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Review

Avian IgY antibodies and their recombinant equivalents in research, diagnostics and therapy

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ABSTRACT

The generation and use of avian antibodies is of increasing interest in a wide variety of applications within the life sciences. Due to their phylogenetic distance, mechanisms of immune diversification and the way in which they deposit IgY immunoglobulin in the egg yolk, chickens provide a number of advantages compared to mammals as hosts for immunization. These advantages include: the one-step purification of antibodies from egg yolk in large amounts facilitates having a virtually continuous supply; the epitope spectrum of avian antibodies potentially grants access to novel specificities; the broad absence of cross-reactivity with mammalian epitopes avoids assay interference and improves the performance of immunological techniques. The polyclonal nature of IgY antibodies has limited their use since avian hybridoma techniques are not well established. Recombinant IgY, however, can be generated from mammalian monoclonal antibodies which makes it possible to further exploit the advantageous properties of the IgY scaffold. Moreover, cloning and selecting the immune repertoire from avian organisms is highly efficient, yielding antigen-specific antibody fragments. The recombinant approach is well suited to circumvent any limitations of polyclonal antibodies. This review presents comprehensive information on the generation, purification, modification and applications of polyclonal and monoclonal IgY antibodies.

1. Introduction

Antibodies are likely to remain the affinity molecules of choice in a wide variety of analytical, biochemical, and medical approaches. This is primarily because they have familiar properties and their use is well-established in many applications. Moreover, their specificities and biological effects can now be readily manipulated using standard molecular biological techniques. In most cases mammalian antibodies are perfectly adequate. Unfortunately, their involvement in the immune response and immune-mediated pathologies along with a high degree of conservation among mammals can, however, make them susceptible to unwanted interactions with conserved proteins, which can in turn hamper their use in certain approaches.

The immunization of chickens provides an attractive alternative [1–3] to using mammals as hosts for antibody production. IgY is the major low molecular weight immunoglobulin in oviparous animals. This type of antibody has distinctive properties which can be exploited in various ways in research, diagnostics and therapy. One important advantage arises from the phylogenetic distance and genetic background that distinguishes birds from mammals. This improves the likelihood that an immune response will be elicited against antigens or epitopes that may be non-immunogenic in mammals. The deposition of IgY into the egg yolks of the immunized bird then provides an elegant source of polyclonal immunoglobulins. Since polyclonal IgY can be recovered from the eggs of laying hens for prolonged periods, this approach provides a long-term supply of substantial amounts of antibodies. In addition, such antibodies exhibit biochemical and structural features, which can render them superior in virtually all types of immunoassays, especially those designed to detect molecules in specimens like mammalian blood or serum [4,5].

Due to the technical difficulties of avian hybridoma techniques, and the problem that existing immortalized B cell lines (such as the ALV-induced bursa-derived lymphoma line DT40) undergo Ig gene conversion during *in vitro* culture [6], the production of chicken antibodies languished somewhat until it became possible to generate monoclonal IgY through the *in vitro* selection from

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combinatorial antibody libraries by phage display [7]. In the chicken, only a single functional V and J segment is present in the light and heavy chain gene loci. As a result, diversification of the avian immune repertoire is introduced into the rearranged V(D)J segments by gene conversion using pseudo V genes as donors. As will be seen, this greatly simplifies the construction of combinatorial recombinant antibody libraries while the selective power of phage display provides a way of accessing unique binders.

This review focuses on the immunological background and novel approaches that have been made possible as a result of avian antibody technologies. It suggests that the chicken can be more widely used for generating both native, and recombinant IgY.

2. Immunological, structural and biochemical characteristics

IgY is the predominant low molecular weight serum immunoglobulin isotype in amphibians, reptiles, and birds. This designation derives from its occurrence in egg yolk, and, as demonstrated in 1893, it transfers immunity from the hen to the developing embryo [8].

Among the three avian isotypes (IgY, IgM and IgA), IgY is the most abundant in serum, with concentrations ranging from 5 to 15 mg/ml in laying hens [9,10] compared to the lower concentrations of IgM (1–3 mg/ml) and IgA (0.3–0.5 mg/ml). In anseriform birds like ducks there exists in addition to IgY, an alternatively spliced version of IgY, the IgY ΔFc. This variant lacks the Fc region and therefore does not have the Fc-mediated secondary effector functions. It is also found in relatively substantial amounts (1–3 mg/ml) [11].

The organs in the chicken responsible for antibody production differ significantly from those in mammals. The central (primary) lymphoid organs are represented by the thymus and bursa of Fabricius (BF), while peripheral (secondary) lymphoid organs include the spleen, Harderian glands, bone marrow, conjunctival-associated lymphoid tissue (CALT), bronchial-associated lymphoid tissue (BALT) and gut-associated lymphoid tissue (GALT). Chickens do not have lymph nodes as such, but instead have lymphoid nodules associated with the lymphatics [12].

The BF is located above the cloaca in the caudal body cavity and plays a cardinal role in avian B cell development and antibody diversification [13]. Following colonization by a small number of B cell precursors, cells expressing surface immunoglobulin undergo rapid proliferation such that at about two months of age there are approximately 10^4 follicles in the BF [14]. A few weeks after hatching, about 5% of the bursal cells migrate each day into the blood and then into the spleen, thymus, and caecal tonsils, where they subsequently produce immunoglobulins. The spleen is the largest secondary lymphoid organ and is important for antigen processing and in the production of antibodies after hatching [15].

Although IgY is essentially an immunoglobulin with characteristics and functions similar to IgG, it possesses a slightly different structure which provides its distinct properties and biochemical behaviour. IgY has a slightly higher molecular mass (approximately 167 kDa) than its mammalian counterpart [16] due to the presence of four constant- and one variable Ig heavy chain domains. The nucleotide sequence of the chicken upsilon (υ) heavy chain [17] reveals that as with the more ancient amphibian IgY [18,19], the avian molecule contains a domain (Cυ2) which is conserved in mammalian IgE, but was condensed to form the flexible “hinge” region in mammalian IgG [17]. An orthologous domain must therefore have existed in the IgY-like ancestor prior to duplication and subsequent divergence from the mammalian lineage.

In mammals, IgG forms immune complexes and facilitates opsonisation, activates the complement system and provides protection for the foetus upon transport across the placenta. IgE can sensitize effector cells and mediates anaphylactic reactions [20]. IgY appears to combine mammalian IgG- and IgE-like functions since it not only provides defence against infections [21], but may also mediate anaphylaxis [22]. In contrast to mammals, basophils are much more numerous in birds than mast cells [23] and antibody-dependent hypersensitivity and fatal systemic anaphylaxis [22,24,25] are mainly mediated by these cells [26]. This constitutes indirect evidence for the presence of IgY receptors on effector cells. IgY binds to monocytes with IgG-like kinetics [27], despite its putative IgE like structure as predicted from the chicken υ heavy chain primary sequence [17]. Recently, the chicken leucocyte receptor complex (LRC) was analysed and four major types of chicken Ig-like receptors (CHIR) were identified: CHIR-A, activating receptors displaying two extracellular C2-type Ig-domains, CHIR-B, inhibitory receptors also displaying two C2-like Ig-domains, and two types of CHIR-AB with one or two C2-like Ig-domains, which are reported to have bifunctional potential, since they display features of both inhibitory and activating receptors [28,29]. So far, CHIR-AB1 and its recently identified homologues are the only receptors in the LRC of known specificity [30]. It functions as a classical Fc receptor expressed on chicken B cells, macrophages, monocytes, and NK cells [31]. In contrast to IgG and IgE receptors, CHIR-AB1 binds in a similar way as FcαRI or FcRn with a 2:1 stoichiometry. Its affinity is comparable with the values reported for IgA binding to its receptor [32]. In contrast to mammalian IgG or IgE the CHIR-AB1 binding site was mapped to the upsilon heavy chain domains 3 and 4 (Cυ3/Cυ4) interface, a finding that together with the phylogenetic relationship of the antibodies and their receptors indicates a substantial shift in the nature of Fc receptor binding during evolution [33,34]. A specific interaction between CHIR-AB1, which provides an inhibitory motif in its cytoplasmic tail and the Fc portion of IgY was shown to enhance calcium release in a chicken B cell line expressing CHIR-AB1 and the common activating γ-chain [31]. The activation required aggregation of IgY suggesting that immune complexes are required to trigger a response [31]. By comparing CHIR-AB1-like sequences in databases, 18 homologues of CHIR-AB1 have been identified and cloned. These comprised non-IgY-binding and IgY-binding isoforms displaying different affinities [30].

An additional FcR-related gene designated *Gallus gallus* FcR (ggFcR) was recently identified [35]. The receptor which selectively binds IgY consists of four extracellular C2-set Ig domains. Surprisingly, ggFcR is closely related to chicken LCR encoded genes, but is located on chromosome 20 distinct from the LCR and FcR gene clusters. Recently a chicken yolk sac IgY receptor (FcRY) responsible for IgY transport from yolk to the embryonic circulation was characterized as a homologue of the mammalian phospholipase A2 receptor (PLA2R), a member of the mannose receptor family [36]. Deposition in the yolk however is mediated by another receptor not yet cloned [10] the specificity of which has been addressed by different approaches. One factor restricting the deposition of IgM and IgA in the yolk appears to be their polymeric nature [37]. The Fc region structure may also be important since anseriform species like ducks preferentially incorporate full-length IgY into the egg yolk over the truncated isoform IgY ΔFc [38]. Site-specific mutagenesis experiments using mammalian IgG sequences and extrapolation of this information to the upsilon heavy chain suggested the Cυ2/Cυ3 interface, especially residues 362–365, and positions 550–553 within Cυ4 as essential for the interaction with the receptor [39]. Besides its function and interaction with Fc receptors, IgY differs from IgG in a variety of aspects which are more directly attributable to the molecule itself. As mentioned earlier, the phylogenetic distance between the avian immune system and mammalian proteins most likely increases the immune response towards the respective antigens. This means that IgY can often be raised against epitopes on highly conserved proteins when

other mammals fail to provide an immunological response [40,41]. The extent to which the overall antibody affinities of mammalian IgG and IgY relate to each other is still under investigation but monoclonal IgY antibody fragments generally exhibit reactivities at least comparable to those of IgG (unpublished observations).

Compared to mammalian IgG IgY is lacking the flexible hinge region and, thus, thought to be a more rigid immunoglobulin. This hinge-less structure is also found in mammalian IgE. IgY therefore exhibits structural features of both mammalian IgE and IgG, a finding also supported by a structural analysis of the IgY Fc portion [42]. Potentially reduced molecular flexibility might be associated with decreased susceptibility to proteolytic degradation or fragmentation. Nevertheless, IgY can be fragmented by papain or trypsin [43]. IgY, like mammalian IgG, is reasonably stable and can be stored for several months under standard conditions [44]. A serious limitation of IgY for therapeutic applications, however, is its reduced stability at low pH [44,45]. In contrast to IgG the antigen binding activity of IgY decreases significantly under acidic conditions. As demonstrated by circular dichroism analyses, loss of activity of chicken IgY is accompanied by significant conformational changes, a fact attributed to fewer intramolecular disulphide linkages than, for example, rabbit IgG [46].

3. Generation of polyclonal and monoclonal IgY

Today, production of polyclonal IgY by immunization of chickens is offered on a routine basis by several commercial companies. In general, this approach is subject to the same constraints as the conventional immunization of mammals. Nevertheless, the advantages of chickens being a non-mammalian species [47] and the bloodless isolation of immunoglobulins have perhaps not yet been fully appreciated and exploited.

Egg yolk collected after immunization can provide concentrations of IgY in the range of 10 mg/ml as starting source for the recovery of the immunoglobulin. Relatively simple methods may be used to extract the antigen-specific immunoglobulin from egg yolk with several commercial kits being available. The decision to use a particular protocol is usually brought about by the intended downstream applications as well as the expertise and equipment available [48]. One of the most frequently used procedures involves protein precipitation with ammonium sulphate, dextran sulphate or polyethylene glycol (PEG). A particularly efficient method comprises two successive precipitations by using 3.5% PEG to remove any lipids, followed by 12% PEG to precipitate the IgY. A variant protocol includes an emulsification step, adding one volume of chloroform to one volume of egg yolk rather than using fractional precipitation [49,50].

IgY can also be purified by conventional ion exchange chromatography [51]. Another strategy, useful as an additional "polishing" step, relies on thiophilic adsorption chromatography (TAC) in which the target protein adsorbs to a sulphone thioether ligand in an interaction mediated predominantly by aromatic residues [52–54]. The elution conditions are very mild compared with conventional methods used to purify antibodies, such as protein A, G, or L, none of which bind to IgY. Nevertheless, purification strategies or polishing steps based on affinity ligands might be helpful, in particular when high purity is desired. Anti-IgY antibodies and synthetic ligands [55,56] are available, but this spectrum of reagents could be broadened by using soluble IgY receptor constructs or other affinity molecules as affinity medium. For instance, SSL7, the superantigen-like protein 7 from *Staphylococcus aureus* can be used for affinity purification of IgY (unpublished data). Additionally, recombinant expression of CHIR in mammalian cells and in *Escherichia coli* is highly efficient and can potentially provide a highly homogeneous protein fraction (unpublished data).

Polyclonal preparations of IgY are suitable for many routine applications. In some diagnostic approaches, however, the use of monoclonal reagents is imperative for accuracy, reproducibility and standardisation. Even though hybridoma technology has been applied to avian species, there are technical obstacles, which together with low secretion rates of fusion lines limit its efficacy [57,58]. These obstacles can, however, be circumvented by using recombinant antibody technologies to address the need for monoclonal IgY.

An antibody essentially represents the sum of its antigen-binding moieties and the Fc portion which is essential for dimerization, effector functions and facilitates detection using conventional secondary reagents. When deciding on a reagent for a particular application, it is important to consider whether an authentic fully avian antibody is required or whether parts may in fact be derived from other species. Both avian IgY and chimeric IgY with avian constant domains and murine binding moieties have recently been produced [59,60]. In these studies, despite their heterologous origins, transfected mammalian cells were able to stably express different IgY-based constructs.

The successful secretion of immunoglobulins from mammalian cells requires that chaperones interact with nascent immunoglobulin chains and guide their folding and assembly. Binding immunoglobulin protein (BiP) binds transiently to most domains of the Ig heavy chain (CH) and some variable regions of the light and heavy chain (VH and VL) [61,62]. CH1 provides a site for covalent attachment of CL and interacts stably with BiP in the absence of light chains [63]. Pronounced differences in the amino acids sequences within the antibody constant domains of antibodies from birds and mammals and the potential loss of specific interaction sites for chaperones with the nascent immunoglobulin chains might conceivably affect the efficiencies with which mammalian cells can secrete avian antibodies. For instance, IgY was found to be more efficiently secreted than IgG1, possibly as a result of less stringent control by the ER secretion machinery [60]. Since having efficient and economic ways of producing antibodies is always desirable, yields might in some cases be improved by using the binding moieties from pre-existing murine hybridomas to generate chimeric IgY. Moreover, the enormous diversity of the synthetic antibody libraries available today means that immune animals are not necessarily needed to derive suitable binders. Indeed, frameworks of synthetic human [64] and avian [65] variable regions have been successfully converted to their IgY derivatives and produced in eukaryotic hosts.

The primary sequence of the IgY heavy chain provides two potential N-linked glycosylation sites, both of which are located in the Fc region, namely Asn308 and Asn407 [17]. Carbohydrate analysis of native IgY revealed mono-glucosylated oligomannose type oligosaccharides, oligomannose type oligosaccharides and biantennary complex type oligosaccharides [66]. The first two of these have been reported as being the major glycoforms in IgY from different species [66–68] and are attributed to the Cυ3 glycosylation site [69]. Additionally, a terminal sialic acid was identified in native IgY. No significant differences between the overall glycosylation pattern of native and recombinant IgY produced in mammalian cells could be detected using common lectins, thereby confirming that recombinant IgY from this source closely resembles the native immunoglobulin [60]. However, recombinant production of IgY in different hosts is likely to result in variable glycosylation patterns. As a result, the biochemical properties of recombinant and native IgY are likely to differ. Since glycosylation of immunoglobulins is not only implicated in a variety of physiological mechanisms but also influences their physicochemical behaviour, recombinant IgY may not always be suitable for all the potential applications envisaged for it.

To summarise, authentic polyclonal IgY is relatively easy to generate while recombinant antibody technology provides access to avian monoclonal antibodies. Moreover, pre-existing antibodies can now be converted to IgY when necessary for specific individual applications.

4. Therapeutic potential of IgY

Eggs constitute a very common component of our diet and are therefore tolerated by the human immune system. Topical administration of IgY may therefore represent an attractive approach to immunotherapy with a reduced risk of toxic side effects. While it is now widely accepted that IgY applied to human mucosal surfaces does not exhibit any immunogenicity, potentially detrimental effects might be anticipated in patients that are sensitized against egg proteins (including IgY), but this aspect is discussed later in the context of assay performance (see Section 6). Although immunogenic when applied systemically, the oral uptake of IgY antibodies opens up new possibilities for therapeutic interventions with respect to a variety of pathologies including, but not limited to, pulmonary or gastrointestinal infections (for overview see Table 1) [70]. Such approaches have been effective in reducing bacterial and viral loads in animal studies as well as in clinical trials in human cohorts [71–73].

Besides being suitable in approaches that target infective processes, IgY has been suggested for blocking, inhibition and delivery in those pathological conditions which demand specific reagents in substantial amounts. Chicken antibodies are well established as anti-toxins and/or for passive vaccination. For instance, specific anti-venom IgY can neutralize bacterial toxins [117] and be used to treat snake bites [118–125]. Indeed, anti-venom IgY can provide a higher bioactivity than antidotes raised in horses [126]. In such applications, egg yolks can provide a continuous supply of potentially superior reagents.

Today's consumers have become increasingly interested in foods that supposedly promote health and reduce the risk of disease. Incorporating egg yolks of immunized chickens into certain foodstuffs, for example drinking yoghurt or mouthwash can provide the consumer with a functional food that can potentially protect against pathogens (so-called "edible vaccines") without him or her having to consume synthetic pharmaceuticals [107,127–130]. A potential drawback of IgY in some therapeutic or prophylactic approaches is its reduced stability under harsh conditions such as an acidic environment. This is especially true for gastrointestinal applications. Different strategies to improve the therapeutic efficacy have therefore been evolved including a variety of techniques for stabilizing or controlling the release of IgY [86,131–133].

So far, the therapeutic interventions mentioned above are all confined to polyclonal native IgY obtained from egg yolk after immunization of hens. In contrast, the therapeutic potential of recombinant monoclonal IgY molecules remains to be explored, but it is perhaps here where the greatest potential of IgY paratopes fused to human Fc regions lies.

5. Avian antibody libraries

Applying combinatorial approaches in biology and chemistry demands high efficiency and where possible, simple and straightforward techniques. Chicken therefore provide an ideal basis for generating large immune antibody fragment libraries as compared to most mammalian species [134]. The inherent complexity of mammalian diversification mechanisms can make it difficult to recover antibody sequences. This is especially true in humans and mice. The genetic organization of these mammals is based on the modular use and recombination of a broad panel of V, D, and J segments which are further diversified by several different mechanisms. Therefore, accessing and amplifying mouse and human repertoires requires a large set of different oligonucleotides to cover the entire set of V segments which is prone to preferential amplification of high abundance transcripts and the potential loss of particular V segments during PCR. In chickens, genetic diversification is achieved differently. Both the heavy and light chain loci consist of single functional V and J genes, (and D segments for the heavy chain) that are rearranged using conventional V(D)J recombination mechanisms. In order to generate a large, diverse antibody repertoire and to allow affinity maturation upon antigen priming avian species utilize a unique mode of DNA recombination, termed gene conversion (reviewed in [135]). In this process, short DNA segments from non-functional V pseudogenes located upstream are inserted into the rearranged gene. These modulate the primary structure and, hence the binding characteristics of the resulting immunoglobulin. However, the 5′- and 3′-ends of the rearranged gene remain unaltered thus allowing the diversity of the chicken humoural immune system to be recovered by the use of only two pairs of primers.

The first avian repertoires were cloned more than a decade ago [134,136]. Somewhat surprisingly, at first little attention was paid to those libraries, a fact that might be attributed to a lack of familiarity with chicken immunization and the need for established recombinant antibody technologies. Over the past few years, chicken libraries have attracted wider interest and accordingly, reports on the isolation of chicken-derived antibody fragments have steadily increased (for an overview see Table 2).

The targets for these antibodies have included difficult antigenic structures such as haptens, highly conserved proteins and complex crude extracts. Interestingly, the avian VH/VL scaffold has been employed not only for the generation of immune but also naïve as well as semi-synthetic single chain antibody (scFv) libraries [143]. This approach allows entirely avian recombinant antibody formats aimed especially at diagnostics.

The exclusive use of single variable region genes makes the humanization of avian antibody fragments more practical than the humanization of rodent antibodies with their plethora of variable region genes. This could be shown for the engineering of an anti-prion and an anti-IL12 antibody [153,154]. Human frameworks and CDR grafting followed by further optimization were used to provide the proof of principle for this approach.

In summary, the generation and use of avian immune repertoire libraries represent a powerful approach with the potential to both complement established methods and to provide novel and original approaches. In addition, the different spectrum of epitopes recognized by the avian immune system could facilitate the development of novel therapeutics, particularly if the technology of chimeric chicken/mammalian fusions can be fully exploited.

6. Performance of IgY in immunoassays

Generally, one of the most intriguing and extraordinary characteristics of IgY is the lack of most, if not any, interactions with mammalian immune components. This makes IgY especially suited to applications in which the use of its mammalian counterparts is prone to unwanted cross-reactivities. For instance, in proteomics, pretreating of serum samples with IgY to specifically neutralize highly abundant serum components was found to improve downstream analyses [155]. In another study the identification of underrepresented serum proteins and disease marker candidate discovery was simplified when specific IgY was used as a blocking reagent [156,157]. This approach was facilitated by the general characteristics of IgY such as the ease of production and the low incidence of cross-reactivity.

Table 1
Overview of therapeutic approaches in humans and animals [71,72,74–117].

Disease	Antigen	Effect	Authors
Bovine coronavirus	Inactivated bovine coronavirus	Passive protection in neonatal calves	Ikemori et al. [74]
Candida albicans	*Candida ssp., C. albicans*	Reduction of inherence and inhibition of biofolm formation	Fujibayashi et al. [75]
	C. albicans antigen	Protection against oral candidiasis in mice	Ibrahim et al. [76]
Cholera	Killed O1 and O139, rCTB	Protection in suckling mice	Hirai et al. [77]
Clostridium difficile	rColonization factor	Inhibition of adherence and protection from infection in hamsters	Mulvey et al. [78]
E. coli	Heat-extracted antigens from ETEC strain 431	Prevention of fatal bovine colibacillosis in neonatal calves	Ikemori et al. [79]
	E. coli B16-4, enterotoxin, colonization factor antigen I	Prevention of diarrhoea in rabbits	O'Farrely et al. [80]
	K88, K99, 987P fimbrial adhesions	Passive protection of neonatal pigs against fatal colibacillosis	Yokoyama et al. [81]
	F18ab-fimbriae	Reduction of diarrhoea and death in infected pigs	Imberechts et al. [82]
	Fimbrial antigens of ETEC K88+	Passive protection in neonatal pigs	Marquardt et al. [83]
	Bacterial suspension of EPEC	Recognition of several bacterial virulence factors	Amaral et al. [84]
	E. coli O78:K80	Improvement of intestinal health indices and immunological responses in chickens	Mahdavi et al. [85]
	ETEC K88+, fimbrial antigen	Reduction of diarrhoea in pigs	Li et al. [86]
	B subunit protein of Stx1	Protection from toxin challenges in mice	Wang et al. [87]
Glioma	Membrane fractions of rat C6 cells enriched in metalloproteolytic activity	Inhibition of spreading, migration and invasion of C6 cells *in vitro*	Hensel et al. [88]
Heliobacter pylori	Cell lysate, H. pylori 58 kDa antigen	Inhibition of infection in mice	Attallah et al. [89]
Human and bovine rotavirus	Purified human rotavirus	Prevention of development of gastroenteritis	Yolken et al. [90]
		Virus neutralization in suckling mice	Hatta et al. [91]
	Human group A rotavirus	Protection in suckling mice	Ebina et al. [92]
	Bovine rotavirus	Protection against homologous BRV in calves	Kuroki et al. [93]
Human enterovirus Type 71	Inactivated human EV71	Reduction of morbidity and mortality in infected mice	Liou et al. [94]
Infectious bursal disease virus	IBDV	Passive protection in chicks	Eterradossi et al. [95]
Inflammatory bowel disease	Tumour necrosis factor (TNF)	Reduction of inflammatory end points in rats	Worledge et al. [96]
Influenza	H5N1 virus vaccines, inactivated H1N1 virus	Protection from infection in mice	Nguyen et al. [97]
	Swine influenza virus vaccine	Neutralizing of virus A/H1N1	Tsukamoto et al. [98]
Listeria monocytogenes	*L. monocytogenes*	Growth inhibition	Sui et al. [99]
Malignant diseases	P110 purified from human stomach cancer MGC-803 cells	Recognition of gastrointestinal system cancers	Yang et al. [100]
P. aeruginosa	Mixture of formalin-treated pathogenic bacteria	Growth inhibition *in vitro*	Sugita-Konishi et al. [101]
	P. aeruginosa	Retention of specific IgY in human oral cavity	Carlander et al. [102]
		Prevention of PA colonization in humans	Kollberg et al. [71]
		Prevention of infection in humans	Nilsson et al. [103]
Porcine epidemic diarrhoea virus	Concentrated PEDV	Immunoprophylactic effect in piglets	Kweon et al. [104]
S. aureus	Mixture of formalin-treated pathogenic bacteria	Inhibition of production of enterotoxin A	Sugita-Konishi et al. [101]
	Staphylococcal enterotoxin B	Passive protection in mice and rhesus monkeys	LeClaire [105]
S. mutans	*S. mutans* MT8148 serotype c	Protection against dental caries in rats	Otake et al. [106]
		Mild reduction of S. mutans in human saliva	Hatta et al. [107]
	S. mutans glucan binding protein B	Protection against dental caries in rats	Smith et al. [108]
	Cell-associated glycosyltransferrases	Reduction of smooth surface lesions and sulcal surface caries	Kruger et al. [72]
Salmonella spp.	Fimbriae of SEF14	Passive protection in mice	Peralta et al. [109]
	S. thyphimurium and *S. dublin*	Protection in neonatal calves	Yokoyama et al. [110]
	S. enteritidis	Inhibition of adhesion and invasion of S.E.	Sugita-Konishi et al. [111]
	S. enteritidis and *S. thyphimurium*	*In vitro* binding and growth inhibition of bacterial cells	Lee et al. [112]
White spot syndrome virus	Inactivated WSSV and DNA	Protection in crayfish	Lu et al. [113]
Xenograft rejection	Alpha-Gal antigen epitopes and other porcine aortic endothelial cell antigens	Inhibition of pig-to-human xenograft rejection	Fryer et al. [114]
	Alpha-gal epitope	Protection against porcine xenograft rejections	Leventhal et al. [115]
Y. ruckeri	Formalin-killed cells of serovar 1 (RS1154) and serovar 2 (RS1153)	Passive protection in rainbow trouts	Lee et al. [116]

In the case of immunoassays, homologous mammalian immunoglobulins may have deleterious effects on the performance of many different types of immunoassays. In particular, approaches using immunoglobulins as bioactive molecules to capture or detect the analyte are often affected by heterophilic antibodies and/or high levels of non-specific binding. In addition, antigen-independent specific binding via immunoglobulin Fc receptors or lectins and non-immunoglobulin-based interactions, e.g. those mediated by complement factors [158], can result in false-positive and false-negative results [159,160]. As recently summarized [159],

Table 2
Overview of established avian libraries [40,134,136–151].

Antigens	Size	Source	Authors
Allergens (rFel d 1, nAmb a 1, YJV extract)	7.2×10^8	Spleen and bone marrow	Finlay et al. [137]
BSA, lysozyme, bovine thyroglobulin	2.7×10^7	Bursal lymphocytes of naïve chicken	Davies et al. [134]
C-reactive protein (CRP)	3×10^7	Spleen and bone marrow	Leonard et al. [138]
Domoic acid-BSA	3.1×10^8	Spleen and bone marrow	Finlay et al. [139]
Fluorescein-BSA	9.6×10^7; 5×10^6 (scFv-libraries), 3.8×10^7 (chimeric Fab-library)	Spleen and bone marrow	Andris-Widhopf et al. [140]
Fragments of SARS-CoV spike protein	5×10^7	Spleen	Lee et al. [141]
Halofuginone	2.5×10^7 (original library) 1.2×10^7 (chain-shuffled library)	Spleen	Fitzgerald et al. [142]
Haptens, proteins, viruses	2×10^9	Naïve bursae	Van Wyngaardt et al. [143]
Human clan III Ig	$>8.5 \times 10^8$	Bone marrow and spleen	Cary et al. [40]
Human LDL	nd	Spleen	Sato et al. [144]
IBDV strain 002/73	1.5×10^5 and 7.5×10^7	Spleen	Sapats et al. [145]
Live endothelial progenitor cells	2.7×10^8	Spleen and bone marrow	Bowes et al. [146]
Mixture of aldolase and actate dehydrogenase of *Plasmodium falciparum*, variant surface glycoprotein of *Trypanosoma sp*., and purified malignant catarrhal fever virus	6.7×10^8	Spleen	Chiliza et al. [147]
Mixture of autoantigens	2×10^8 and 1×10^8	B cells from bone marrow and peripheral blood lymphocytes	Hof et al. [148]
Murine serum albumin	1.4×10^7	Spleen	Yamanaka et al. [136]
Non-structural protein (NSP) 3ABC from foot-and-mouth disease virus (FMDV)	1×10^7	Spleen	Foord et al. [149]
Alpha-enolase	2.4×10^4, 3.5×10^5	Spleen	Leu et al. [150]
HA from H5N1	1.65×10^8	Spleen	Pitaksajjakul et al. [151]
Synthetic peptide	5×10^7	Bone marrow and spleen	Meyer et al. [152]
TNP conjugate Insect venoms	5×10^6	Spleen	Greunke et al., Manuscript in preparation

estimates of the prevalence of assay interference by heterophilic antibodies range from 1 to 80% [161–166]. A more thorough analysis of over 11,000 sera in an anti-CEA assay format revealed that about 4% of the results were potentially false [167]. Approaches to eliminate heterophilic antibody interference include the removal or inactivation of interfering immunoglobulins, e.g. by precipitation with PEG [168], the use of various buffer additives [167], or the modification of assay antibodies by proteolytically removing of Fc fragments [167,169]. It is unlikely, however, that a single method can resolve these problems [170]. Alternatively, recombinant modifications like the humanization of animal derived antibodies [171] and the use of single-chain fragments [172] can be useful. Other possibilities include shifting to non-immunoglobulin affinity systems [173] such as affibodies or aptamers. Each of these approaches is of course likely to suffer from the particular drawbacks inherent in these molecules.

Taking the foregoing into account, chicken-derived antibodies, either as polyclonal or monoclonal preparations, offer several obvious advantages over their mammalian homologues in certain applications since they do not interact with rheumatoid factor (RF), human anti-mouse IgG antibodies (HAMA), complement components or mammalian Fc receptors [174].

Recombinant avian library-derived antibody fragments, such as scFvs have low functional affinities since they are monovalent. The first group to express IgY in mammalian cells was able to produce heterotetrameric IgY antibodies in CHO cells by recloning IgY heavy and light chains from a chicken hybridoma cell line [59]. Additionally, Greunke *et al.* could demonstrate that mammalian cells can express a variety of artificial IgY constructs including chimeric IgY antibodies and homodimeric scFv-constructs with the latter showing increased secretion efficiencies [60].

Antibody constructs larger than individual scFvs may be more effective in some immunoassay systems. Chicken scFvs from a large semi-synthetic phage displayed library that recognised the 65 kDa heat-shock protein (HSP65) of *Mycobacterium bovis* were converted into larger bivalent constructs which more closely resemble IgY molecules. These "gallibodies" could be used for immunocapture in ELISA and could be readily conjugated to colloidal gold nanoparticles [66].

Although most currently used immunotests are based on murine monoclonal antibodies, we have recently provided further evidence for the potential of the use of monoclonal IgY [60,175] as a way of avoiding interference by RF and heterophilic antibodies in human serum samples [175]. Work in our laboratory has shown that monoclonal and polyclonal IgY antibodies bind neither to mammalian Fcγ receptors CD64 and CD16A [175] nor to the human high affinity IgE receptor, despite similarities in the amino acid sequences of human IgE and avian IgY. The low degree of relationship between mammalian and avian Fc receptors [27,31,176,177] explains these findings.

For some diagnostic applications, the advantages of IgY may be undermined by the prevalence of anti-chicken antibodies in certain individuals. Although various hen-egg proteins were implicated in allergies by both *in vivo* and *in vitro* investigations as early as 1912 [178], reports on the occurrence of human IgY-specific antibodies are scarce and are focussed on IgE-mediated hypersensitivity reactions. One study [179] demonstrated that 15 in 28 egg-allergic patients exhibited specific IgE binding against one or more egg yolk-derived antiviral chicken immunoglobulins. In contrast, according to another study the overall allergenic potential of IgY in animal models appears to be low [180]. To what extent IgY-specific antibodies of IgG, the isotype that is most relevant in immunological analyses, occur in individuals sensitized to egg yolk remains to be established.

Yet another potential source of unwanted interference in immunoassays might result from the interaction of carbohydrate binding serum proteins such as mannose-binding lectin (MBL) with N-linked glycostructures in the IgY Fc region [17,181]. However, such problems largely depend on the particular expression host needed to produce the IgY and could conceivably be counteracted

by deleting the particular asparagine residues responsible for the interaction. Both N-glycosylation sites can be eliminated in recombinant IgY without severely affecting binding behaviour and production efficiency (unpublished observation).

Although it may well be worthwhile to convert many of the existing hybridoma-derived antibodies used in problematic immunoassays into recombinant IgY, the biochemical characteristics of such murine/avian chimeras might differ from those of the authentic avian antibody. Interference may still arise from proteins that interact specifically with rodent immunoglobulin variable regions such as HAMA, a potential consequence of therapeutic interventions using chimeric therapeutic antibodies. Today, however, since humanized antibodies are becoming more readily available for clinical applications, a decrease in the prevalence of HAMA can be expected.

Another approach to improving the reliability of immunoassays based on IgY and one of its receptors was developed very recently. In immunoassays aimed at detecting circulating Ig species specific for pathogens or other antigens, pools of human sera represent the immunologist's first choice as controls. These are sometimes not readily available, are usually expensive and vary in quality. Instead, artificial substitutes for human reference sera specific for virtually any protein of interest could easily be established (unpublished results) by using avian polyclonal or (under certain circumstances) monoclonal IgY complexed with the IgY-specific CHIR-AB1 ectodomain which has been genetically fused to human Ig Fc domains, as the binding moiety.

7. Conclusions

In summary, the ready availability of polyclonal egg-yolk immunoglobulins and the rise of recombinant technologies that can generate monoclonal IgY have focussed attention on the useful characteristics of avian antibodies. Moreover, the fact that monoclonal IgY and IgY-like constructs can now be obtained from combinatorial libraries, sometimes without immunisation, is likely to make IgY in all its manifestations much more widely used in research, diagnostics and therapeutics.

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