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The Immunoglobulins: New Insights, Implications, and Applications

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Abstract

Immunoglobulins (Igs), as one of the hallmarks of adaptive immunity, first arose approximately 500 million years ago with the emergence of jawed vertebrates. Two events stand out in the evolutionary history of Igs from cartilaginous fish to mammals: (*a*) the diversification of Ig heavy chain (IgH) genes, resulting in Ig isotypes or subclasses associated with novel functions, and (*b*) the diversification of genetic and structural strategies, leading to the creation of the antibody repertoire we know today. This review first gives an overview of the IgH isotypes identified in jawed vertebrates to date and then highlights the implications or applications of five new recent discoveries arising from comparative studies of Igs derived from different vertebrate species.

1. INTRODUCTION

Somatic hypermutation (SHM): mutation in V-region DNA of rearranged Ig gene to produce Igs with subtle difference in specificity and antigen affinity

Isotype: also known as class, H or L chain defined in terms of the type of constant region it has

Cμ: C region of the H chain of IgM or the gene encoding the C region of the H chain of IgM

Class-switch recombination (CSR): a nonhomologous gene recombination in activated B cells that replaces the *Cμ* gene with a different CH gene

The adaptive immune system first evolved at the dawn of the vertebrates, approximately 500 million years ago, through the rearrangement of antigen receptors. In contrast to jawless vertebrates, which assemble their variable lymphocyte receptors (VLRs) via the differential insertion of leucine-rich repeat cassettes into an incomplete germline VLR gene (1, 2), jawed vertebrates have evolved a very different strategy to generate considerably more diverse repertoires of antigen receptors. This strategy depends on the recombination-activation gene (RAG)-mediated rearrangement of the variable (V), diversity (D), and joining (J) segments within the immunoglobulin (Ig) or T cell receptor (TCR) gene loci (**Figure 1**), followed by the activation-induced cytidine deaminase (AID)-mediated somatic hypermutation (SHM) of the rearranged V(D)J region. Over the years, diverse Ig isotypes have been characterized extensively in different vertebrate species. New and exciting information regarding the diversity of Ig isotypes and the genes that encode them is rapidly emerging from research on organisms other than humans and mice. These new insights not only have extended our understanding of how flexible the Igs are in defending the host against pathogens but also have potential applications in many other areas of research. In this review, we first summarize the Ig heavy chain (IgH) isotypes identified in jawed vertebrates and then focus on several major findings recently derived from comparative studies of Igs.

2. THE IgH ISOTYPES IDENTIFIED IN JAWED VERTEBRATES

Cartilaginous fish are phylogenetically the oldest living animals that employ Igs as part of their adaptive immune response. They express two conventional IgH isotypes, IgM (μ) and IgW (orthologous to IgD), and a lineage-specific isotype, IgNAR, which is a heavy chain homodimer that naturally does not associate with Ig light (L) chains (3) (**Table 1**). The IgH genes are arranged in hundreds of clusters dispersed throughout the genomes of cartilaginous fish. Each cluster represents an individual isotype and consists of a V segment, several D segments, and a J segment, as well as a constant (C) region gene (4) (**Figure 2**).

In bony fish, the cluster organization of IgH loci, a characteristic of cartilaginous fish, has been replaced with a translocon configuration. Three IgH isotypes, IgM, IgD (δ), and the specialized mucosal antibody IgT (τ), have been identified in the ray-finned bony fish species studied to date (**Table 1**). In some species, such as zebrafish, the IgH loci show an archetypic architecture, in which the *Dτ-Jτ-Cτ* cluster is located between the VH gene cluster and the *Dμ/δ-Jμ/δ-Cμ-Cδ* locus, which resembles the mammalian *TCRα/δ* locus (5) (**Figure 2**). Owing to successive genome duplications and gene deletions, multiple IgH loci variants have been successively identified in other bony fish species (reviewed in 6) (**Figure 2**). However, the IgH isotypes expressed in the few extant species of lobe-finned bony fish seem to be different from those found in ray-finned bony fish. For instance, no τ ortholog has been identified in the African lungfish (*Protopterus annectens* and *Protopterus aethiopicus*), and both the *Cμ* and *Cτ* seem to be absent in the African coelacanth (*Latimeria chalumnae*) (7, 8) (**Table 1**).

Compared with fish, tetrapods have a single IgH locus, arranged in a typical translocon organization, which contains multiple tandemly organized VH, D, and JH segments, followed by a series of C genes, encoding diverse H chain isotypes or subclasses associated with different effector functions (**Figure 2**). This organization of the IgH locus enables a B lymphocyte to replace its IgM with downstream Ig isotypes, equipped with identical antigenic specificities but different effector functions, in a phenomenon termed conventional class-switch recombination (CSR). Like typical amphibians, clawed frogs (*Xenopus*) express five H chain isotypes: IgM, IgD, IgX (χ), IgY (υ), and IgF (9) (**Table 1**). IgX, which is orthologous to IgA, is the major mucosal isotype employed by amphibians. Meanwhile, IgY is the predominant serum antibody used for systemic immunity and

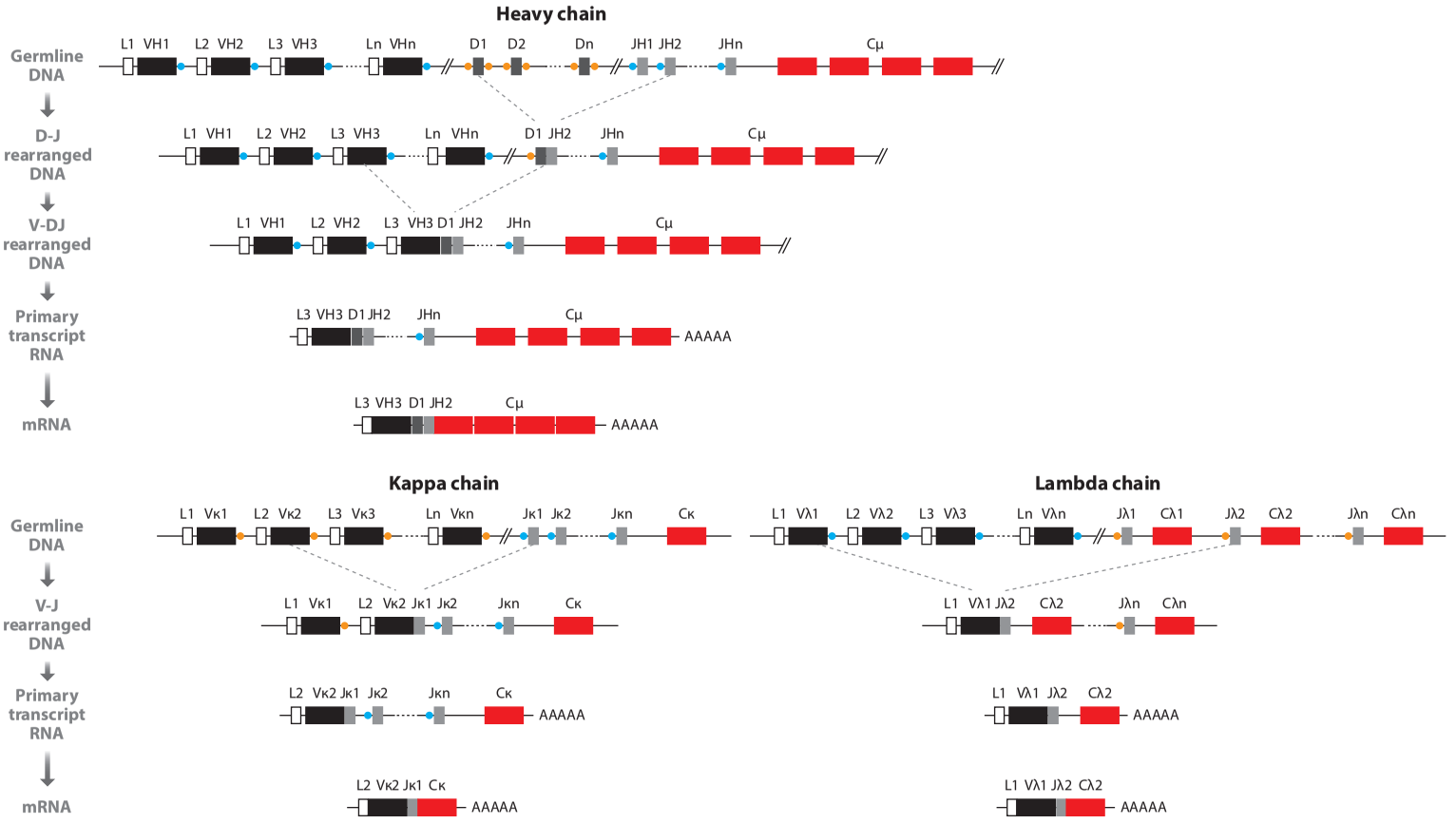


Figure 1

Schematics of V(D)J recombination and expression of the IgH and IgL (κ and λ) loci. V(D)J recombination is a process to assemble the functional variable exons of the Ig gene from different germline gene segments during B lymphocyte development. The germline IgH locus contains multiple copies of V (*black boxes*), D (*dark gray boxes*), and J (*light gray boxes*) gene segments, and the D gene segments are absent from the germline DNA of the IgL (κ and λ) loci. Each V gene segment is preceded by an exon encoding a leader peptide (L, *hollow boxes*). V(D)J recombination is directed by recombination signal sequences (RSSs), which flank the coding sequences of V, D, and J gene segments. An RSS is composed of conserved heptamer and nonamer sequences separated by a non-conserved spacer of 12 or 23 bp. The RAG proteins can recognize the RSSs and mediate the D-JH and VH-DJH rearrangements in the IgH locus or VL-JL rearrangement in IgL loci according to the 12/23 rule, which means a gene segment flanked by an 23-bp RSS (*blue circles*) can be joined only to one flanked by a 12-bp RSS (*orange circles*).

Table 1 IgH isotypes in the different vertebrate classes

Vertebrate class		IgM	IgD/W[a]	IgT[b]	IgX	IgA[c]	IgY	IgG	IgE	Others[d]
Cartilaginous fish		+	+	–	–	–	–	–	–	IgNAR
Bony fish	Ray-finned bony fish	+	+	+/–	–	–	–	–	–	–
	Coelacanth	–	+	–	–	–	–	–	–	–
	Lungfish	+	+	–	–	–	–	–	–	–
Amphibians		+	+	–	+	–	+	–	–	IgF
Reptiles	Lizards	+	+	–	–	?	+	–	–	–
	Snakes	+	+	–	–	–	+	–	–	–
	Turtles	+	+	–	–	–	+	–	–	–
	Crocodiles and alligators	+	+	–	–	+	+	–	–	–
Birds		+	+/–	–	–	+	+	–	–	–
Mammals		+	+/–	–	–	+	–	+	+	IgO

[a]IgD2 has been found in some lizard, turtle, and crocodile species.
[b]+/– indicates the corresponding isotype has been absent in some species of the corresponding vertebrate taxa.
[c]? indicates that an IgA-like gene has been identified only in the leopard gecko but not in other lizard species so far.
[d]IgH isotypes that are present only in particular vertebrate taxa or species.

is considered to be the precursor to mammalian IgG and IgE (discussed in Sections 5.1 and 6.1). IgF is the only IgH isotype known to contain a separately encoded hinge region in nonmammalian vertebrates. However, IgF is also termed a dead-end isotype, because it has been identified only in Western clawed frog (*Xenopus tropicalis*) and not in urodeles (9).

Amniotic vertebrates are a clade of tetrapods that can be subdivided into the reptilian lineage (composed of birds and reptiles) and mammals. For decades, IgD was thought to be absent in birds. However, based on genome and transcriptome data, Han et al. (10) recently showed that the *Cδ* gene is absent only in certain bird species, such as chickens and ducks (**Figure 2**). Therefore, four IgH isotypes, IgM, IgD, IgY, and IgA (α), have been found in birds and reptiles to date (**Table 1**). Furthermore, the subclass diversification of *Cμ*, *Cυ*, or *Cα* genes was reported to have occurred in some species of birds and reptiles (10–19) (**Figure 2**).

In contrast with the reptilian lineage, the genomic organization of IgH loci, as well as the structural features of its associated isotypes, has been studied extensively in extant species of mammals. Most mammals express five IgH isotypes: IgM, IgD, IgA, IgG (γ), and IgE (ε), whereas the duck-billed platypus expresses another dead-end isotype, IgO (discussed in Section 5.1) (20) (**Table 1**). The continuing diversification of the mammalian IgH loci is evidenced by the differences in the number of IgG and IgA subclasses (**Figure 2**) and in the structures of IgD, IgA, and IgG, in which the CH2 domain has been replaced with a short and flexible hinge region. Similar to those of IgM, IgT, IgY, IgX, and the primitive IgA, the H chain of IgE has retained the four-CH-domain structure. Notably, the *Cδ* gene has been either pseudogenized or lost in several mammals, including rabbits, opossums, camels, elephants, and guinea pigs (21–25).

Hinge region: a flexible domain that lies between Fab and Fc regions, allowing for some degree of movement of two Fab arms

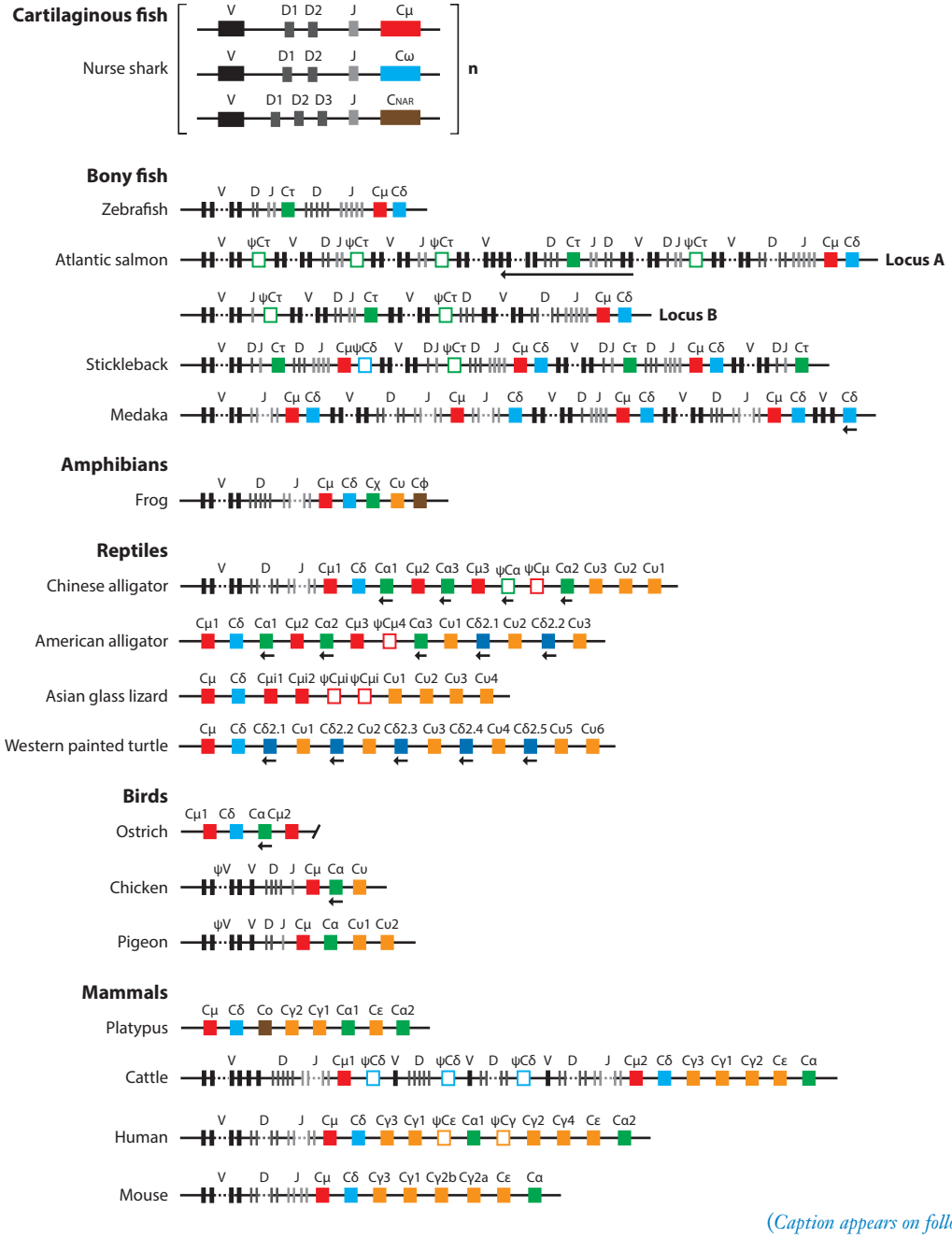
3. IgM: THE PRIMORDIAL ISOTYPE

3.1. An Overview of IgM Structure and Function

As the primordial isotype, IgM has been found in nearly all jawed vertebrates examined to date, with the exception of the coelacanths. The four-μCH-domain structure of IgM is highly conserved. In bony fish, however, the transmembrane IgM shows a remarkable diversity of splicing

patterns, including only two or three μCH domains, the functional reasons for which are unknown (reviewed in 6). In tetrapods, IgM is the first isotype expressed during B cell development and the immunological response against cognate antigens. The transmembrane IgM exists as a monomer and forms the B cell receptor (BCR), the expression of which is essential for both B cell survival and the subsequent expression of other IgH classes (as exemplified by the IgD-BCR, discussed in

CH domains: protein domains that make up the C regions of each H chain



(*Caption appears on following page*)

Figure 2 (*Figure appears on preceding page*)

Schematic structure of the genomic organization of IgH loci in representative vertebrate species. The schematics are not drawn to scale and depict only the genomic configuration of V (*black boxes*), D (*dark gray boxes*), J (*light gray boxes*), and CH gene segments. The genomic regions containing V, D, and J gene segments are not shown in American alligator (*Alligator mississippiensis*), Asian glass lizard (*Ophisaurus gracilis*), Western painted turtle (*Chrysemys picta bellii*), ostrich, and platypus. Except for chicken and pigeon, functional V and pseudo V (ψV) segments are not distinguished in schematics of other species. Functional J (or D) and pseudo J (or D) segments are not distinguished in schematics of all species. Different CH genes are represented as different colored boxes: *Cμ* (*red*), *Cδ/ω* (*blue*), *Cδ2* (*dark blue*), *Cα/τ/χ* (*green*), *Cυ/γ/ε* (*orange*), and *Cϕ/o/NAR* (*brown*). The pseudo CH genes are indicated by a ψ and represented as hollow boxes with corresponding colors. The gene segments with the opposite transcriptional orientation to the whole gene locus are indicated as an arrow. Asian glass lizard IgH: *Cμi* indicates the duplications of the *Cμ* gene; ostrich IgH: *Cυ1* and *Cυ2* genes have not been mapped to this locus because they are located very far downstream of the *Cμ2* gene.

Section 4.2.1). Secreted IgMs (sIgMs), including natural and immune (antigen-specific) IgMs, are predominantly expressed as multimeric forms, which endows sIgM with an extraordinary ability to multivalently bind antigens, receptors, and complement (C′). The sIgMs of bony fish exist primarily as tetramers (not associated with the J chain), whereas tetrapods most commonly employ pentameric or hexameric forms of sIgM (26) (**Figure 3**). The sIgMs of cartilaginous fish exist in both multimeric and monomeric states (**Figure 3**). However, it is the sIgM monomers rather than the multimers that seem to contribute most prominently to the specific, antigen-driven response of cartilaginous fish (26, 27).

In humans and mice, sIgM is involved in a variety of pathophysiologies, including infection, B cell homeostasis, inflammation, atherosclerosis, and autoimmunity (reviewed in 28 and 29 and not addressed further in this review). At present, the functions of IgM in nonmammalian species have not been systematically investigated. As the major serum isotype in both bony and cartilaginous fish, IgM may have evolved to fulfill cognate-comparable functional diversity that is achieved by the complementary functions of different isotypes, as seen in other vertebrates. For instance, the IgM of fugu (*Takifugu rubripes*), like the mammalian IgG/E, can bind to basophils and induce their degranulation (30).

3.2. The Coexistence of Multiple *Cμ* Genes in the IgH Loci of Some Tetrapod Species

Multiple *Cμ* genes are found in the IgH loci of a limited number of tetrapod species, including crocodiles (*Crocodylus siamensis* and *Crocodylus porosus*), alligators (*Alligator sinensis* and *Alligator mississippiensis*), Squamata species, ostriches, and emus, and in cattle (the only recorded case among mammals to date) (10, 11, 15, 17, 18, 31) (**Figure 2**). In some aforementioned species, the downstream *Cμ* genes can be expressed via CSR (11, 31). However, the roles of these IgM subclasses in B cell differentiation and the antibody response remain unclear. Quantitative real-time polymerase chain reaction analyses revealed that the downstream *Cμ* genes were expressed at much lower levels than the *Cμ1* gene in various crocodile and ostrich tissues (10, 11).

Complement (C′): a collection of plasma proteins that interact with each other to active different pathways to kill pathogens

J chain: a polypeptide chain that promotes polymerization of IgM or IgA by linking to cysteine in the tailpiece of a μ or α chain

However, in cattle, the downstream *Cμ2* gene was predominantly expressed in most tissues throughout the different developmental stages (31). The preferential expression of the *Cμ2* gene is probably due to the specific organization of the bovine IgH chain gene locus. Because both the *Cμ1* and *Cμ2* genes contain D and J segments in their respective upstream sequences, independent D–J recombination events may occur when the two genes are expressed. However, a functional *Cδ* gene is found downstream of only the *Cμ2* and not the *Cμ1* gene (**Figure 2**). Thus, only when the *Cμ2* gene is expressed first can both the surface IgM and IgD be dually expressed by alternative splicing of a precursor mRNA on antigen-naïve mature B cells, promoting B cell survival

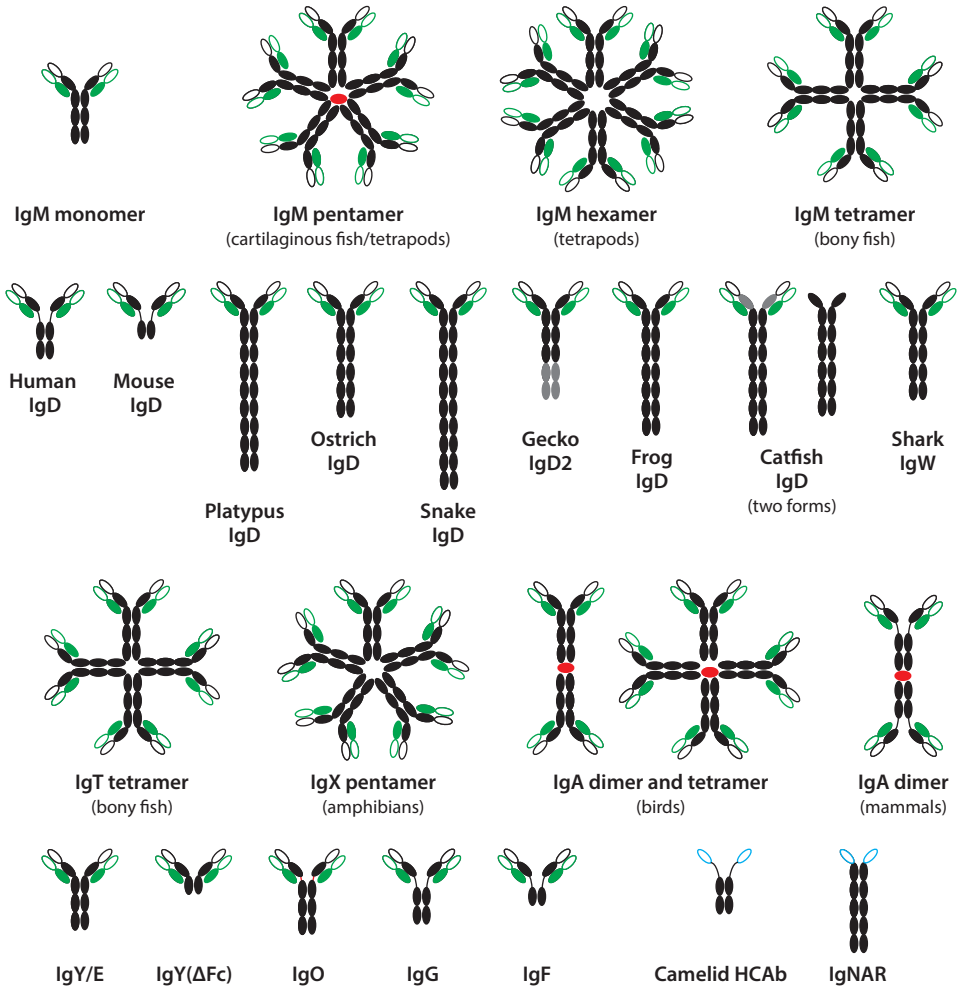


Figure 3

Schematic representation of the domain architectures of all Ig isotypes. The conventional VH, VL, CH, and CL domains are represented by black hollow ellipses, green hollow ellipses, black ellipses, and green ellipses, respectively. The VHH domain of camelid HCAb and V-NAR domain of IgNAR are shown with blue hollow ellipses. J chains are indicated by red ellipses and hinge regions by connecting lines. All disulfide bonds, including those between two H chains or those linking monomers to the J chain as well as to each other, are not shown. Owing to the marked structural plasticity of IgD/W, only a few representative structures of IgD/W are shown here. The IgA-like domains (CH5 and CH6 domains) of IgD2 and the μCH1 domain of the catfish IgD are indicated by gray ellipses. The hinge regions of IgO are emphasized in red, because this hinge region is encoded by the 5′-terminal sequence of the *oCH2* exon but not encoded by a separate exon as those of IgG.

(discussed later in Section 4). Interestingly, the bovine *Cμ2* can be expressed either through independent V(D)J recombination or through CSR by the recombination between switch region *μ1* (*Sμ1*) and *Sμ2*. Furthermore, compared with the third complementarity-determining region of the heavy chain (CDR H3) of IgM1, the CDR H3 of bovine IgM2 not only is on average longer but also displays a much greater length variability (31 and discussed in Section 7.2). Because the Fc region sequences of IgM1 and IgM2 are almost identical (99.2% amino acid homology), the

Switch region: stretch of repetitive DNA located upstream of each C region gene that functions in CSR

major functional difference between the secreted forms of these two IgMs seems to lie within their V regions, which exhibit different binding preferences for various antigenic epitopes. It is highly likely that the bovine IgM2, expressed via CSR, would hold an advantage over IgM1, in terms of both its higher antigen affinity (via SHM) and its avidity. The functions of the class-switched IgM2 would therefore merit considerable attention.

CDR H3: the most varied portion of the Ig molecule that is derived from DNA rearrangement of V, D, and J gene segments

In contrast to those in cattle, a comparison of the Fc regions of multiple *Cμ* genes derived from both the Siamese crocodile and the ostrich yielded less than 80% shared amino acid sequence identity (10, 11). This implies that the structural variation within the IgM Fc regions may offer a means of achieving diverse effector functions with a single isotype. For example, the Fc regions of different IgM subclasses may specifically bind to different IgM receptors or may bind the same receptor with different affinities. Transcriptome studies of the leopard gecko (*Eublepharis macularius*) also found that IgM2/3 are expressed only in the lung and the intestine, sites of the mucosal immune response, implying that these additional IgM subclasses may act as antibodies during the secondary immune response (17). Because reptiles seem not to form germinal centers in their secondary lymphoid tissues, and because the antigen-specific antibody titer does not increase following a second exposure to the same antigen (32), we can speculate that the subclass of crocodile IgM expressed via CSR may not undergo classical affinity maturation. This process is dependent upon the antigen-driven selection of the somatically hypermutated V(D)J repertoire, although evidence for SHM has been found in the variable regions of Ig genes belonging to reptiles (32). Interestingly, under denaturing and nonreducing conditions, the crocodile IgM1 is present as both pentamers and hexamers, whereas IgM2 exists as a tetramer, the sIgM polymer adopted by bony fish (11). In bony fish, antigen-sensitive B lymphocytes can modulate the level of sIgM disulfide polymerization to optimally tailor their function to the level of antigen affinity. This affinity-driven polymerization seems to be a unique feature of the IgM isotype (33, 34). It is therefore possible that a comparable situation may exist in the subclasses of crocodile IgM.

4. IgD: THE MOST PLASTIC ISOTYPE PRESENT IN ALL CLASSES OF JAWED VERTEBRATES

4.1. The Structural Plasticity of IgD

Similar to IgM, IgD is present in all vertebrate lineages. In the species possessing translocon IgH loci, transmembrane IgD or IgD-BCRs are coexpressed alongside IgM-BCRs through alternative splicing of a precursor mRNA, composed of V(D)J, *Cμ*, and *Cδ* fragments. In humans and mice, the IgD-BCR emerges during the latter phase of B cell ontogeny, mostly at the transition to the mature B cell stage. In these same organisms, after encountering antigen in the secondary lymphoid organs, a small subset of B cells undergoes CSR (via both the alternative nonhomologous end-joining and the homologous recombination pathways) to express secreted IgD (sIgD) through the recombination between the *Sμ* and a noncanonical switch-like region *σδ* (35, 36). Interestingly, the $IgD^{+}IgM^{-}$ B cells, identified in rainbow trout, express sIgD through a novel strategy in which the splice site at the end of the last *Cδ* exon is ignored and transcription continues into the intron until a stop codon is reached, resulting in a secretory tail (37).

In contrast to IgM, IgD displays marked structural and functional plasticity (**Figure 3**). The structural plasticity of IgD arises from the following three features: (*a*) varying numbers of germline *δCH* exons, (*b*) various splicing forms, and (*c*) varying copy numbers of *Cδ* genes. The *Cδ* gene of placental mammals possesses no more than three *δCH* exons, whereas the number of *δCH* exons varies from 4 to 11 in the platypus and other tetrapods (9–11, 14–18, 20, 38–40). In fish, this variation is even greater owing to the frequent duplication and deletion of certain *δCH*

exons (reviewed in 6). The longest-expressed *Cδ* transcript, composed of 19 *δCH* exons, has been cloned from a Siberian sturgeon (*Acipenser baerii*), although the genomic structure of the *Cδ* gene has not yet been determined for this species (41). In addition to the varying numbers of *δCH* exons, extensive alternative RNA splicing events also contribute to the heterogeneous *Cδ* transcripts found in most jawed vertebrate species, an event that occurs relatively rarely in mammals (7, 11–13, 41–44). Interestingly, alternative splicing also generates chimeric IgD H chains containing a μCH1 domain, followed by several δCH domains, which has been observed in ray-finned bony fish and pigs (6, 45) (**Figure 3**). Another unusual VH-less IgD transcript, in which the leader is spliced directly to the δCH1 domain, has been identified in channel catfish (*Ictalurus punctatus*) and spiny dogfish (*Squalus acanthias*), suggesting a completely different effector function for this isoform (46, 47) (**Figure 3**). In some bony fish species, such as catfish, medaka (*Oryzias latipes*), and stickleback (*Gasterosteus aculeatus*), multiple copies of *Cδ* genes, each located downstream of a *Cμ* or pseudo *Cμ*, were arranged in tandem within the IgH locus owing to duplications of large DNA regions (reviewed in 6) (**Figure 2**). A variant of IgD, designated IgD2, has been found in the leopard gecko, the Chinese crocodile lizard (*Shinisaurus crocodilurus*), and various turtle and crocodile species but seems to be absent from other Squamata species (**Table 1** and **Figure 2**). IgD2, which, just like other isotypes, is most likely expressed via CSR, appears to have arisen via duplication of the *Cδ* gene and subsequent recombination with a *Cα*-like gene (15, 16, 18, 38) (**Figure 3**).

Based on the presented evidence, we can speculate that the different IgD structures observed in a variety of species may execute specific effector functions by adapting to the specific immune environments that each species or genus is confronted with. As mentioned in Section 2, the *Cδ* gene is present in all classes of jawed vertebrates and missing only in some species. The evolutionary conservation of the *Cδ* gene highlights the need for a deeper exploration of the role of IgD in B cell development and the wider immune response. Indeed, some novel functions of IgD, which are discussed in the next section (Section 4.2), have recently been discovered in humans and mice.

4.2. The Functional Plasticity of IgD

Despite its evolutionary conservation from cartilaginous fish to mammals, IgD remains an enigmatic antibody isotype, because its biological functions have only recently begun to be elucidated. Recent studies suggest that IgD plays a role in various aspects of humoral immunity in human and mice. These advances are briefly discussed below.

4.2.1. The role of the IgD-BCR in B cell development and self-tolerance. Earlier studies have suggested that a broad functional overlap exists between the IgM- and IgD-BCRs. A comparison of mice expressing either IgM or IgD alone revealed that IgM- and IgD-BCRs were equally capable of supporting (*a*) normal B cell development, (*b*) the initiation of immune responses to T-dependent and T-independent antigens, and (*c*) the induction of self-tolerance in vivo (48–51). However, the expression pattern of the IgD-BCR differs significantly from that of the IgM-BCR. IgM-BCR expression begins immediately after the completion of light chain rearrangement in the bone marrow and persists until the initiation of CSR, following B cell activation in the periphery. IgD-BCR expression, in contrast, starts at the transitional B cell stage and progressively increases until it reaches peak levels on mature naïve B cells, as a result of alternative *Cμ* and *Cδ* mRNA splicing. This suggests that IgD may be pivotal to mature naïve B cell function. In recent years, increasing evidence has suggested that the co-expression of the IgM- and IgD-BCRs on the surface of mature naïve B cells contributes to the regulation of B cell selection and self-tolerance mechanisms. In a reporter mouse model, in which BCR responsiveness was monitored

by a specific readout for antigen-dependent BCR signaling in vivo (Nur77-eGFP), the majority of mature B cells in the spleen had experienced varying degrees of stimulation by endogenous antigens. Moreover, this self-antigen exposure tuned the responsiveness of BCR signaling by selectively downregulating IgM-BCRs but not IgD-BCRs (52). Indeed, multiple studies in mice and humans have proved that the $IgD^{high}IgM^{low}$ phenotype is a common feature of naturally occurring autoreactive B cells, which maintain functional unresponsiveness or anergy in the mature B cell pool (53–55). Anergic B cells were analyzed in three mouse models: (*a*) mice lacking IgD; (*b*) mice with a missense mutation in IgD; and (*c*) mice with an inactive IgD-splicing factor, Zfp318. The authors found that the predominant expression of the IgD-BCR, combined with the downregulation of the IgM-BCR, plays a crucial role in attenuating the response to self-antigen and promoting an accumulation of mature anergic B cells in the periphery (56). In addition, a more recent study using Nur77-eGFP to monitor BCR signaling in mice that express either IgM- or IgD-BCRs provided direct evidence that the IgD-BCR is less sensitive than the IgM-BCR to bona fide endogenous antigens in vivo (57). This defect is evidenced by the fact that IgD-only B cells favored marginal zone cell development, which needs relatively weak BCR signals, but disfavored B1a cell development, which requires stronger BCR signals (57). This raises the question of why the IgD-BCR is less efficient than the IgM-BCR in responding to endogenous antigens. By using an in vitro system and corresponding mouse models, Jumaa and colleagues (58) first showed that IgM-BCRs respond to both monovalent and polyvalent antigens, whereas IgD-BCRs bind exclusively to polyvalent antigens. This difference in binding is attributed to the differences in the structural properties of the hinge region between the two isotypes (58). Whereas IgM has a rigid Y structure, owing to a short and inflexible hinge region, IgD has a longer and highly flexible hinge region, which enables the two V-regions to bind antigen epitopes with a wider range of angles and distances. The flexibility of IgD may partly contribute to its mode of antigen binding (59–61).

Recently, super-resolution techniques, including direct stochastic optical reconstruction, transmission electron microscopy, and proximity ligation assay, were used to monitor the nanoscale organization of the IgM- and IgD-BCRs, as well as their differential interactions with the co-stimulatory CD19 co-receptor, in resting and activated B cells (62–64). The results revealed two things. First, in resting B cells, the IgM- and IgD-BCRs reside in separate membrane domains (called protein islands or nanoclusters) with a distinct protein and lipid composition. The IgM nanoclusters were smaller and contained lower numbers of BCRs than the IgD nanoclusters, with the CD19 molecules residing closer to the IgD-BCRs, on resting B cells. Second, upon B cell activation, the BCR nanoclusters became smaller and more dispersed so that the IgM- and IgD-BCRs could move closer to each other, accompanied by the dissociation of the CD19 molecules from the IgD-BCR and their association with the IgM-BCR, on activated B cells. Moreover, in addition to the well-studied co-receptors, such as CD19, other immune receptors expressed on B cells, including the inhibitory co-receptor CD22, the chemokine receptor CXCR4, and the BAFF receptor, have recently been shown to interact with the BCR and modulate its function (65–67). Together, these observations suggest that the isotype-related nanoscale organization of the BCRs in B cell signaling and activation, as well as the selective association of IgM- and IgD-BCRs with additional co-receptors and immune receptors, may contribute to the differential antigen-binding modes of the IgM- and IgD-BCRs.

The potential biophysical and biochemical mechanisms for the differential efficiency of antigen sensing, attributed to the IgM- and IgD-BCRs, have been well summarized in a review by Noviski & Zikherman (60). The selective downregulation of IgM-BCR, but not IgD-BCR, is likely to represent a tolerance mechanism, enabling mature autoreactive B cells to accumulate in the periphery. It is unclear why the majority of autoreactive B cells maintain anergy instead of undergoing clonal deletion by apoptosis. Recent studies have shown that the autoantibodies

produced by anergic B cells can undergo SHM and BCR glycosylation within the germinal centers to (*a*) attenuate self-reactivity and (*b*) acquire a higher affinity for foreign antigen (68–70). The so-called autoantibody redemption mechanism, just described, serves to reduce the potential for pathogens, such as the human immunodeficiency virus (HIV), lymphocytic choriomeningitis virus, and Lassa fever virus, to evolve antigens that mimic certain self-antigens, which could be recognized only by preimmune autoantibodies that had been deleted or edited in bone marrow (70). Consistent with the above view, anergic $IgD^{high}IgM^{low}$ B cells appear to enter the germinal center more efficiently than naïve $IgD^{+}IgM^{high}$ B cells in response to antigen (56, 68). IgD alone is sufficient to promote B cell differentiation in the germinal center but is less efficient than IgM alone at generating short-lived plasma cells in response to endogenous antigen (57). These data suggest that predominant IgD expression may prevent autoreactive B cells from differentiating into plasma cells capable of secreting autoantibody. On the other hand, however, it may facilitate entry of autoreactive B cells into the germinal center to attenuate autoreactivity via the autoantibody redemption program (60).

Polymeric Ig receptor (pIgR): a receptor expressed by mucosal and glandular epithelial cells that transports polymeric Igs into secretions

4.2.2. The functions of sIgD. The primary function of sIgD is to participate in mucosal immunity. In humans, sIgD is released from class-switched $IgD^{+}IgM^{-}$ plasmablasts, which have been detected in the upper respiratory mucosa but rarely found in the spleen, bone marrow, and non-respiratory mucosal sites (71). The serum concentration of sIgD is much lower than that of IgG, IgA, and IgM but is higher than that of IgE (72). $IgD^{+}IgM^{-}$ plasmablasts can most likely migrate through the circulatory system from the inductive sites in the upper respiratory mucosa to the distal mucosal sites, including the middle ear as well as mammary, salivary, and lacrimal glands (61, 71). At these effector sites, sIgD antibodies can bind to pathogenic bacteria, such as *Moraxella catarrhalis* and *Haemophilus influenzae* and their products, including *M. catarrhalis*–IgD-binding protein, lipopolysaccharide, and capsular polysaccharide, through both conventional V-mediated and unconventional *Cδ*-mediated mechanisms (71, 73, 74). In bony fish like the rainbow trout, sIgD has been shown to coat gill microbiota, albeit at a significantly lower level than sIgT. Moreover, trout sIgD seems to have the ability to interact with polymeric Ig receptor (pIgR), which is required for its transport. This is the first description of a pIgR being implicated in IgD secretion in a vertebrate respiratory tract (75).

In addition to mediating mucosal immunity, human sIgD can bind to various innate immune cells, especially basophils and mast cells (71). However, the receptors involved are yet to be identified. The binding of IgD to granulocytes is an evolutionarily conserved immune pathway, observed even in a subset of catfish granulocytes (71). In humans, the crosslinking of basophil-bound IgD with a specific monoclonal anti-IgD antibody induces basophils to produce (*a*) B cell–activating factors, such as IL-4, IL-13, BAFF, and APRIL; (*b*) antimicrobial factors, such as β-defensin 3, cathelicidin, SPAG11, PTX3, and CRP; and (*c*) proinflammatory and immunostimulatory factors, such as TNF, IL-1β, IL-8, and CXCL10 (71). In a recent study, sIgD protein levels were shown to increase in the sinonasal mucosa of patients with chronic rhinosinusitis (76). IgD-activated mast cells can facilitate local IgE production and exacerbate eosinophilic inflammation (76). Moreover, the proinflammatory function of IgD was further supported by the analysis of autoinflammatory disorders associated with hyper-IgD production. Compared with healthy individuals, patients with autoinflammatory disorders had more class-switched $IgD^{+}IgM^{-}$ plasmablasts and mucosal IgD-armed basophils (and probably also more mast cells), which suggests that abnormal sIgD levels may enhance the mucosal homing, proliferation, and activation of basophils and mast cells, leading to an immune system imbalance (71). Overall, the secretion of sIgD by class-switched $IgD^{+}IgM^{-}$ B cells may serve to arm the basophils (and probably also mast cells), enabling them to

undertake evolutionarily conserved immune surveillance at the interface between immunity and inflammation.

5. IgY, IgG, AND IgE: THE ISOTYPES INVOLVED IN SYSTEMIC IMMUNITY

5.1. The Evolution and Function of IgY, IgG, and IgE

IgG and IgE represent two isotypes that are exclusively found in mammals. The γCH2 and γCH3 domains of IgG-Fc are homologous in sequence and similar in structure to the εCH3 and εCH4 domains of IgE-Fc. Therefore, the εCH2 domains of IgE are located in the position equivalent to that occupied by the hinge region of IgG (77). In healthy humans, IgG has four subclasses, and its serum concentration is 10^4 times greater than that of IgE. In contrast, IgE is encoded by only a single gene copy in all mammalian species known to date (77). Although the γCH domains of the human IgG subclasses share more than 90% of their amino acid sequence identity, the affinity and specificity of different IgG subclasses to antigen and their effector molecules (including C1q, various FcγRs, and alternative receptors for IgG) vary considerably, reflecting the distinct biological functions of each IgG subclass. Differences in the structure, glycosylation, and effector mechanisms of human IgG subclasses, as well as those of IgE, have been extensively reviewed by others (77–79) and are not further discussed here. It is worth noting that some important livestock animals, such as pigs and horses, have multiple *Cγ* genes, which were generated by recently occurring duplication events within the IgH locus, following the process of speciation (80, 81). The retention of duplicated *Cγ* genes implies that each expressed IgG subclass has a specific and vital function. The generation of different IgG subclass-specific antibodies will enable us to carry out a thorough examination and comparison of their various effector functions in livestock animals.

Like mammalian IgG, IgY is the predominant serum antibody found in birds but not in reptiles and amphibians. The functions of IgY have not as yet been extensively studied, but it is known that IgY is expressed in a T-dependent manner (82). IgY not only is the major systemic antibody involved in opsonization and C′ fixation but also may mediate anaphylactic reactions (83, 84). It is widely believed that IgY is also the precursor of IgE and IgG. Not only does it seem to combine mammalian IgG- and IgE-like functions, but there is considerable sequence homology and structural similarity between the three Ig isotypes (20, 85). For instance, the crystal structure of Fcυ3–4 has features that are common to both IgG-Fc and Fcε3–4. Further evidence is provided by the presence of the IgO isotype in the platypus. In the platypus IgH locus, the IgO-encoding gene, *Co*, which is physically located in the same position as the *Cγ* genes usually found in the IgH locus of eutherians (**Figure 2**), encodes four oCH domains and a hinge attached to the oCH2 N terminus (**Figure 3**). The presence of the hinge region, together with the sequence homology between the *Co* and *Cγ* genes, suggests that IgO may be an evolutionary intermediate between the ancestral IgY and the present IgG and IgE (20). The origin of IgY, as a primitive antibody isotype specialized in orchestrating systemic immunity, is still unclear. When the first two or the last two CH domains of different isotypes were separately used to construct phylogenetic trees, χ/αCH1 and χ/αCH2 had a clear homology with υCH1 and υCH2, whereas χ/αCH3 and χ/αCH4 were homologous with μCH3 and μCH4. This homology pattern was also observed in the IgA-like gene of the leopard gecko (discussed in Section 6.2) and the IgX of the *Xenopus* and the axolotl (*Ambystoma mexicanum*) (86, 87). Other amino acid sequence phylogenetic analyses of the entire CH region of IgM, IgD/W, IgA/X, and IgY/G/E, performed with different methods and using different models, further show that IgA/X either first clustered with IgM and then with IgY/G/E or first clustered with IgY/G/E and then with IgM (10–13). These findings support Zhang et al.'s (88) hypothesis that IgM first gave rise to the mucosal antibodies IgA/X, and IgY subsequently

evolved from both IgM and an ancestor of IgA/X. Because IgA/X appears to be a transitional isotype connecting IgM and IgY/G/E, there is bound to be a functional overlap between IgM and IgA/X or between IgA/X and IgY/G/E, which is discussed in Section 6.2.

Fc receptors: a family of cell-surface molecules that bind the Fc fragments of different Igs

5.2. The High Prevalence of IgY(ΔFc) in Reptiles and Birds

A truncated isoform of IgY, designated as IgY(ΔFc) and initially found in ducks, is characterized by a missing Fc region (including the loss of the υCH3 and υCH4 domains) (89) (**Figure 3**). IgY(ΔFc) has subsequently been identified in many other species, such as lizards (*Anolis carolinensis*) (90), turtles (*Pseudemys scripta* and *Trachemys scripta elegans*) (12, 91), snakes (*Elaphe taeniura*) (13), salamanders (*Andrias davidianus*), and geese (*Anser cygnoides orientalis*) (40, 92). Different species seem to adopt different strategies for generating IgY(ΔFc). In ducks and geese, IgY(ΔFc) is generated by the alternative splicing of the same gene (encoding the intact IgY antibody) by using different transcriptional termination sites (83, 92). However, in turtles, IgY and IgY(ΔFc) are encoded by separate genes (12). More interestingly, our recent study indicates that in some snakes, IgY and IgY(ΔFc) are expressed from the same gene, giving rise to identical transcripts, which are then processed differently by posttranslational mechanisms (data not shown).

IgY(ΔFc) is also expressed in many species of amphibians, reptiles, and anseriform birds. As this Ig is a natural $F(ab')_2$ analog, with only antigen binding activity, the major question of what immunological advantages the expression of these truncated antibody forms offers the animal host needs to be addressed. The ratio of IgY to IgY(ΔFc) in duck serum is approximately 3:5, and the truncated form appears predominant later in the immune response, at least in animals repeatedly immunized with the same antigen (93, 94). However, although not systematically studied, ducks seem unable to mount a stronger secondary immune response on repeated immunization (95). When the ducks experienced transient exposure to antigen via repeated immunization, the magnitude and duration of the specific antibody dose did not significantly increase. Instead, the antibody underwent affinity maturation, although the increase in affinity was sometimes negligible (95). Based on the above observation, Magor (96) suggested that the accumulation of higher-affinity IgY(ΔFc) later in the immune response could serve as an immunomodulatory mechanism, as the immune response gradually weakens. On the one hand, IgY(ΔFc) cannot facilitate the macrophage-mediated clearance of opsonized pathogens or the uptake of pathogens for antigen presentation to T cells. On the other hand, IgY(ΔFc) may limit virus infection, because the Fc-mediated virus internalization may promote viral entry into target cells and the mobilization of virus throughout the host.

Meddings et al. (97) proposed for the first time that antibody-dependent enhancement (ADE) of viral infection may serve as an evolutionary selection pressure for the IgY(ΔFc) isotype. The ADE of viral infection occurs when virus–antibody complexes interact with specific molecules (e.g., Fc receptors, C1q, C3, and C′ receptors, and viral receptors and co-receptors) on target cells, promoting viral attachment and subsequent entry (98). Importantly, the titer and affinity of the full-length antibody will theoretically affect the risk of ADE, depending on the neutralizing or non-neutralizing nature of the antibody. Viruses captured by a high titer of non-neutralizing antibodies or subneutralizing concentrations (that are no longer sufficient for neutralization) of neutralizing antibodies are theoretically likely to increase infection by ADE. Higher full-length antibody affinities for viral antigens, whether neutralizing or non-neutralizing, will further increase the risk of ADE (99). Although the quantitative relationship between the subneutralizing concentrations of neutralizing antibodies and ADE is less clear, a study using monoclonal antibodies specific for epitopes of the West Nile virus E protein evaluated the upper and lower threshold quantity of neutralizing antibody capable of mediating ADE. While the neutralization threshold

governs the upper limit of ADE stoichiometry, the minimum quantity of antibodies required for ADE was approximately half of the quantity required for neutralization (100).

Based on the mechanism of ADE, Meddings et al. (97) suggested that, at least in ducks, truncated Ig (lacking an Fc region) such as IgY(ΔFc), which appears predominant later in the immune response and possesses higher affinity for the same antigen, would outcompete the full-length antibody for the same antigen. This mechanism is thought to be in place to reduce the levels of antigen-bound full-length antibody below the lower threshold levels required for ADE. However, because (*a*) different types of antibodies, including neutralizing, enhancing, non-neutralizing, and nonenhancing antibodies, are produced in parallel in virus-infected hosts (98) and (*b*) the binding of antibody to virus is further determined by antibody affinity, the location of the epitope, and epitope accessibility (99), it remains to be seen whether IgY(ΔFc) really contributes to limiting Fc receptor- and C′-mediated ADE. This hypothesis will need to be proven by experiments designed with strictly controlled variables.

6. IgA, IgX, AND IgT: THE SPECIALIZED MUCOSAL ISOTYPES PRESENT IN DIFFERENT CLASSES OF JAWED VERTEBRATES

6.1. The Evolution and Function of Specialized Mucosal Igs

To date, IgA has been identified in mammals, birds, and Crocodylia species. In humans, IgA exists as a dimer and represents the most abundant isotype in the whole body, particularly concentrated at the mucosal sites. IgA is also the second most abundant isotype in the serum, where it is present as a monomer. The main function of IgA has long been considered in passive immunity at mucosal sites, through immune exclusion, neutralization, and antigen excretion. However, it has recently become clear that IgA is also able to induce active immunity by modulating the production of various cytokines, at both mucosal and nonmucosal sites (reviewed in 101). Another research hotspot is the function of IgA in host-microbial mutualism (reviewed in 102, 103).

Although IgA is absent in lower-jawed vertebrates, amphibians and bony fish express specialized mucosal antibody isotypes (IgX and IgT), independently, by convergent evolution. In *Xenopus*, IgX- but not IgY-positive B cells are abundant in the intestinal epithelium, whereas they are hardly detected in the spleen, indicating that IgX may be a major constituent of mucosal immunity (104). This role was subsequently confirmed by a study demonstrating that IgX expression was upregulated in the plasma of frogs receiving oral but not systemic immunization (105). Subsequent studies found that larval thymectomy abolished the expression of IgY but did not affect IgX levels or gut bacterial communities, consistent with IgX's T-independent role in the mucosal immunity of amphibians (82).

Since 2005, new IgT and IgT subclasses have been successively identified in various ray-finned bony fish. However, the biochemical functions of IgT have been systematically studied only in rainbow trout. A series of studies showed that IgT is the main isotype induced in response to pathogenic challenge in mucosal tissues, including the gut, skin, olfactory epithelium, and gill, where it also plays a prevalent role in coating the microbiota found on these surfaces (75, 106–108). Moreover, a B cell lineage expressing only surface IgT (IgT^+ B cells) represents the predominant B cell subset localized to the lymphoid tissues of the trout mucosal surfaces (75, 106–108). A recent study provided the first evidence that the local activation and proliferation of IgT^+ B cells, as well as the production of pathogen-specific IgT, did occur within the nasal mucosa upon parasite infection (109). Parasite-specific IgT is therefore the main isotype responsible for orchestrating nasal-adaptive trout immune responses (109). In summary, the IgT of bony fish represents the most primitive Ig isotype equipped to carry out specialized functions at mucosal surfaces.

Interestingly, mucosal antibodies all have multimeric structures: secreted IgA (sIgA) is typically a dimer (associated with the J chain) in mammalian secretions and is believed to be a dimer, tetramer, or trimer (associated with the J chain) in avian secretions (110, 111); secreted IgX is a pentamer or hexamer (not associated with the J chain); and secreted IgT is a noncovalently connected tetramer (not associated with the J chain) (26, 104, 106) (**Figure 3**). Moreover, the epithelial transcytosis of polymeric IgA and IgT is mediated by the pIgR, because a secretory component–like polypeptide has been found to be associated with polymeric IgT in rainbow trout and polymeric IgA in birds (106, 112). It is worth noting that the *Cτ* gene is absent in some ray-finned bony fish species, including catfish and medaka (113, 114) (**Figure 2**). Coincidentally, the natural absence of the *Cα* gene has also been reported in many reptile species, indicating that the functions of specialized mucosal antibodies may not be completely independent, as discussed in the next section.

6.2. The Natural Absence of the *Cα* Gene in Many Reptiles

So far, no IgA-encoding gene has been identified in turtles, snakes, or Squamata species (**Table 1** and **Figure 2**), suggesting that the *Cα* gene is naturally absent in these species (12–14, 16–18, 90). However, two IgA-like genes (*Cα*-like) with four CH domains are found in the leopard gecko (17, 86) (**Table 1**). Both genes contain a cysteine codon within the secretory tailpiece of the protein, which is used for generating polymers. Whereas one of the IgA-like isotypes is expressed only in the lung, the other is expressed only in the intestine, suggesting that they are functionally equivalent to mammalian IgA (17, 86). Although the total amino acid sequences encoded by the *Cα*-like genes share more similarities with IgA than with other isotypes, the *Cα*-like genes are likely produced by a similar recombination pattern as IgX, as the CH1-CH2 and CH3-CH4 domains show a clear homology with IgY (υCH1 and υCH2) and IgM (μCH3 and μCH4), respectively (17, 86). So far, no *Cα*-like genes have been identified in other Squamata species, suggesting that this gene evolved by an independent recombination event within the Eublepharidae branch of the Gekkota order (17). Considering the crucial functions of IgA in both passive and active immunity, the compensatory mechanism for the loss of IgA as a host defense mechanism against mucosal pathogens and a means of maintaining intestinal homeostasis is worthy of further investigation.

Studies based on mouse models and human subjects with selective IgA deficiency (sIgAD) indicate that other isotypes, particularly sIgM, which shares evolutionary, structural, and functional similarities with IgA (including sequence homologies between the Fc portion and the tailpiece, the presence of a J chain, the formation of polymers, and the reliance on the same pIgR for transport across the mucosal epithelial cells), can compensate for the loss of IgA. Compared with wild-type mice, mice with a targeted deletion of the IgA switch and constant regions demonstrate normal lymphocyte development, proliferative responses, and cytokine production but show a compensatory rise in the mucosal and serum levels of IgM and IgG (115). IgA-knockout mice also display normal rates of primary infection clearance as well as equivalent levels of protection against secondary infection with vaginal herpes simplex virus type 2, gastric *Helicobacter pylori*, and possibly rotavirus and influenza virus (conflicting results obtained from different models of infection) (116–121). IgA-deficient mice, generated via AID knockout (*Aicda*$^{-/-}$), also elicit a compensatory IgM response that targets commensal microbes in the small intestine, which are normally coated with IgA (122). Furthermore, mice with a J-chain or pIgR deletion, which affects the normal assembly and transport of sIgA and sIgM, respectively, have been shown to display defective mucosal immunity and the deterioration of epithelial barrier functions (123–126). In humans, sIgAD is one of the most common types of primary antibody deficiency, with a variable prevalence from 1/100 to 1/22,000 among different populations (127). Compared with other immunodeficiencies, sIgAD has a relatively mild clinical phenotype, including recurrent respiratory

and gastrointestinal infections, allergy, and autoimmunity, with the majority of patients remaining asymptomatic (127, 128). Similar to IgA-deficient mice, the compensatory mucosal IgM count is elevated in these patients (129, 130). Together, these data indicate that, in humans and mice, IgM can largely compensate for the loss of passive immune functions with IgA deficiencies.

In contrast, it is still less clear which isotype can compensate for the active immune functions of IgA, because this is unlikely to rely on IgM. Hansen et al. (101) proposed that in humans, IgG may compensate for IgA in inducing inflammatory cytokine production, as evidenced by (*a*) the broad distribution of IgG throughout various tissues, including the lamina propria of the intestine, blood, and skin, and (*b*) the co-expression of FcγRs and FcαRI, both of which promote similar subsequent immune responses and cytokine profiles, by numerous immunocytes (101). In this regard, it is interesting that sIgAD patients with associated antibody deficiency, such as IgG2 subclass deficiency, have a higher risk of suffering from more severe infections and complications (131).

In addition to IgM and IgG, evidence also suggests that sIgD has some overlapping functions with IgA in response to microbiota. CSR-derived sIgD was shown to increase in response to commensal microbe innate sensing pathways, such as Toll-like receptor signaling. Similarly, IgD secretion decreases in germ-free or antibiotic-treated mice. These observations suggest that, similar to IgA, sIgD plays a role in the recognition of commensal microbial flora and may contribute to the homeostatic regulation of the microbial community (36).

In summary, because the actions of IgA can be compensated for by IgM, and possibly even IgG and IgD, the question of why the immune system has evolved to consume considerable energy in the daily production of several grams of IgA remains a mystery. Thus, it appears as though, in humans and mice, the functions of IgA may be redundant. In reptiles such as the lizard *A. carolinensis* and the turtle *T. scripta elegans*, which naturally lack IgA, a high expression of IgM is reported in the intestine, which is even higher than in the spleen (12, 90). In the snake *E. taeniura*, IgM is also expressed at much higher levels than the total titer of IgY (IgY1 and IgY2) found in the lung, stomach, and intestine (13). These data suggest that, in mammals and above-mentioned reptile species, IgM may play an important role in orchestrating mucosal defense in the absence of IgA. In IgA-absent reptile species, it is common to find multiple IgY or IgD subclasses. In these species, the active immune function of IgA may also be compensated for by one or more IgY or IgD subclasses.

7. ANTIBODIES WITH UNUSUAL STRUCTURES AND THEIR APPLICATIONS

7.1. Heavy Chain–Only Antibodies

The conventional Ig molecule is a heterotetramer, consisting of two identical H chains and two identical L chains linked together by interchain disulfide bonds. In addition to producing conventional Igs, camelids and cartilaginous fish produce functional homodimeric antibodies composed of only two H chains (HCAbs). The HCAb antigen-binding site consists of a single variable domain, referred to as the VHH or the nanobody in camelids and the V-NAR of the IgNAR in cartilaginous fish (**Figure 3**). The IgH locus of camelids contains intermixed organized germline VH and VHH genes (designated as *IGHV* and *IGHVH*), followed by D and JH gene pools and the C genes (including *Cγ* genes dedicated to the production of the HCAbs and other *Cγ* genes used to exclusively produce the classical antibodies) (23). The VHH domains are generated using unique *IGHVH* genes and promiscuous *IGHV* genes (genes that can produce the H chains for both the HCAbs and the classical antibodies), which are rearranged to the same D-J clusters that are shared with conventional VH domains (23, 132). In contrast to camelids, in which the genetic

elements required for the generation of the H chains belonging to the two antibody types are located in the same locus, the V(D)J rearrangement of *IgNAR* occurs almost exclusively within the *IgNAR* cluster (3). Each *IgNAR* cluster consists of a single V segment, three D segments (or a fusion of D1 and D2 segments followed by a D3 segment), one J segment, and a single *C-NAR* (**Figure 2**). Thus, the V-NAR domains are produced by three or four rearrangement processes, which result in VD1D2D3J assembly. Although the number of *IgNAR* clusters was much lower than that of *IgM* clusters, both the sequence and the length of V-NAR CDR3 show great variability, which is imparted by the abundant junctional diversity seen at the V-D, D-D, and D-J junctions, as well as the exceptionally high level of SHM, which occurs in an antigen-driven manner (3, 133).

CDRs: three loops (CDR1, CDR2, and CDR3) in the V domain that determine the antigen-binding site and contribute to Ig diversity

Although the camelid HCAbs and IgNAR are two entirely different HCAb isotypes, they seem to have undergone remarkable convergent evolution independently in the ancestors of camelids and cartilaginous fish. Both HCAbs and IgNAR use similar strategies or mechanisms to avoid interacting with the L chain to produce soluble V domains in the absence of a VL partner, as well as to generate a highly variable antigen-binding repertoire in the absence of VH-VL combinatorial diversity (including longer CDR1 and CDR3, extensive SHM, and complex intraloop and interloop disulfide bonds). Further details regarding these fascinating and unusual Ig isotypes can be found in an elegant review by Flajnik and colleagues (134).

Of particular note, both VHH and V-NAR domains are characterized by their small size, increased solubility and stability, and multiple structural topologies. These features allow them to target antigen epitopes that are inaccessible to conventional antibodies, even in harsh physiological conditions (134). Ever since their discovery, HCAbs (and especially nanobodies) have been extensively applied in basic research, as well as in therapeutic, diagnostic, and other biotechnological areas. Many examples, highlighting the value of nanobodies and V-NAR domains, have been documented elsewhere (133, 135–138) and are not further discussed here.

7.2. The Bovine Ultralong CDR H3

Unlike humans and mice, cattle have a significantly limited VH gene repertoire composed of only 12 potentially functional VH genes, all of which belong to the same *IGHV1* subgroup and share more than 90% nucleotide sequence identity (31). However, a subset (~10%) of bovine H chains have unusually long CDR H3 structures, reaching more than 70 amino acids in length, which is considerably longer than the longest CDR H3 regions found in humans, camelid HCAbs, and IgNARs to date (31, 139–141). The crystal structures of five bovine antibodies with unrelated ultralong CDR H3 sequences have shown that they all assume unique minifolds architecture composed of a β strand stalk, which supports a disulfide-bonded knob domain. Both the stalk and knob domains can accommodate significant structural variation, such as diverse disulfide-bond patterns and loop structures in the knob (140, 142). In traditional antibodies, the antigen-binding site is formed by the CDRs of both the H and L chains. In antibodies with an ultralong CDR H3, the CDRs H1 and H2, as well as the CDRs of a restricted set of λ chains, are not used to bind antigen but instead provide structural support for the CDR H3 stalk (140, 142).

The bovine ultralong CDR H3 is exclusively a product of a single germline variable gene, *IGHV1–7* (previously referred to as VHBUL), which is rearranged to the longest D gene segment, *IGHD8–2* (previously referred to as DH2) (141). Both *IGHV1–7* and *IGHD8–2* have unique sequence features that favor the formation of the CDR H3 stalk and knob structures. An eight-nucleotide duplication, beginning at or just after the canonical second cysteine at position 104 of the *IGHV1–7* gene, encodes a 'CTTVHQ' motif instead of the traditional 'CA(R/K)' motif, found at the C terminus of most vertebrate IGHV regions. The 'CTTVHQ' motif plays an integral role in the formation of the ascending stalk portion of the ultralong CDR H3 (141, 142). The germline

IGHD8–2 sequence encodes 48 amino acids, including 4 cysteines and Gly-Tyr-Gly repeats. Furthermore, as many as 19 AID hotspots (RGYW or reverse WRCY) are distributed throughout the *IGHD8–2* sequence, which make up more than 80% of the 48 *IGHD8–2* residues and have the potential to mutate to a cysteine via a single nucleotide change (143). Consistent with its sequence features, high mutation rates were observed within the *IGHD8–2* of ultralong CDR H3, making these regions extremely divergent from the germline *IGHD8–2* and rich in non-germline-encoded cysteines (142). Interestingly, in-frame deletions of 1 to 18 codons were identified in the internal region of the *IGHD8–2* in nearly 50% of the rearranged ultralong CDR H3 transcripts. Meanwhile the *IGHD8–2* 5′ and 3′ ends, which encode the structurally conserved motifs (CPDG turn at the 5′ end and alternating aromatic amino acids YxYxY at the 3′ end) were unaffected. Thus, in addition to the numerous SHM-induced mutations that alter the amino acid content, including the formation of non-germline-encoded cysteines, deletions can also modify the cysteine number and their positions. These features act to further diversify the disulfide-bonded loops and change the topology of the antigen-binding site (141). In contrast to conventional CDR H3, the level of amino acid variability produced by SHM is significantly lower in the CDR H1 and CDR H2 of the V regions within ultralong CDR H3, suggesting that the antigen-binding function is solely attributed to CDR H3 (141).

Exactly what advantages the ultralong CDR H3 confers to Ig function remains intriguing. For the cattle themselves, the ultralong CDR H3 may have developed as an alternative mechanism for achieving a maximum level of diversification when faced with a limited V(D)J combinatorial repertoire. Alternatively, the ultralong CDR H3 may have evolved to optimize the binding to antigens originating from rumen microorganisms or certain bovine-specific pathogens, which are not typically encountered by other vertebrates (141–143). Interestingly, because the *IGHD8–2* is located downstream of the *Cμ1* gene in the bovine IgH locus, V regions containing an ultralong CDR H3 seem to be completely associated with IgM2, so that the CDR H3 of IgM2 has a much greater length and amino acid variability than those of IgM1 (31). Thus, the bovine IgM1 and IgM2 subclasses may show different preferences in binding to certain antigens (discussed in Section 3.2). A recent study showed that cattle can reliably and rapidly elicit a broadly neutralizing antibody response against the well-ordered HIV BG505 SOSIP envelope (Env) trimer. The monoclonal antibody isolated from immunized cattle harbored an ultralong CDR H3 that can easily access and bind to the CD4 cell binding sites, which is effectively immunoquiescent in most humans (144).

The knob domains of the ultralong CDR H3, which has a molecular weight even lower than that of the VHH or the V-NAR, may independently bind to antigen epitopes that are more difficult to access with typical antibodies. This unusual structural feature of the ultralong CDR H3 thus provides a novel approach for generating engineered antibodies against challenging antigenic targets. A knob-replacement strategy, whereby the knob domain is replaced by another protein or peptide, such as GCSF, EPO, or a CXCR4 peptide antagonist, resulted in the formation of functional fusion proteins with desired pharmacological properties (145–147). Given the versatility of the bovine ultralong CDR H3, it provides a structural platform for the development of a new generation of diagnostics, therapeutics, vaccines, and immunomodulating drugs.

8. CONCLUSION

With the rapid development of new reagents and powerful sequencing technologies, we are beginning to gain more insight into the structural characteristics, effector functions, and diversity mechanisms of specific Ig isotypes in vertebrate species. On the one hand, these new discoveries could help us understand more comprehensively how Ig molecules evolve to adapt to the unique

physiological environment of certain species; on the other hand, they serve to provide fresh ideas for the development of novel therapeutic and diagnostic applications, for which there is growing interest.

DISCLOSURE STATEMENT

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