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Chicken egg yolk antibodies (IgY) as an alternative to mammalian antibodies

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Abstract

Chicken eggs present an ideal alternative antibody source to mammals, as the IgY in the chicken's blood is transported to the egg and accumulates in the egg yolk in large quantities. The existence of an IgG like molecule in avian eggs, referred to as IgY, has been well documented in recent studies and extensive research has been carried out on its characterization, production and purification. The yolks of eggs laid by immunized chicken have been recognized as an excellent source of polyclonal antibodies for over a decade. This simple non invasive approach presents an appealing alternative to conventional polyclonal antibody production methods. The use of immunoglobulin therapy broadens the arsenal available to combat pathogens in medicine and IgY is a promising candidate, both as an alternative to antibiotics and as a useful tool in research and diagnostics.

Keywords: Chicken antibody IgY; antivenom; passive immunotherapy; *Salmonella*.

Introduction

Antibodies presently available for research, diagnostic and therapies are mostly mammalian monoclonal or polyclonal antibodies. Traditionally, bigger animals such as horses, sheep, pigs, rabbits and guinea pigs were used for the production of polyclonal antibodies, while mice and rats were used for the production of monoclonal antibodies. Nowadays, most frequently chosen mammals for polyclonal and monoclonal antibody production are rabbits and mice respectively. Both technologies have their advantages but also disadvantages. Major problem of monoclonal antibody production is that some antigens are weakly or not at all immunogenic for mice. In polyclonal antibody production purification of antibodies from mammalian blood has been found to be low yielding and laborious in many cases. Both technologies also involve some steps each of which causes distress to the animals involved i) the immunization itself, ii) collecting of blood samples and iii) bleeding, which are a prerequisite for antibody preparation (Mojca Narat, 2003). During the past 20 years, the use of chickens instead of mammals for antibody production has increased. A major advantage of using birds is that the antibodies can be harvested from the egg yolk instead of serum, thus making blood sampling obsolete. In addition, the antibody productivity of an egg-laying hen is much greater than that of a similar sized mammal (Hau & Hendriksen, 2005).

Purification of immunoglobulin from mammalian blood is time-consuming and expensive. Today, hens are recognized as a convenient and inexpensive source of antibodies. It has been reported that the amount of immunoglobulin that can be yielded from one egg of an immunized hen is as much as that can be obtained from 300 ml of rabbit blood. Chicken egg yolk antibodies (IgY) have been applied successfully for scientific, diagnostic, prophylactic and therapeutic purposes. Because of the phylogenetic distance between birds and mammals, mammalian proteins are often more immunogenic in birds than in other mammals and antibody synthesis readily stimulated in hens (Bizhanov *et al.*, 2004). Laboratory production of antibodies involves immunization and bleeding of animals, causing distress to them. However, use of chickens for the purpose offers two advantages. The use of chicken for the antibody production, as opposed to mammals, represents both a refinement and a reduction in animal use. It is a refinement in that the second painful step, the collection of blood is replaced by antibody extraction from egg yolk. It enables the reduction in the number of animals used because chickens produce higher amounts of antibodies than laboratory animals. Larsson *et al.* (1993) found that IgY is more highly concentrated in egg yolk than it is in serum. The chicken is an excellent producer of antibodies, but despite this, is still an underused resource. This may be due to lack of information concerning the different methods and applications where IgY is more advantageous compared to the traditional mammalian IgG antibodies (Table 1). This review is on the subject of IgY, where basic properties as well as direct applications of IgY, both in assays and in therapy have been studied.

Chicken immunoglobulin (IgY)

Three immunoglobulin classes, analogues to the mammalian immunoglobulin classes have been shown to exist in chicken, IgA, IgM and IgY (IgG). The presence of antibodies homologous to mammalian IgE and IgD has also been proposed but has not been proven (Burns & Maxwell, 1981; Chen *et al.,* 1982). The molecular

Indian Journal of Science and Technology Vol. 3 No. 4 (Apr. 2010) ISSN: 0974- 6846

weights, morphology and immunoelectorophoretic mobility of chicken IgA and IgM are similar to mammalian IgA and IgM. IgY is the major low molecular weight serum immunoglobulin in oviparous (egg laying) animals. The overall structure of IgY is similar to mammalian IgG, with two light (L) and two heavy chains.

Table 1. Comparison of mammalian IgG & chicken IgY (Schade et al., 1991)

Animals	Rabbit (IgG)	Chicken (IgY)
Source of antibody	Blood serum	Egg yolk
Kind of antibody	Polyclonal	Polyclonal
Antibody sampling	Bleeding	Collecting eggs
Antibody amount	200 mg/bleed (40 ml blood)	100 ± 150 mg/egg
Quantity of antibody (per year)	1400 mg	40, 000 mg
Amount of specific antibody	~5%	2 ±10%
Protein A/G binding	Yes	No
Interaction with mammalian IgG	Yes	No
Interaction with rheumatoid factor	Yes	No
Activation of mammalian complement	Yes	No

Structure and characteristics of avian IgY versus mammalian IgG

Initially, avian serum immunoglobulins were classified as IgG-like immunoglobulins that are transferred to the egg yolk. In 1969, Leslie and Clem showed experimental data proving profound differences in their structure and proposed the name IgY. Among all birds, chicken IgY is most frequently studied, best described and characterised. General structure of IgY molecule is the same as of IgG with 2 heavy (Hv) chains with a molecular mass of 67-70 KDa each and two light (L) chains with the molecular mass of 25 KDa each. The major difference is the number of constant regions (C) in H chains: IgG has 3 C regions (Cv1 - Cv3), while IgY has 4 C regions (Cv1 - Cv4). One additional C region with two corresponding carbohydrate chains has a logical consequence in a greater molecular mass of IgY compared to IgG *i.e.* 180 and 150 KDa, respectively. IgY is much less flexible than IgG due to the absence of the hinge between Cv1 and Cv2, which is a unique mammalian feature. There are some regions in IgY (near the boundaries of Cv1-Cv2 and Cv2-Cv3) containing proline and glycine residues enabling only limited flexibility. IgY has isoelectric point 5.7-7.6 and is more hydrophobic than IgG. Regarding the relatively high core body temperature of chickens, which is 41°C, it is not surprising, that half-life time of IgY is in months and that they retain their activity for 6 months at room temperature or for one month at 37°C. Structure differences are reflected in different molecular and biochemical interactions. Most biological effector functions of immunoglobulins are activated by the Fc region, where the major structure difference between IgG and IgY is located. That is why typical IgG-Fc dependent functions are essentially different in IgY molecule. First, IgY do not activate the complement, second, IgY do not bind to protein-A and G, third, IgY do not bind to mammalian antibodies *i.e.* to rheumatoid factors (RF, an autoantibody reacting with the Fc portion of IgG) or to HAMA (Human Anti-Murine Antibodies), and fourth, they do not bind to cell surface Fc receptor. These differences in molecular interactions bring great advantages to the application of IgY antibodies that were successfully applied in a variety of methods in different areas of research, diagnostics, medical application and biotechnology.

Serum IgG antibodies of immunized chicken were efficiently transported and accumulated in the egg yolk (Patterson *et al.,* 1962; Rose *et al.,* 1974; Bar-Joseph & Malkinson, 1980). High levels of antibody activity in egg yolk were maintained for several months by periodic immunization. Furthermore, vaccination of small animals such as chickens can be performed easily (Polson *et al.*, 1985; Altschul *et al.,* 1984). Chicken antibodies recognise more epitopes on a mammalian protein than the corresponding rabbit antibody does, making it advantageous to use IgY in immunological assays of mammalian proteins. This is especially true when the antigen is a highly conserver protein, such as a hormone (Gassmann *et al.*, 1986; Gourele *et al.,* 1990). Chicken antibodies do not react with anti-mammalian antibodies in human serum, such as rheumatoid factors and human anti-mammalian anti-IgG. In immunological assays the interference caused by these antibodies can be problematic, particularly as the sensitivity of the assay increases. Thus, if chicken antibody is used, interference by anti-mammalian IgG antibodies is eliminated (Lindmark *et al.*, 1983). Larsson and Sjoquist (1989) developed a novel latex agglutination method with chicken anti-protein-A for detection of *Staphylococcus aureus* infections. The assays utilized latex particles coated with chicken anti-protein-A antibodies. These assays were reported to have sensitivity exceeding 99% for *Staphylococcus aureus* and they didn't give any false positive results with other staphylococci. Jensenics *et al.* (1981) suggested that chicken antibodies might also be of interest for developing other bacteriological assays when there is a risk of false positive results owing to reactions between Fc region of mammalian antibodies and staphylococcal protein A or streptococcal protein G. Akita *et al.* (1998) reported that immunoaffinity chromatography using immobilized IgY used for the isolation of IgG or the individual subclasses for supplementation in immunotherapy or selective removal of antibodies from milk if clinical studies confirm the potential adverse effect of IgG. The chicken egg yolk anti-pPGalMan antibody-antigen interaction was not as sensitive as that from the

Indian Journal of Science and Technology Vol. 3 No. 4 (Apr. 2010) ISSN: 0974- 6846

rat on fungal (Galactomannan), the use of chickens may have utility in some situations in which antibody can be stored in the egg for long periods (Tuekem *et al.,* 1998).

The non-invasive approach offers the ease of isolation and purification of antibody. IgY antibodies specific for *Salmonella* OMP, LPS and Fla may protect mice from experimental salmonellosis when passively administered orally. They also reported that anti-outer membrane protein (anti-OMP) exhibited highest level of protection *in-vitro* and *in-vivo* (Hidekai *et al.,* 1998). Calzado *et al*. (2001) reported that the highest titres were observed four weeks after the first immunization, and these remained stable for up to seven weeks for the intravenous route. Positive reactivity against human erythrocyte antigens A, B and O was demonstrated in de-lipidated supernatants from the egg yolks of immunised hens. The strongest reaction was observed against blood group O Rh+ (O+). Treatment with hyperimmune egg yolk (HEY) against four human rotavirus strains resulted in modest improvement of diarrhea associated with earlier clearance of rotavirus from stools. These results indicate an encouraging role of HEY in the treatment of rotavirus-induced diarrhea in children. The proven rotavirus diarrhea was treated with immunoglobulin extracted from eggs of chicken immunized with human rotavirus strains (Sarker *et al*., 2001). The passive antibody to *S. mutans* GBP-B can have a protective effect against cariogenic *S. mutans* infection and disease. Furthermore, this decrease in infection and disease did not require continuous antibody administration for the duration of the infection period (Smith *et al.,* 2001). The specific activity and IgY contents when purified by ammonium sulphate were 1.6-2.0 and 1.6-1.8 fold higher than those purified by the Zinc sulphate or cadmium sulphate method (Bizanov *et al.,* 2003). Hodek *et al.* (2003) reported chicken antibodies as superior alternative for conventional immunoglobulins. Antibodies purified in large amount from egg yolks were found to be suitable for passive immunization against pathogenic microorganisms and toxins. Bizhanov *et al.* (2004) developed a novel method, based on lithium sulfate precipitation for purification of chicken egg yolk immunoglobulins (IgY), applied to immunospecific antibodies against Sendai virus. They developed IgY purification methods based on salt precipitation using lithium sulfate and sodium citrate. These methods were compared with polyethylene glycol precipitation and chloroform extraction methods. The results indicate that the purification of IgY by lithium sulfate results in very pure IgY in high quantities. Raj *et al*. (2004) reported that caprylic acid precipitation method gave the highest recovery of IgY and with high purity. The anti-infectious bursal disease -IgY (anti-IBD-IgY) was found to possess immunodiagnostic potency as assessed by agar gel precipitation test and counter immunoelectrophoresis for replacing the use of conventional antibody (Shanmugasamy *et al*., 2005). IgY to rabies recombinant proteins could serve as a reagent for diagnosis of rabies virus infection (Motoi *et al*., 2005). The post-immunization IgY might be considered a prophylactic agent or possibly an adjunct to antifungal therapy. Pre-immunization IgY appeared to contain factors that prolonged survival, but did not prevent dissemination of the fungus (Alexander *et al*., 2006). Anti-Pseudomonas IgY has great potential to prevent *P. aeruginosa* infections (Nelson *et al*., 2008). Zhen *et al*. (2009) reported that the cure rates by chicken antibodies (IgY) for experimental and clinical mastitis were 83.3% and 50%, respectively he also reported that the IgY served as an alternative therapy for mastitis caused by *S. aureus*.

Antivenom

It has been estimated that 5 million snake-bite cases occur worldwide every year, causing about 100,000 deaths (Brunda *et al*., 2006). Antivenom is the specific antidote for snakebite envenomation. Until now, anti snake venom for neutralizing snake bite venom has been made by injecting horses with small quantities of venom to produce an immune reaction. Antibodies are then harvested from the animal's blood. Products of animal serum can produce adverse side effects such as anaphylactic reactions and serum sickness. Thalley and Carroll (1990) described a new avian source of antivenom that precludes these complications and an efficient method for preparing antivenoms composed solely of venom specific antibodies. Scientists at Vittal Mallaya Scientific Research Foundation generated antivenom specific antibodies in white leghorn chicken and their egg yolks. They recently reported that the purity, efficacy and ease of manufacture of avian antivenoms and their inability to react with mammalian complement make them an attractive alternative to equine antivenoms. He also specified that over 10-15 mg of venom specific antibodies can be obtained from an immunized chicken's egg yolk. Adult white leghorn hens hyperimmunized with Brazilian snake venoms produced antibodies capable of recognizing, combining with and neutralizing the toxic and lethal components of the venoms (Almeida *et al*., 1998). The antibodies are present in the egg for upto 100 days after the immunization. The antivenom purified from immunized chicken egg yolk is biologically active (Devi *et al*., 2002 a,b). Paul *et al*. (2007) reported this system as a more convenient alternative to current conventional production of equine anti-snake venom. Antibodies raised in chicken could effectively neutralize the pharmacological effects induced by venoms and chickens therefore present an alternative and cheaper source of specific antibody generation, he also indicated that the antivenom generated in chicken could be used for therapeutic purposes in case of snakebite envenomation (Meenatchisundaram *et al*., 2008 a,b).

Immunization

Different immunization protocols, using different adjuvants, antigen dose and volume, route of injection,

Indian Journal of Science and Technology Vol. 3 No. 4 (Apr. 2010) ISSN: 0974- 6846

vaccination frequency and interval were described. Basically different immunizing protocols for each antigen and for each animal species have to be tested to find out which method induces the highest serum and egg yolk antibody titer. Usually 10–100 µg of protein antigen in a final volume of 1ml is applied intramuscularly in the breast muscle at two or three injection sites of 7 to 8 week-old chicken. To avoid an eventual local tissue reaction the Freund's incomplete adjuvant could be efficiently used even for the first immunization. Vaccination frequency and interval depend on the immunogenic potential of antigen itself and on adjuvant used (Leenaars *et al*., 1999) (Table 2).

Table 2. Comparison of rabbit and chicken polyclonal antibody yield during a two-week period following the second immunization (Leenaars et al., 1999)

	Rabbit	Chicken
No. of animals	1	1
Method of sampling	Bleeding (20 ml/week)	daily collecting of eggs
Sample volume (in 2 weeks)	40 ml of blood	14 eggs= 210 ml of egg yolk*
Amount of total antibodies	200 mg	1120mg**
Amount of specific antibodies	5% (10 mg)	2-10% (22.4-112mg)
Rabbits/chicken-total***	5 -6	1
Rabbits/chicken-specific****	2-11	1
Presence of other Ig	IgM, IgA, IgE	None

**average volume of egg yolk is 15mL; **average amount of IgY is 80mg per one egg yolk; ***No. of rabbits that produce an equal amount of total antibodies as one chicken in a two-week period; ****No. of rabbits that produce an equal amount of specific antibodies as one chicken in a two-week period*

The presence of yolk antibodies should be checked two weeks after the second immunization. When the antibody titer decreases booster immunizations can be given during the whole laying period. A laying hen produces five to six eggs per week. Average volume of egg yolk (15 ml) contains 50–100 mg of IgY, of which 2 to 10% are specific antibodies.

Isolation and purification methods for IgY

Several methods were described in the 1950s for purifying IgY based on the strategy of separation of proteins (levitins) from lipoproteins (lipovitellins) and the rest of the yolk lipids using extraction with organic solvents with rather low yields of antibody. However, purification methods based on organic solvents like chloroform remain in use (Kovacs *et al*., 2004). Other methods are based on affinity chromatography or on dilution of the yolk followed by a freezing-thawing process after which the process consists of ion exchange chromatography and salt precipitations often combining a number of salts like for e.g. polyethylene glycol (PEG), dextran sulfate, dextran blue, sodium sulfate, ammonium sulfate, caprylic acid and sodium citrate. More recently methods combining chloroform removal of lipids with ammonium sulfate precipitation techniques have been shown to result in a good yield of antibodies of high purity. The effect was pronounced with λ–carrageenan and the lipid content in the supernatant after removal of the resulting precipitate was less than 0.4% of that of egg yolk. Hatta (1990) reported that the IgY remaining in this supernatant was isolated by DEAE-Sephacel column followed by salting-out with sodium sulfate resulting in almost pure IgY (98%) and the yield was 70-100 mg per egg. Water dilution method found to be superior in terms of ease of use and large scale production of IgY. This is simple rapid and efficient means of purifying IgY with high activity (Akita & Nakai, 1993). The IgY recovery from thiophilic interaction chromatography is close to 100%, providing a simple and efficient means for purifying IgY from egg yolk. They also determined that the amount of specific antibody present in egg yolk from an immunised chicken is around 1% of total IgY. A particularly efficient method consists of two successive precipitations in PEG, using 3.5% PEG to remove fatty substances, and then 12% PEG to precipitate the IgY (Per Hansen *et al.,* 1998). Purification of IgY by PEG and ammonium sulphate yielded very pure IgY at high quantities (93% ± 5% of total egg yolk protein), which was also capable of neutralizing toxic and lethal components of the *E. carinatus* venom (Meenatchisundaram *et al*., 2010). An improvement of this method incorporates an emulsification step, adding one volume of chloroform rather than using 3.5% PEG. It is generally assumed that about 100 mg of IgY can be recovered per egg yolk (Kovacs *et al*., 2004).

Adjuvant effects on antibody production

Adjuvants are used to stimulate the immune response of experimental animals; the desired antigen is applied in combination with various adjuvant compounds. The ideal adjuvant can be characterized as a substance which stimulates high and sustainable antibody titers even with small quantities of antigen (Hodek *et al.,* 2003). Water-in-oil emulsions, which include Freund-type adjuvants, are the adjuvants most commonly used to produce pAbs in laboratory animals. Freunds Complete Adjuvant (FCA) is composed of inactivated and dried mycobacteria (usually *Mycobacterium tuberculosis)* and the incomplete form (FIA) is the same adjuvant, but without the mycobacterial components. Freund's complete adjuvant may be used only for the first (priming) antigenic dose. The incomplete adjuvant may be used for subsequent immunizations. Montanide ISA 740 adjuvant is composed of highly purified mannitol octadecenoic esters (Montanide ISA 80) as surfactant, in a mixture of metabolisable oil and a

Indian Journal of Science and Technology Vol. 3 No. 4 (Apr. 2010) ISSN: 0974- 6846

refined non-metabolisable light mineral oil classified pharmacologically as an excipient. This mixture can form a stable emulsion (especially under nitrogen storage), in the weight ratio 70:30 Montanide ISA 740: aqueous phase antigen. When properly formulated, the emulsion will remain in a single phase for at least 2 years. This adjuvant emulsion is easy to inject and is well-tolerated by the recipient animal. When injected subcutaneously into mice or guinea-pigs in accordance with the European Pharmacopoeia, there are no serious adverse effects. Aluminum adjuvants in the form of aluminum hydroxide or aluminum phosphate hydrated gels can be injected subcutaneously or intramuscularly for priming an immune response in the recipient. These adjuvants are generally regarded as safe and they have been used for human vaccination for more than 50 years. The biological function of these adjuvants is related to their ability to adsorb protein antigens, thereby ensuring that soluble proteins will be taken up as particulate antigens by antigen-presenting cells. Due to this adsorption/function relationship, it is strongly recommended that investigators ascertain that adsorption of the antigen to the gel has been successfully accomplished prior to its injection. Saponins are triperpene glycosides which are derived from the bark of the *Quillaja saponaria* tree and which have detergent and adjuvant properties. Saponin preparations intended for use as immunological adjuvants are purified to reduce the presence of components which cause adverse local reactions. Food-grade saponin preparations should not be used for immunization schemes. In general, saponins should not be injected intraperitoneally or intravenously, but only subcutaneously or intramuscularly, due to their hemolytic activity. Just recently, application of lipid nanoparticles causing only minor tissue irritation at the injection sites appears to be a promising alternative to Freund's Complete Adjuvant (Leenaars *et al*., 1999). Meenatchisundaram *et al.* (2009) reported that Freund's adjuvant works well without causing any side effects to birds and the antibody production also good. He also reported that Freund's adjuvant is the preferred adjuvant to generate chicken egg yolk antivenom antibodies to treat snake bite envenomations.

Antibody stability

IgY is fairly heat stable and most antibody activity remain after 15 min at 70°C. Incubation of IgY at pH above 4 is well tolerated, but at pH 2 and 37°C the activity is rapidly decreased. The rapid activity loss is probably due to conformational changes, as the polypeptide is not broken down as observed by SDS-PAGE. The immunological activity of IgY is not affected by pasteurization at 60°C for 3.5 min. Addition of high concentrations of sucrose stabilizes IgY regarding heat denaturation, acid environment as well as high pressure. IgY fractions have been stored in 0.9% NaCl, 0.02% NaN_3 at +4°C for over 10 years without any significant loss of antibody titer. Affinity-purified and 21 biotinylated antibodies have after 5 years of storage at +4°C retained high activity. The purified antibodies also retained their antigen binding capacity after 6 months at +20°C or 1 month at +37°C. An egg can be stored in +4°C, with just a small loss of IgY activity for at least six months (Carlander, 2002).

Conclusion

Since it is possible to produce antibodies in chicken against a vast array of antigens and epitopes, there exists scope for raising antibodies against any number of bacterial, viral, or biological antigens. The significant potential of avian antibodies for further use in immunodiagnostics and identification of disease markers, immunotherapy and the treatment and prevention of disease is expected. Since lot of benefits of IgY technology and its universal application in both research and medicine, it is expected that IgY will play an increasing role in research, diagnostics, and immunotherapy in the future.

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Indian Journal of Science and Technology Vol. 3 No. 4 (Apr. 2010) ISSN: 0974- 6846

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