reviewed in Ref. [125]). Their immunoglobulin loci exist in a multiple cluster organizations throughout the genome [126]; yet sharks are still able to utilize class switching as well as haplotype exclusion [127]. A unique feature of cartilaginous fish is that some of the immunoglobulin loci are inherited from their parents in a partially (VD–J) or completely (VDJ) joined state [128]. The fusion of V, D, and J elements in the germline, as opposed to a somatically developing lymphocyte, may be a product of gonadal RAG expression in sharks [129]. RAG expression has been demonstrated in shark gonad, explaining how some of the many shark immunoglobulin loci can become V(D)J rearranged in the germline. One specific germline-joined IgM locus is preferentially used in young sharks [130]. Although shark IgH arise from simpler loci with fewer elements, sequence differences between the multiple genomic loci in the shark "multi-cluster" (as opposed to translocon of tetrapods) organization and junctional diversification by non-template and palindromic additions produce a repertoire thought to be as diverse as other vertebrates [131, 132].

In cartilaginous fish, like shark, the immunoglobulin new antigen receptor (IgNAR) is a unique heavy chain-only antibody. In young sharks, low serum levels of IgNAR are present. These serum levels slowly rise during the shark's first year of life. The affinity maturation of the molecule has suggested that this isotype serves a similar role in the shark's immune system as the mammalian IgG does in mammals and that it may be T-dependent [133, 134]. A memory response by the IgNAR isotype is characterized by specific antigens and is clearly present in the nurse shark [135]. Additionally, upon antigen exposure, IgNAR is a target of significant somatic hypermutation, leading to affinity maturation [136].

Surface IgNAR has one amino terminal variable domain. This single variable domain antibody's general quaternary structure has independently evolved at least twice in the vertebrate natural history: once in cartilaginous fish, and again in camelids (Figure 4.2) [137]. IgNAR also contains three or five constant domains determined by alternate splicing. IgNAR commonly exists as a monomer, but it has been found to multimerize in some species such as the spiny dogfish [138]. Similar to the structure found in C1-type immunoglobulin superfamily domains, the complementarity-determining region 2 of the heavy chain (CDR H2) in IgNAR can form a belt around the side of the domain instead of projecting away from the constant domain with the other CDR [139]. Additionally, IgNAR CDR H2 can have selected hypermutations and can be important for antigen binding [140].

The IgNAR variable region is 12 kDa, making it the smallest antigen-binding variable region known in the animal kingdom [141].

4.5.2.3 Antibody Isotypes

Significant differences exist between the shark and mammalian immunoglobulin isotypes (Table 4.1). IgM is found in sharks and nearly all jawed vertebrates, with the coelacanth being the only known exception [9]. IgW is present in sharks and is orthologous to IgD of other vertebrates. Good evidence exists for an unusual form of class-switch recombination between IgM and IgW [142]. Lastly, IgNAR is a lineage-specific isotype of cartilaginous fish that does not associate with light chains (Figure 4.2) [143]. However, in cartilaginous fish, IgM and IgW have four light chains to pair with: κ, λ, σ, and σ-cart (Table 4.2) [94]. Currently it is unclear whether the additional light chains serve to enhance diversity of the immune repertoire or whether they have evolved for other specific functions.

4.5.2.4 Therapeutic Application

Because of its small structure and binding ability, the single variable domain from the IgNAR represents an opportunity to bind different epitopes than traditional antibodies. Ossianix Inc. is targeting several different targets with their VNAR platform. Utilizing the transferrin receptor, Ossianix Inc. has also demonstrated that they can transfer their VNAR antibodies across the blood–brain barrier [144].

4.6 Conclusions

Significant differences in antibody genetics, structure, and function exist across species. These distinctions may provide a clue to evolutionary relationships as well as to the pathogens that may have provided selection pressure on the immune system. Evolution has led to incredible diversity among the immune systems of modern species, yet comparatively little research has been done to provide an understanding of the unique differences. Interestingly, IgM is widely conserved among vertebrates, with the interesting exception of the coelacanth. The majority of mammals possess the five major immunoglobulin isotypes IgM, IgD, IgG, IgA, IgE; however, unique immunoglobulin isotypes and structures are also present in other species (Table 4.1). Although the cow contains all five major immunoglobulin isotypes, within these isotypes is a subset of ultralong antibodies that comprise a “stalk” and “knob” domain structure not found in other species [37]. Camels and sharks provide insight into convergent evolution. In cartilaginous fish and camels, the single variable domain antibody structure appears to have independently evolved in each species [137]. The immunoglobulin IgNAR in sharks is encoded at dedicated loci, and camels use an IgG variant that has evolved to encode certain structural modifications; yet, both antibody types lack an associated light chain [47]. An additional example of convergent evolution is

present in teleost. Bony fish lack IgA yet possess the mucosal antibody IgT [111]. In avian species, which lack IgG, IgD, and IgE, the IgY isotype appears to be evolutionarily related to both mammalian IgG and IgE [59]. In ancient reptiles, similarities exist between IgD2 and avian IgA; however, it does not appear that IgD2 is a true ortholog to IgA, but rather IgD2 and IgA may share a common ancestor similar to the amphibian IgX [79]. Additionally, Xenopus contain three light chains (κ, λ, σ) as opposed to κ and λ found in most species (Table 4.2) [91–93]. Yet, in teleosts and sharks, a fourth immunoglobulin light chain has been described, σ-cart, that is an ortholog of the amphibian σ light chain [94]. While the function of some of these unusual constant regions, like IgY and IgT, can be surmised, others like IgD2, IgW, and so on, have not been studied well. The function of these unusual constant regions perhaps could shed light on new mechanisms of disease resistance or pathogen removal in these species.

In addition to the differences in isotype evolution, some species like camel, shark, cows, and perhaps chickens have evolved antigen-binding structures that are atypical compared to traditional antibodies derived from mice or humans. Camelid antibodies have been produced with novel paratopes targeting G-protein-coupled receptors (GPCRs). Given the small size and protruding nature of the antibodies devoid of light chains (camels and sharks), these antibodies may have evolved the ability to bind concave epitopes. Similarly, the ultralong CDR H3s of cows provide a remarkably protruding paratope, suggesting that these may also "reach" into cavities or crevices within an antigen surface. Further detailed study on the antigens and epitopes bound by these novel structures could shed further insight into the evolutionary drivers behind their selection. Given the importance of antibodies as research reagents, diagnostics, vaccines, and recombinant therapeutics, unusual antibodies derived from more exotic species might provide an important niche for targeting antigens that are challenging for traditional antibodies derived from other species.

The complexity of the evolution of the immune system is remarkably vast throughout nature. Significant diversity exists in isotype evolution as well as in the genetic and structural properties of antibody binding regions. Remarkably, relatively few species have been studied in detail in this regard, yet many unusual and interesting antibody properties have already been uncovered. The differences and biologically unique approaches to immune defense may provide answers to some of today's most challenging diseases.

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