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Chicken Antibodies

A Clinical Chemistry Perspective

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ABSTRACT

The chicken immune system has been studied for many years and these studies have contributed substantially to our understanding of the fundamental concepts of immunology and the development of different immunoglobulin classes. It is thus surprising that only a small fraction of the antibodies presently used in laboratories are of avian origin. A laying hen produces more yolk antibodies than a rabbit can produce during the same time period, and the animal care costs are lower for the chicken compared to the rabbit.

Chicken antibodies offer many advantages to the traditional mammalian antibodies when used for the detection of mammalian antigen. Due to the evolutionary difference chicken IgY will react with more epitopes on a mammalian antigen, which will give an amplification of the signal. Chicken antibodies can also be used to avoid interference in immunological assays caused by the human complement system, rheumatoid factors, human anti-mouse IgG antibodies (HAMA) or human and bacterial Fc-receptors. The antibodies can be purified in large amounts from egg yolk, making laying hens highly efficient producers of polyclonal antibodies.

THE CHICKEN IMMUNE SYSTEM

Studies of the chicken immune system have contributed substantially to our understanding of the immune response, including separation of the T- and B-cell lineages. The chicken immune system consists of the bursa of Fabricius, bone marrow, spleen, thymus, the Harderian gland, lymph nodes, circulating lymphocytes and lymphoid tissue in the alimentary tract. The studies of Glick et al (12) showed that antibody synthesising cells (B-cells) were produced by the bursa of Fabricius. The chicken bone marrow is the source of bursal and thymic stem cells while the spleen is the centre for plasma cell proliferation and memory B-cells (42). Birds without spleen have a lower antibody production (1). The thymus is a maturation centre where stem cells differentiate into T-lymphocytes. The activities of chicken T-lymphocytes are similar to those in mammals.

CHICKEN ANTIBODIES

Three immunoglobulin classes (IgA, IgM and IgY) have been shown to exist in the chicken (4,28,29). The presence of antibodies homologous to mammalian IgE and IgD have also been proposed (7,10). The molecular weights, morphology and immunoelectorophoretic mobility of chicken IgA and IgM are similar to mammalian IgA and IgM.

The low molecular weight (LMW) serum antibody found in birds, reptiles, amphibia and probably also in lungfish is the IgY whereas mammals have the IgG. Most species that have IgY are oviparous and IgY is usually a systemic rather than a secretory antibody and IgY is the main serum immunoglobulin in chicken. Chicken IgY is transported from the mother to the embryo via the egg yolk and the egg yolk thus contains high concentrations of chicken IgY. Other Ig classes are present only in negligible amounts in the egg yolk.

Chicken IgY (28), or chicken IgG as it is also called, is the functional equivalent of mammalian IgG in birds but it differs in many functional aspects to mammalian IgG (43). Chicken IgY consists of two light and two heavy chains and has a molecular weight of approximately 180,000 Da, the heavy chain (ypsilon, υ) has a weight of 66,000 Da and the light chain 22,000 Da. The υ-chain of IgY consists of four constant regions and one variable region and the light chain is composed of one variable and one constant domain. The Cυ3 and Cυ4 of the IgY are most closely related to the Cγ2 and Cγ3 of IgG respectively and the Cυ2 domain is absent in the γ chain. The Cυ2 region was probably condensed to form the hinge region of IgG as recent studies have shown that IgY is an ancestor to mammalian IgG and IgE and also to IgA.

ANTIBODY DIVERSITY

The antibody diversity in chicken differ from mammals and is mainly due to somatic hyper-conversion. Rearrangement apparently contributes little to the diversity as both the heavy and the light chain loci consist of only one functional V gene (38,44). There seems to be a deficiency in the mechanism for selecting higher-affinity somatic mutants.

Chicken antibody has the valency of 2.0 and sometimes higher which might be due to large antigen-binding sites (43). Most chicken IgY bind antigen strongly but display precipitation properties only at raised salt concentrations (16). The poor precipitation properties might be due to steric hindrance of the Fab arms to crosslink epitopes of two large antigens. The conditions permitting precipitation (salt ~1.5 M) might loosen the restricted movement of the Fab arms and give functional independence to the binding sites.

ADVANTAGES OF CHICKEN ANTIBODIES

As the difference between the antigen and the immunized animal increases, the immune response usually increases. There is a vast phylogenetic difference between avian and mammalian species compared to the difference between two mammalian species. This evolutionary spread means that there is no immunological cross-reactivity between chicken IgY and mammalian IgG (18). Thus, chicken should be a better choice than e.g. rabbits for the production of antibodies against mammalian proteins. Due to evolutionary differences chicken antibodies will bind to more epitopes on a mammalian protein than the corresponding mammalian antibody. It has been shown that 3-5 times more chicken antibody than swine antibody will bind to rabbit IgG (17), which will amplify the signal (35). Chicken antibodies also recognize other epitopes than mammalian antibodies (41). This gives access to a different antibody repertoire than the traditional mammalian antibodies.

Cross-reactivity occurs between IgG from different mammalian species. An increased background binding may result if a secondary anti-mammalian IgG antibody is used. The secondary antibody may cross-react with IgG that is present in a histological section or with bovine IgG in the bovine serum albumin solution often used for blocking purposes. Because chicken IgY is so different from mammalian IgG, no cross-reactivity occurs between the two. Thus, contrary to an anti-rabbit IgG antibody, a secondary anti-chicken IgY antibody will not react with mammalian IgG in the tissue and this may reduce background staining (Fig. 1).

Immune complexes formed with chicken antibodies are probably slightly different to those formed with rabbit antibodies. The precipitation curve is steeper and the antigen excess effect on immune complex formation is more pronounced. The antibodies also seem to scatter slightly less than the corresponding rabbit antibody in nephelometry or turbidimetry.

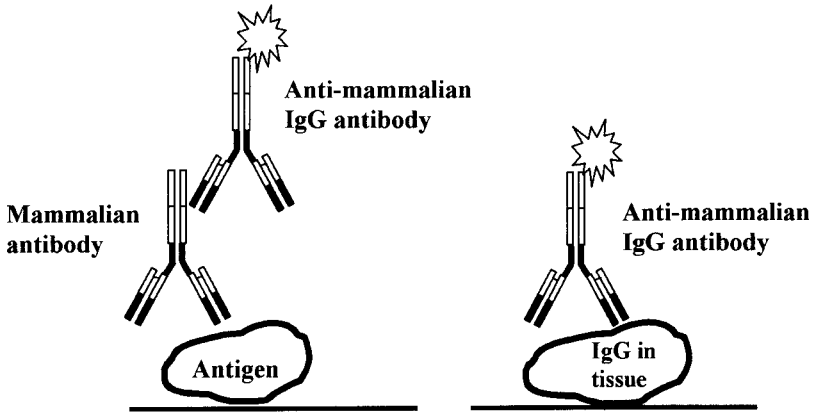

Fig. 1. Increased background in histochemical staining due to crossreactivity of the anti-rabbit IgG antibody used as second antibody with mammalian IgG in the tissue.

IgY REDUCES INTERFERENCE PROBLEMS IN IMMUNOLOGICAL ASSAYS

Complement activation

Mammalian antibodies bound to a solid phase and antigen-antibody complexes containing mammalian antibodies will activate the human complement system (26). Activated C4 is bound to the Fab region of IgG and thus may interfere with the antigen binding (8). Complement components may also solubilize precipitated immune complexes and prevent soluble immune complexes from precipitating (3,33). Such complement activation was shown to interfere in an immunometric TSH assay and depressed the TSH values by up to 40% (22).

In clinical laboratories, most analyses are performed on serum samples. A newly obtained serum sample contains an active complement system, but the activity declines during storage and handling. Thus, the complement activity may vary between different patients and also between different samples from the same patient. However, most of the standards and controls used have been stored and thus contain an inactive complement system. This difference in activity between the samples and the standards will cause erroneous results. Because, chicken antibodies do not activate the human complement system, they can be used as capture antibodies to reduce interference by complement activation (27).

Rheumatoid factor and human anti-mouse IgG antibody (HAMA) interaction

Rheumatoid factor (RF) and human anti-mouse IgG antibodies (HAMA) are probably the most well known causes of false positive or false negative reactions in immunological assays (6,14). RF is an autoantibody that reacts with the Fc part of mammalian IgG. The disease most often associated with RF is rheumatoid arthritis, but RF can be found in serum from patients with many other diseases and also in 3-5% of healthy blood donors (20). An increasing number of patients are treated in vivo with mouse monoclonal antibodies. This treatment often evokes an antibody response in the patient resulting in production of HAMA. HAMA may also be found in serum from patients who have not been treated with antibodies. However, the increasing use of monoclonal and polyclonal antibodies in vivo will increase the number of patient samples which contain HAMA.

RF or HAMA may react with both the capture antibody and the detection antibody in a sandwich assay, thus mimicking antigen activity (Fig. 2). They may also react with the detection antibody, resulting in formation of an immune complex. This immune complex may influence the activity of the detection antibody. HAMA may also react with the antigen-binding epitopes and inhibit the antigen binding. The problem of RF and HAMA interference will increase as the sensitivity of the assay increases. Interference by anti-IgG antibodies have been demonstrated in

approximately 40% of serum samples from healthy individuals in an immunoradiometric assay (5). RF and HAMA will also give erroneous results in nephelometry and turbidimetry as they change the size of antigen-antibody complex (9).

Chicken IgY do not react with RF or HAMA and can be used to avoid interference due to these factors (24,25).

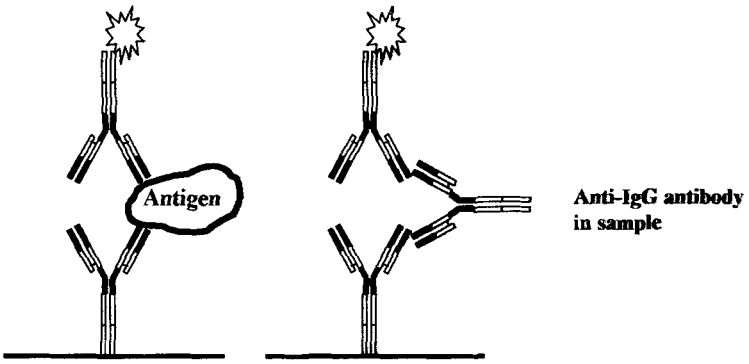

Fig. 2. A true positive reaction in a sandwich-ELISA (left), and a false positive reaction caused by rheumatoid factor or human anti-mouse IgG antibodies (right). The false positive reaction occurs when both capture and detection antibody are of mammalian origin.

Human Fc and complement receptor interaction

Whole mammalian IgG molecules contain the Fc portion of the antibody. Fc binds to Fc receptors, which are found on many types of blood cells (46). Human FcγRI has a high affinity for monomeric mammalian IgG, while FcγRII and FcγRIII mainly bind mammalian IgG complexes. There is often some aggregated IgG formed during the purification of IgG or during the labelling procedures which will increase the binding to FcγRII and FcγRIII receptors. Interaction with Fc receptors may cause an increased background staining. When working with live cells the interaction with Fc receptors may cause cell activation and changes in the expression of surface proteins. It has been shown that mammalian antibodies used in flow cytometry form immune complexes that cause platelet activation and changes in the expression of the GpIIb-IIIa receptor (30,39). No activation was observed when chicken antibodies were used (30). Immune complexes containing mammalian IgG may also stimulate the production of cytokines (46).

Complement receptors can also be found on many types of blood cells. When the mammalian antibody reacts with the antigen, an immune complex is formed which may activate the complement system. Also, the antibody aggregates formed during the preparation of the antibodies may activate the complement system and the interaction with complement receptors cause platelet activation (31).

Chicken antibodies do not react with human Fc receptors and do not activate the human complement system and can thus be used to avoid these problems (30).

Bacterial Fc-receptor interaction

Staphylococcal protein A and Streptococcal protein G are Fc-binding bacterial proteins which are widely used for their ability to react with mammalian IgG. Whole bacteria of the Staphylococcus aureus Cowan 1 strain and group C Streptococcus sp. are also used as immunoadsorbent for mammalian IgG. Staphylococci and Streptococci are often found in bacterial specimens. When present, they may bind detection antibodies with specificities for other bacteria and cause erroneous results. Chicken antibodies do not react with protein A or protein G (13) and thus can be used to reduce interference problems due to bacterial Fc receptors.

There are also other bacteria with Ig-binding capability (11,21). The binding of IgG to the Fc receptor probably have a protective function for the bacteria. Bacteria isolated from human specimens will have Fc receptors with affinity for human immunoglobulin. Due to the immunological similarities these Fc receptors will often bind other mammalian immunoglobulin but not avian IgY. On the other hand, bacteria isolated from avian specimens will probably have Fc receptors for avian immunoglobulin instead of mammalian IgG.

PRODUCTION OF CHICKEN ANTIBODIES

Antibody production normally requires the use of laboratory animals. The procedure involves two steps which causes distress to the animals: a/ the immunisation and b/ bleeding, which is a prerequisite for production of mammalian polyclonal antibodies.

The use of chicken egg yolk for antibody production represents a reduction in animal use as chickens produce larger amounts of antibodies than laboratory rodents. It also makes it possible to eliminate the collection of blood wich is painful for the animal. The European Centre for the Validation of Alternative Methods (ECVAM) thus recommends that yolk antibodies should be used instead af mammalian antibodies for animal welfare reasons (40). Antibodies can be produced by using chickens bred for commercial egg production as well as specific pathogen free (SPF) chickens. SPF chickens generally give higher antibody titres (15).

IMMUNISATION OF CHICKEN

Various protocols for raising antibodies in chickens have been reported but it is often difficult to compare the different methods. Chickens kept under field conditions are usually vaccinated intramuscularly in the breast muscle but it is also possible to use subcutaneous immunization. We usually use 25-100 µg antigen per immunization but it is possible to obtain a good immune

response with 1 μg/immunization (23). The antigen used for immunization is emulsified with an equal volume of Freund's adjuvant. The first immunization is performed with Freund's complete adjuvant and the booster immunizations with Freund's incomplete adjuvant. The hens are immunized intramuscularly in the breast muscle with 0.5-1 mL of emulsified antigen. After the initial immunization the animals receive 2-3 booster injections with 2-week intervals.

Chickens can be used for antibody production throughout their entire egg laying period. Animals that are used for antibody production for more than three months are given booster immunizations every other month so that the antibody titre remains high. Other types of adjuvant than Freund's adjuvant can also be used, such as Specol and the lipopeptide Pam_3-Cys-Ser-$(Lys)_4$ (40).

Antibody responses of lower vertebrates have a restricted diversity and limited affinity maturation (45). Despite this limitation chicken have developed the ability to produce very effective antibodies. We have immunized laying hens with approximately 100 different antigens and we routinely obtain a good immune response.

PURIFICATION OF CHICKEN IgG

To obtain material from which chicken antibodies can be isolated, either bleeding of the animal or collecting eggs is necessary. However, as the chicken has very fragile veins, bleeding is often difficult and results in large haematoma formation. Poor clot retraction can also limit the amount of serum obtained. Sometimes, only 100 μL of serum is obtained from 2 mL of blood. Thus, plasma is more useful than serum. A better way to obtain antibodies is to purify them from the yolk. Several methods can be used to purify chicken IgY from egg yolk (2,19,37). These methods can all be used for large scale purification of functionally active chicken antibodies. Over 100 mg of purified IgY can be obtained from a single egg (2). As a laying hen produces approximately 20 eggs per month, over 2 gram IgY per month can be isolated. The IgY concentration in chicken serum is approximately 5-7 mg/mL (32), therefore 2 gram of egg yolk IgY corresponds approximately to the IgY content of 300 mL of serum or 600 mL of blood. Only large mammals can produce equal amounts of serum antibodies.

It is also possible to purify the specific antibodies by affinity-chromatography. The antibodies are applied at a neutral pH to a column where the antigen of interest is bound to the matrix. The column is then washed with PBS (0.02 M Na_2HPO_4, 0.15 M NaCl, pH 7.2) to remove unspecific IgY. Bound antibodies are then eluted with 0.1 M glycine pH 2.25.

Monoclonal chicken antibodies have also been reported (34). So far, the number of different chicken monoclonal antibodies is limited, but is certain to increase in the future. This will allow the advantages of monoclonal antibodies to be combined with those of chicken antibodies.

LABELLING OF CHICKEN IgY

Chicken IgY can be labelled with the same methods that are used for mammalian antibodies. There are optimized labelling procedures described for biotin (35), FITC (30) and horse-radish peroxidase (23).

ANTIBODY STABILITY AND AFFINITY

Chicken antibodies purified from egg yolk appear to be very stable. We have stored IgY fractions in 0.9% NaCl, 0,02% NaN_3 at +4°C for over 10 years without any significant loss of antibody titre. We have also used affinity-purified and biotinylated antibodies after 5 years of storage at +4°C which have retained high activity (35). The purified antibodies also retained their antigen binding capacity after 6 months at +20°C or 1 month at +37°C.

The few investigations on the affinity constant of chicken IgY report a high affinity for mammalian proteins. The K-value for a chicken anti-α-fetoprotein antibody was found to be 2×10^{11} L/mole and for a chicken anti-albumin antibody 5.5×10^{11} L/mole (36). These K-values are higher than for most mammalian antibodies.

CONCLUSION

Chicken egg yolk antibodies can be produced more cheaply and with less suffering for the animal than for mammalian antibodies. Chicken IgY also have several biochemical advantages when used for quantifying mammalian or bacterial antigens. Egg yolk antibodies could thus replace traditionally produced mammalian serum antibodies in many immunoassays.

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