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Avian IgY Antibody

In vitro *and* in vivo

BY

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ABSTRACT

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Immunoglobulin Y (IgY) is the major antibody found in eggs from chicken (*Gallus domesticus*). IgY can be used as an alternative to mammalian antibodies normally used in research, and its use in immunotherapy has recently been proposed. Compared to mammalian antibodies, IgY possesses several biochemical advantages and its simple purification from egg yolk prevents a stressful moment in animal handling, as no bleeding is necessary.

Small amount of antigen (1 μg) can be used to elicit an immune response in chickens and there are low intra-individual differences regarding antibody concentration found in yolk. By studying two chicken breeds and their cross, a genetic correlation was shown regarding the IgY concentration, which implies a possibility by breeding to increase IgY concentrations. By using IgY instead of goat antibody as capture antibody in ELISA, it is possible reduce interferences by complement activation. After oral administration of IgY to healthy volunteers, IgY activity was present in saliva 8 hours later, indicating a protective effect. This effect has been studied in an open clinical trial with cystic fibrosis patients. Specific IgY against *Pseudomonas aeruginosa* given orally prolongs the time of intermittent colonization by six months, decrease the number of positive colonizations and might be a useful complement to antibiotic treatment. Immunoglobulin therapy may diminish the development of antibiotic resistant microorganisms. 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.

Key words: Antigen production, chicken, cystic fibrosis, egg, ELISA, IgY, immunotherapy, *Pseudomonas aeruginosa*, yolk.

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Omne vivum ex ovo

All living from the egg

Wherever you go, there you are

This thesis is based on the following papers, which are referred to by their roman numerals:

I. Larsson, A., Carlander, D. and Wilhelmson, M.
Antibody response in laying hens with small amounts of antigen.
Food and Agricultural Immunology, 10, 29-36. 1998.

II. Carlander, D., Wilhelmson, M. and Larsson, A.
Limited day to day variation of IgY levels in eggs from individual laying hens.
Food and Agricultural Immunology, 13, 87-92. 2001.

III. Carlander, D., Wilhelmson, M. and Larsson, A.
Immunoglobulin Y Levels in Egg Yolk from Three Chicken Genotypes.
In manuscript 2002.

IV. Carlander, D. and Larsson, A.
Avian Antibodies Can Eliminate Interference Due To Complement Activation In ELISA.
Upsala Journal of Medical Sciences, accepted 2001.

V. Carlander, D., Kollberg, H. and Larsson, A.
Duration of specific yolk Ig Y in the human oral cavity.
Submitted 2002.

VI. Carlander, D., Olesen, H., Kollberg, H., Johanesson, M., Wejåker, P.-E. and Larsson, A.
Oral IgY antibodies prevents colonization of *P. aeruginosa* in cystic fibrosis.
Submitted 2001.

LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
CH	Constant, Heavy region of Ig
EDTA	Ethylene diamine-tetraacetate
ELISA	Enzyme Linked ImmunoSorbent Assay
Fab	Fragment, antigen binding
Fc	Fragment, crystallizing
FCA	Freund's Complete Adjuvans
FIA	Freund's Incomplete Adjuvans
FITC	Fluorescein isothiocianate
Ig	Immunoglobulin
IgY	The major immunoglobulin found in egg yolk
kDa	Kilo Dalton
MHC	Major Histocompatibility Complex
NK-Cells	Natural Killer Cells
PAGE	Poly Acrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline (pH 7.2)
PBS-T	Phosphate Buffered Saline with 0.1 % Tween 20
PEG	Polyethylene Glycol

1. INTRODUCTION

Avian IgY antibody, *in vitro* and *in vivo*

Without the use of antibodies in research many of the discoveries that have led us to our present knowledge of nature and man would not have been possible. The almost extreme properties of antibodies to recognize small specific structures on other molecules have made them a very useful tool in studying other molecules as well as complex reactions. As the number of hostile invaders is immensely huge, evolution have created a mechanism that provides the host with an enormous diversity of antibodies, capable of attacking the invaders. Different species have different strategies to create variations in their antibody repertoire and this gives rise to species specific antibodies. In the chicken, the major antibody is called IgY. It was in 1893 that Klemperer first described the acquisition of passive immunity in birds, by demonstrating the transfer of immunity against tetanus toxin from the hen to the chick [1]. This immunity was due to transfer of IgY from the mother to the offspring. 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. This thesis 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.

Immune system

An immune system is present in all species in the animal kingdom and is a defense against intruding organisms, molecules and malignant cells. The purpose is to protect its host from foreign (i.e. non-self) substances. In a broad sense the immune system in birds is no different from the immune system found in mammals. The immune system can be divided into two parts: the innate (non-adaptive) and the acquired (adaptive) immune system. The innate response compromises many functions and acts as a first line of defense against infections

whereas the adaptive immune response is highly specific for a particular antigen. Antibodies belong to the adaptive immune system.

The innate immune system

The skin and mucous membranes act as barriers to prevent invasion of microorganisms. Cilia, mucus and the cough reflex expel inhaled material from the respiratory tract. In the gastrointestinal tract the low pH in the stomach and the normal bacterial flora in the gut also have important protective roles. The normal bacterial flora acts by competing for essential nutrients or by producing inhibitory substances against the invading organisms, thereby suppressing the growth of many potentially pathogenic bacteria. When antibiotics or conditions like diarrhea disturb the normal bacterial flora the susceptibility for opportunistic microorganisms is increased.

Pathogens may also trigger activation of the complement system, which results in the formation of the membrane attack complex, facilitating cell lysis and cell death. There are also several white blood cells involved in the innate immune system. Avian natural killer (NK) cells are large granular lymphocytes that are morphologically similar to mammalian NK cells. The NK cells selectively identify and kill virus-infected and tumor target cells and do not need prior antigenic exposure for target recognition [2], [3]. Unlike cytotoxic T-cells the NK cells are not MHC-restricted [3].

Another important component of avian innate immunity is the monocytes-macrophage system. Macrophages are an important part of the innate immune defense, operating immediately when a microorganism enters the body, thereby limiting the growth of the pathogen [4]. It is also an effector cell during the late phase of the acquired immune response. Macrophages have microbicidal, phagocytic and tumoricidal functions but they also acts as regulatory cells through cytokines and other metabolites. Monocytes are the major phagocytic cell in chicken blood while tissue macrophages are present in almost all organs. Macrophage phagocytic function appears within the first two weeks of chicken embryonic development [5]. The non-specific mechanisms respond rapidly to a

foreign invasion but it does not have the ability to respond with increasing strength to repeated challenges from the same organism.

The acquired immune system

Specific immunity depends on the ability to recognize foreign substances, respond to them and memorize the information in case of repeated exposure. The specific immune system functions through two interacting mechanisms, the humoral and cellular responses.

The humoral response involves interaction of B-cells with an antigen and the subsequent proliferation and differentiation into antibody-secreting plasma cells with or without the help of T-helper-cells. Two basic types of lymphocytes are involved in an antigen-specific response. The B-lymphocytes express surface immunoglobulins that are specific to an epitope on the antigen and T-lymphocytes that recognize processed antigens on antigen-presenting cells. The antibody-secreting plasma cells produce soluble antibodies that are identical to the surface immunoglobulin on the original B-cell. The cellular immune response involves interaction of the T-cell receptor and processed antigen. There are two main pathways. The first is reaction of T-cells with antigen and lymphokine secretion that attracts macrophages to the site, which will fagocyte the antigen. The second route is interaction of cytotoxic T-cells with processed antigen presented by MHC class I cells which eventually leads to cell lysis [6].

IgY in the chicken

Immunoglobulins in the chicken

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 [7], [8].

The molecular 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.

Chicken IgY is a systemic rather than a secretory antibody but IgY is also found in duodenal contents, tracheal washings and seminal plasma. It is called IgY rather than IgG to distinguish it from its mammalian counterpart [9]. The argument was that the heavy (H) chain of the molecule is larger and antigenically different from the mammalian heavy chain. There is no immunological similarity between chicken IgY and mammalian IgG, and the DNA sequence of chicken IgY resembles more the sequence of human IgE. There is a lower content of β-sheet structures in IgY that may indicate that the conformation of the IgY domain is more disordered and less stabile compared to that of rabbit IgG domains [10].

The overall structure of IgY is similar to mammalian IgG, with two light (L) and two heavy chains. The molecular mass has been reported to be 167 250 Da, slightly larger than IgG (~160 kDa) [11]. Interestingly, the light chain is lighter than its mammalian counterpart. The H chain (Mw 65 105 Da), called upsilon, υ, (capital letter Y) has one variable (V) region and four constant (C) regions. The light chain (Mw 18 660 Da) 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 studies have shown that IgY is an ancestor to mammalian IgG and IgE and also to IgA [12]. The Fc region of IgY mediates most biological effector functions in the chicken, such as complement fixation and opsonization. IgY is a skin-sensitizing antibody that can mediate anaphylactic reactions, a function that is attributed to IgE in mammals [13]. In many ways IgY combines the functions associated with mammalian IgG and IgE in the chicken.

Immunoglobulin diversity in chickens

Studies of the chicken immune system have contributed substantially to the 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 antibody-synthesizing cells (B-cells) are produced by the bursa of Fabricius. The chicken bone marrow is the source of bursal and thymic stem cells while the spleen is the center for plasma cell proliferation and memory B-cells [14]. Birds without spleen have a lower antibody production [15]. The thymus is a maturation center where stem cells differentiate into T-lymphocytes. The activities of chicken T-lymphocytes are similar to those in mammals.

The mechanism of antibody diversity in chicken differs from mammals and is mainly due to somatic hyper conversion. Rearrangement contributes little to the diversity as both the heavy and the light chain loci consist of only one functional V (variable) gene [16], [17]. There also seems to be a deficiency in the mechanism for selecting higher-affinity somatic mutants. The chicken has solved this deficiency by using three mechanisms that diversify the limited germ-line repertoire: gene hyper conversion [18], [17], V-J flexible joining [19] and somatic point mutations [20]. Gene hyper conversion starts around day 15-17 of incubation after the immature B-cell progenitors migrate to the bursa of Fabricius. During this process blocks of DNA are transferred from pseudo-V genes to the recombined variable regions of the Ig genes resulting in the production of mature B-cells competent to form a functional humoral immune system [21].

The υ heavy chain gene is encoded by three exons separated by only two introns, as there is no intervening DNA sequence between the CH1 and CH 2 alleles. The immunoglobulin heavy-chain constant regions of IgY, IgA and IgM are all located on chromosome E18C15W15. The IgA gene is located upstream the IgY gene in an inverted transcriptional orientation. The distances between the IgA, IgY and IgM genes are about 18 and 15 kilobases, respectively. The size of the whole chicken IGHC locus is approximately 67 kilobases [22]. Furthermore there are 16 alternative diversity (D) segments in the heavy chain locus. However, only V(D)J joining in the chicken can not produce the combinatorial diversity of the

large numbers of V and joining (J) segments seen in mammals. Instead, an equivalent degree of diversity is achieved by successive partial conversions of the rearranged V(D) segments by templates in an upstream array of pseudo-V(D) genes [23]. The variable and joining segments of both the heavy and light chain loci undergo V(D)J rearrangement [24]. The entire naive B-cell repertoire of the adult chicken is produced in the Bursa of Fabricius of the young bird [24].

Transport of IgY from maternal serum to the offspring

The transport of IgY from the hen serum to the offspring is a two-step process. First IgY is transported from the serum to the egg yolk in analogy to the cross-placental transfer of antibodies in mammals. The second step is the transmission of IgY from the yolk sac to the developing embryo.

The concentration of IgY in the yolk is essentially constant through the oocyte maturation, and at maturity the yolk will contain about 10-20 mg/ml IgY. Looking at the egg, IgY is not present in the egg white while IgA and IgM is not present in the yolk [25]. There is about 100-400 mg IgY packed in the egg. Labeled IgY binds specifically to yolk sac tissue from day 7 up to at least day 18. This binding is saturable, Fc-specific, pH-dependent and reversible [26].

There is both a high and a low affinity receptor for IgY on the embryo. The low affinity receptor, (K_D 3.4×10^{-7}), is present at day eight, whereas the high affinity receptor (K_D 3.0×10^{-8}) is detected at day 18. The low affinity receptor has a constant density as the total weight of the yolk sac increases, which implies that the rate change is due to an increase in tissue mass [26]. The receptor binds some heterologous IgY such as pigeon IgY but in a less efficient manner. Molecules such as bovine serum albumin, phosvitin, conalbumin, chicken IgM and chicken Fab fragments does not bind [26]. The IgY receptors on the oocyte bind and move all populations of IgY from the hen serum to the egg [27]. The populations of IgY are transported according to their concentration in the maternal serum. There is no selection nor destruction of IgY during transport and the yolk IgY has the same amount of sialic acid as the serum IgY.

The amount of IgY transported is independent of egg size and known to be proportional to the maternal serum IgY concentration [27]. A delay of three to four days is found between the appearance of IgY in serum until it is found in the yolk. The concentration of IgY in the yolk is by a factor 1.23 to the serum concentration [28]. The density of yolk is about 1.1 g/ml. About 50 % of the yolk is non-aqueous material.

The total amount of IgY in the hatched chick has been estimated to be only 2-3 mg, compared to the 100-400 mg present in the yolk [29]. The major part of the IgY probably serves only as nutrition for the developing embryo. In the newly hatched chick the IgY concentration in circulation is about 1-1.5 mg/ml and the circulating half-life of IgY is about 36 h. IgY secreting cells in the offspring are not detectable until six days after hatching [30].

IgY in vitro

Biochemical properties of IgY

The valency of IgY is two, same as for mammalian antibodies [12]. In place of the hinge region of mammalian IgG, IgY has a sequence that is more rigid, giving IgY limited flexibility. This probably is the reason for many of the different properties of chicken IgY in comparison to mammalian IgG.

The restricted mobility of the hinge region (Cυ2) in IgY heavy chain makes the antibody more rigid. This affects the capability of the antibody to precipitate or agglutinate antigens. Only part of chicken antibodies is precipitated at physiological salt concentrations and approximately 25% of the antibodies remain in the supernatant at maximum precipitation. The precipitation curve resembles the curve obtained with horse antibodies with a rapid decline with antigen excess. The precipitation improves at 1.5 M NaCl [31]. The poor precipitation properties might be due to steric hindrance of the Fab arms to cross link epitopes of two large antigens. The conditions permitting precipitation might

loosen the restricted movement of the Fab arms and give functional independence to the binding sites.

Orally given IgY is generally not immunogenic but IgY injected intravenously is an immunogen and elicits a typical anti-IgY IgM and IgG response in mice [32]. IgY applied to other endothelial surfaces then the gastrointestinal tract is probably immunogenic but not yet sufficiently tested. The stability of IgY under acidic conditions and toward pepsin digestion is slightly lower than that of bovine IgG [33]. However, IgY is fairly stable against digestion by internal proteases such as trypsin and chymotrypsin. There appears to be one subpopulation of IgY resistant to papain digestion [27].
Immune complexes formed with chicken antibodies are slightly different to those formed with rabbit antibodies [34]. The precipitation curve is steeper and the antigen excess effect on immune complex formation is more pronounced.

Advantages of IgY

As the difference between the antigen and the immunized animal increases, the immune response usually increases. There is a greater 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 [35]. As a result, chicken is a better choice than e.g. rabbits for the production of antibodies against conserved mammalian proteins [36]. Due to this evolutionary difference, 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 which will amplify the signal in an immunological assay [36], [37]. Chicken antibodies also recognize other epitopes than mammalian antibodies [38]. 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. Therefore, 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 [39].

The use of chicken egg yolk as a source 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, which is painful for the animal. The European Centre for the Validation of Alternative Methods (ECVAM) recommends that yolk antibodies should be used instead of mammalian antibodies for animal welfare reasons [40].
IgY can be used in many immunological assays giving a better result than mammalian antibodies used by tradition. Prevalence of human anti-mammalian antibodies varies from 1-80 % in the general population [41]. These human anti-mammalian antibodies may cause interferences in immunological assays.

IgY avoid complement activation

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 [42]. Accordingly, the complement activity may vary between different patients and also between different samples from the same patient. To avoid activation of the complement cascade EDTA is often included in tubes used for blood sampling. EDTA prevents coagulation by sequestering the calcium ions required for clotting. Most of the standards and controls used have been stored and contain an inactive complement system. This difference in activity between the samples and the standards will cause erroneous results. Mammalian antibodies bound to a solid phase and antigen-antibody complexes containing mammalian antibodies will activate the human complement system [43]. Activated C4 bind to the Fab region of IgG and may

interfere with the antigen binding [44]. Complement components may also solubilize precipitated immune complexes and prevent soluble immune complexes from precipitating [45], [46]. Such complement activation was shown to interfere in an immunometric TSH assay and depressed the TSH values by up to 40 % [47]. Because chicken antibodies do not activate the human complement system, they can be used as to reduce interference by complement activation [48].

IgY avoid RF and 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 [49]. RF is an auto-antibody 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 [50]. 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 that contain HAMA.

RF or HAMA may react with both the capture antibody and the detection antibody in a sandwich assay, thereby mimicking antigen activity. A reaction with the detection antibody, results 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 and antibody-binding substances have been demonstrated in approximately 40% of serum samples from healthy individuals in an immunoradiometric assay [51]. RF and HAMA will also give erroneous results in nephelometry and turbidimetry as they change

the size of antigen-antibody complex [52]. Chicken IgY does not react with RF or HAMA and can be used to avoid interference due to these factors [53], [54].

Human Fc receptor interaction

Intact mammalian IgG molecules contain the Fc portion of the antibody. Fc binds to Fc receptors, which are found on many types of blood cells [55]. 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 labeling procedures that will increase the binding to FcγRII and FcγRIII receptors. Interaction with Fc receptors may cause an increased background staining. When working with living 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 [56], [57]. No activation was observed when chicken antibodies were used [56]. Immune complexes containing mammalian IgG may also stimulate the production of cytokines [55]. Chicken antibodies do not react with human Fc receptors and their use will avoid these problems [56].

Bacterial Fc-receptor interaction

Staphylococcal protein A and Streptococcal protein G are Fc-binding bacterial proteins which are widely used for their ability to bind to IgG. 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 samples. 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 and can be used to reduce interference problems due to bacterial Fc receptors [58], [59], [60]. There are also other bacteria (e.g. *Peptostreptococcus magnus, Streptococcus suis* and *Actinobacillus actinomycetemcomitans)* with Ig-binding capability [61], [62] [63]. 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.

Antibody production

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 should be given booster immunizations every other month to assure that the antibody titer remain high. Chickens can produce high avidity antibodies already after one immunization, compared to sheep whose avidity becomes similar after four immunizations [28]. In response to monthly re-immunizations sheep have been found to produce ten times more specific antibodies than chicken, probably due to the size difference of the animals. Another reason for this might be the differences in antibody half-lives. In sheep the half-life is about 15 days, compared to 36 hours in the chicken. The species may therefore produce immunoglobulins at a comparable rate. The high catabolic rate of the chicken may prevent the accumulation of high titers. However, the avidity in both chickens and sheep after four immunizations was 10^9 to 10^{10} l/mol.

Freund´s complete adjuvans is quite well tolerated in chickens, as the characteristic local inflammatory response seen in mammals is often not observed [64]. Other types of adjuvant than Freund´s adjuvant can also be used, such as Specol, Hunters TiterMax, and the lipopeptide Pam_3-Cys-Ser-$(Lys)_4$ [65]. After immunization with human serum albumin the highest serum IgY titer is found seven to nine days after a single intravenous or intraperitoneal injection [66]. Intramuscular immunization shows a higher antibody level from day 28 after immunization and the specificity is almost more than 10 times higher compared to sub-cutaneous immunization [66], [67]. Non-reimmunized chickens

investigated more than 200 days later showed a similar high IgY level of specific antibodies [66]. The presence of IgY in the yolk is detected four to seven days after the appearance in the serum.

Monoclonal chicken antibodies have also been reported [68]. 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. There are still not many cell lines well suited for fusion with avian cells.

The amount of antigen specific antibodies of the total pool of antibodies in an egg has been reported to be up to 10 % [69], [70]. However, the actual amount of specific antibodies probably varies depending on the individual animal, immunization procedures and the immunogenicity of the antigen itself.

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, therefore 2 gram of egg yolk IgY corresponds approximately to the IgY content of 300 ml of serum or 600 ml of blood. Only larger mammals can produce equal amounts of serum antibodies and compared to rabbits, the chicken antibodies are ten times less expensive [71].

Purification methods

There are several methods of purification of IgY described. The choice of method is a matter of yield and purity desired, final use of the IgY as well as material cost and labor skills.

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 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. Plasma is therefore more useful than serum.

The best way to obtain antibodies is to purify them from the yolk. Several methods can be used, even for large-scale purification, of functionally active chicken antibodies from egg yolk [72], [73], [74], [75], [76], [77]. Over 100 mg of purified IgY can be obtained from a single egg [75]. It is also possible to purify 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 or 2.8 [78]. Other methods, such as thiophilic interaction chromatography have also been evaluated for IgY purification [74], [79]. Synthetic ligands, such as TG19318 and 22/8 also binds to IgY [80], [81]. However, their use in research is still limited.

Antibody stability, affinity and labeling

IgY is a protein and as such, is sensitive to denaturation. However, IgY is fairly heat stable and most antibody activity remain after 15 minutes 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 [33]. Incubation of IgY and Fab' fragments at pH 2 for 4 h have led to a 16 fold decrease in neutralizing activity but there where still substantial neutralization titers retained [82]. The immunological activity of IgY is not affected by pasteurization at 60 °C for 3.5 minutes [83].

Addition of high concentrations of sucrose stabilizes IgY regarding heat denaturation, acid environment as well as high pressure [84]. Sucrose syrup with a high sugar content (50 % w/w) has been suggested as an additive to preserve IgY when refrigeration is not possible.

IgY fractions have been stored in 0.9% NaCl, 0,02% NaN_3 at +4^{o}C for over 10 years without any significant loss of antibody titer [37]. Affinity-purified and

biotinylated antibodies have after 5 years of storage at +4 ^{o}C retained high activity [37]. The purified antibodies also retained their antigen binding capacity after 6 months at +20^{o}C or 1 month at +37^{o}C. An egg can be stored in +4 °C, with just a small loss of IgY activity for at least six months [85].

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 x 10^{11} L/mole and for a chicken anti-albumin antibody 5.5 x 10^{11} L/mole. These K-values are higher than for most mammalian antibodies [86].

Chicken IgY can be labeled with approximately the same methods that are used for mammalian antibodies. There are optimized labeling procedures described for biotin [37], FITC [56] and horseradish peroxidase [87].

IgY in therapy

Oral administration of antibodies specific to host pathogens is an attractive approach to establish protective immunity, especially against gastrointestinal pathogens both in humans and animals. The increasing number of antibiotic-resistant bacteria emphasizes the need to find alternatives that complement antibiotics. Immunotherapy may also be used as a complementary treatment against pathogens that are difficult to treat with traditional antibiotics. Eggs are normal dietary components and there is practically no risk of toxic side effects of IgY given orally. However, caution should be taken to give IgY to persons with known egg allergy. IgY has biochemical properties that make them attractive for peroral immunotherapy. As mentioned earlier, they neither activate mammalian complement nor interact with mammalian Fc-receptors that could mediate inflammatory response in the gastrointestinal tract.

Activated complement components are potent inflammatory mediators. Theoretically, immune complexes containing mammalian antibodies may also interact with Fc- and complement-receptors in the mammalian gastrointestinal tract, causing cell activation. Immune complexes containing IgY do not activate

the mammalian complement system and do not interact with mammalian Fc- and complement-receptors. As a result, yolk IgY seems to be well suited for peroral immunotherapy.

Oral delivery of immunoglobulins

Orally administered antibodies are subjected to denaturation by the acidic pH of the stomach and degradation by proteases, such as pepsin, trypsin, chymotrypsin, carboxypeptidase and elastase [88]. Studies have shown that part of the antibodies remains intact in pepsin and trypsin digests but there is a considerable cleavage of the antibodies into Fab, Fab'2 and Fc fragments. However, Fab'2 and Fab fragments still have the capability to bind to the antigen and exhibit neutralizing activity [82]. By encapsulation in liposomes, coating with polymers, addition of acid neutralizing agents or stabilization with sugars it is possible to increase the stability of immunoglobulins given orally against proteolysis [89]. The transit time for orally administered antibodies in the gastrointestinal tract is between 12 and 36 hours in infants and children [90], [91]. In newborns, the gastric fluid has a pH close to neutral but the pH rapidly decreases to below 3.0 during the first days that will denature the antibodies.

Despite the degradation in the gastrointestinal tract, active antibodies can still be detected in stool samples [92]. The percentage antibody which in the stool remain active varies between very low levels (<0,01 %) to up to 40 % in different studies [93], [91], [94]. These differences are probably due to differences in the pH of the stomach, enzyme activity, presence of antigen and the gastrointestinal passage time. The methods used to detect the antibodies in stool may also influence the results. As a large portion of the antibodies is digested to Fab'2 or Fab fragments these fragments still bind to the antigen, but many immunological tests give different results with fragments than with entire antibodies.

Antibodies are absorbed from the intestine in young piglets and calves (<48 h old). Low levels of IgY can be detected in the circulation if calves or piglets are treated orally with yolk antibodies during the first 24 to 48 hours post natum [95]. After this time period there will be no absorption of active antibodies from

the gastrointestinal tract. No absorption of intact antibodies has been shown in humans. Blum *et al.* and Eibl *et al.* showed that there was no increase in serum immunoglobulin levels after oral administration of human immunoglobulins to infants [96], [97]. Also, Losonsky *et al.* could not detect any high molecular weight radioactivity in blood after oral administration of ^{125}I-labelled IgG [91]. In conclusion, no systemic effects can be expected after oral administration of yolk antibodies to humans. In an *in vitro* study with sera from 28 egg-allergic children 15 had IgE antibodies specific for one or more of five antiviral IgY preparations [98]. Caution should therefore be taken when administering IgY antibodies to egg allergic persons.

Prevention and treatment of bacterial infections

IgY have been produced against opportunistic, invasive and toxin producing bacteria (*Pseudomonas aeruginosa, Salmonella enteritidis* and *Staphyloccocus aureus* respectively) [99]. The IgY asserted a growth inhibition effect on *Pseudomonas aeruginosa*, but did not prevent growth. Staphylococcus enterotoxin A production was suppressed, but not inhibited. Heat pasteurization at 65°C for 15 minutes of an IgY preparation against an *E. coli* toxin did not affect the neutralization activity of the antibody [82]. Growth of *E. coli* is decreased in the presence of specific IgY against the bacteria, but no inhibition is seen with non-specific IgY [33].

Animals

Oral administration of spray dried yolk antibodies specific against *Salmonella typhimurium* or *S. dublin* was shown to prevent Salmonella infections in calves. The antibodies were administered three times a day for 7 to 10 days after inoculation with *S. typhimurium* or *S. dublin*. All calves in the control group died whereas only diarrhea and fever was observed in the group treated with the highest antibody titer. These antibodies gave protection against fatal salmonellosis [100]. Yolk preparations from chickens immunized with purified outer membrane proteins, lipopolysaccharide, flagella and fimbriae of

Salmonella has also been tested in mouse models of experimental salmonellosis. The mice treated with specific antibodies had a significantly higher survival rate than mice fed normal egg-yolk antibodies [100], [101].

Humans

A mouth rinse containing egg yolk antibodies to *Streptococcus mutans* has been used to reduce the establishment of these bacteria in dental plaque of humans. The antibodies inhibited *S. mutans* adherence to saliva-coated hydroxyapatite discs in vitro and decreased the percentage of *S. mutans* per total streptococci in vivo [102].

Prevention of viral infections

Animals

Oral IgY against human rotavirus administered within 24 h after virus challenge to suckling mice was still effective and decreased the incidence of diarrhea [103]. Egg yolk and colostrum powders containing specific antibodies against bovine coronavirus (BCV) antigen was evaluated in a challenge model with a standardized dose of virulent BCV strain. Daily treatment with these antibody preparations started 6 h until 7 days post-challenge. Control calves which received no antibody had severe diarrhea and all died within 6 days after infection. In contrast, calves fed milk containing egg yolk or colostrum with high antibody titers all survived and had positive weight gain. The study showed that specific antibody preparations protected against BCV-induced diarrhea in neonatal calves and that the egg yolk used provided a higher degree of protection compared to colostrum powder on a titer basis [104].

A field trial with oral administration of chicken IgY specific for bovine rotavirus (BRV) resulted in a significantly increased mean body weight ($P < 0.05$) and a decrease in number of calves shedding high titer of BRV in stool compared to control calves ($P < 0.01$) [105]. Passive oral immunization with yolk immunoglobulins has also been shown to protect mice against experimental bovine rotavirus-induced diarrhea [106], [107], [108].

Humans

There are differences in using avian or bovine immunoglobulins. In a randomized, placebo controlled clinical trial, children with rotavirus diarrhea was treated with IgY from hens immunized with Wa, RV5, RV3 and ST3 rotavirus strains. There was a significant reduction in stool output (g/kg/day) and viral clearance in the group treated with specific IgY compared to placebo. However, there was no difference in diarrhea duration [109]. Oral treatment with bovine anti-rotavirus colostrum resulted in a reduction of rotavirus-associated diarrhea in infants [107], [110], [111]. It also reduced the infection rate in children [112].

Prevention of other infections or maladies

In vivo studies on mice have shown that IgY can be an alternative to horse serum antivenoms to rattlesnake and scorpion toxin [69], [113]. Cryptosporidium is a zoonotic pathogen that infects the respiratory or gastrointestinal tracts of a large number of hosts including fish, birds, reptiles, and mammals. Cryptosporidium infections are usually associated with immunosuppressed hosts but it may also occur in individuals with a normal immune system. The use of yolk preparations with high anti-Cryptosporidium activities caused a significant parasite reduction in a neonatal mouse model [114]. Treatment with bovine anti-Cryptosporidium colostrum immunoglobulin has also been shown to induce a 100-fold reduction of oocyte excretion in healthy human volunteers [115]. A maternal transmission of parasite specific IgY against the protozoan *Eimeria maxima*, that cause coccidiosis in poultry, are highly protective, mediating up to 97 % reduction in oocyst excretion in challenged hatchlings [116]. Bovine anti-*Candida albicans* antibodies have been used as a prophylaxis in bone marrow transplanted patients [117]. The results show that a there was a treatment related reduction in candida colonization in a majority of the patients.

Brief introduction to Cystic fibrosis

Cystic fibrosis is the most common fatal genetic disease in Caucasians in Europe and United States. The basic defect in CF is mutations in the cystic fibrosis

transmembrane conductance regulator (CFTR) gene on chromosome seven [118], [119], [120]. These mutations result in faulty transport of chloride, sodium and water that leads to abnormally thick mucus. The abnormal mucus in the lungs secondarily leads to the respiratory infections which are the major causes of morbidity and mortality in CF patients [121], [122], [122]. Although several bacteria may cause respiratory infections in patients with CF (e.g. *Staphylococcus aureus*, *Haemophilus influenzae* and *Burkholderia cepacia*), chronic *Pseudomonas aeruginosa* infections ultimately occur in virtually all patients. Once a chronic *P. aeruginosa* infection has been established, eradication of the bacteria is seldom possible [123].

2. MATERIALS AND METHODS USED

Animals used

The animals used in work I, II, and III where reared at the Swedish Agricultural University. The animals used in paper IV, V and VI where reared in a research animal facility by Ova Production AB, (Morgongåva, Sweden). The hens were housed in single or three hen cages in ordinary batteries in a three-tier system. The hens have been fed water and all mash laying feed *ad lib*. The animals have been reared according to the rules and regulations of the National Veterinary Institute.

Immunization procedures used

Chickens are usually vaccinated intramuscularly in the breast muscle but it is also possible to use subcutaneous immunization. Usually 25-100 µg antigen per immunization is used but it is possible to obtain a good immune response with 1 µg/immunization as we show in paper I. The antigen used for immunization was emulsified with an equal volume of Freund´s adjuvant. The first immunization was performed with Freund´s complete adjuvant and booster immunizations with Freund´s incomplete adjuvant. The hens were immunized intramuscularly in the breast muscle with 0.5-1 ml of emulsified antigen. After the initial immunization the animals received 2-3 booster injections with 2-week intervals. In most of the experiments the animals where given the antigen mixed with Freunds complete adjuvans (FCA), for the first immunization, and antigen mixed with Freunds incomplete adjuvans (FIA) for the booster immunizations.

Purification methods used

Water dilution method

For paper V and VI a water dilution method was used, that gave a fairly crude antibody preparation. This method is based on the procedure first described by Akita and Nakai in 1992 [75]. Briefly egg yolks where separated from the whites,

and then diluted 1:10 with deionized water and mixed. The solution was then left to settle at 4°C for at least 6 hours, often over night. The supernatant was then filtered to remove any larger particles. The solution was then filled into 70 ml aliquots in 100 ml plastic bottles and frozen at -20 °C. This method also rids most of the cholesterol.

PEG-Ammonium sulphate method

Another method used is the polyethylene glycol-ammonium sulphate method described by Polson in 1980. In this method the yolk is diluted with a 3.5 % PEG solution and stirred for 20 minutes. Then follows a 30 minutes centrifugation (2000 g) and the supernatant is saved. To the supernatant more PEG is added to a final concentration of 12 % followed by a new centrifugation. The supernatant is discarded and the pink colored precipitate is dissolved in PBS.

To this solution is added saturated ammoniumsulphate to a final concentration of 40 %. After centrifugation (15 minutes, 13 000 g) the pellet is washed three times with 40 % ammoniumsulphate. The solution is then dialyzed thoroughly (three buffer changes, at least 150 times volume) against PBS in a dialyzing tube with molecular cut off weight (MCOW) of 14 KDa. The obtained solution contains about 95 % pure IgY. Where needed the PBS contained 0.1 % azide (NaN_3) to prevent bacterial growth.

Analyzing methods used

Concentration determinations

The factor 1.36 was used as the extinction coefficient to calculate the IgY concentration at 280 nm. Leslie and Clem reported an average extinction coefficient for IgY to 1.34466 in 1969 [9]. Linden *et al.* have reported a slightly higher extinction coefficient of 1.4 [124].

Nephelometry

The IgY concentration in paper II and III was determined by rate nephelometry on a Beckman Array protein system (Beckman Instruments, Bream, CA, USA).

Rabbit anti-IgY was obtained from Immunsystem AB (Uppsala, Sweden). The antibody was diluted 1:5 in 0.9% NaCl, 0,2 % NaN_3. The samples were diluted in the same buffer. A standard curve was produced with a highly purified IgY sample from Immunsystem AB. The IgY concentrations in the samples were calculated against the standard curve.

ELISA

In most of the work, enzyme linked immuosorbet assays (ELISA) were used to study the IgY activity. The ELISA principle is that an antigen is coated to a surface. The desired antibody in a sample is then allowed to attach itself to the antigen. The bound sample antibody is then detected by another, labeled antibody. The amount of the sample antibody can then be correlated to the labeled antibody in the assay.

More detailed, this is an example of how the ELISA is performed. Microtitre plates were coated with 100 µL antigen, diluted to the desired concentration, usually 2-10 µg/ml, in 0.1 M $NaHCO_3$, pH 9.5. The plate was incubated for one hour at 37°C on an orbital shaker or over night in 4°C. The plate was washed three times with 0.15 M NaCl, 0.02 M Na_2HPO_4, 0.01% Tween 20, pH 7.2 (PBS-T) utilizing an ELISA washer (ImmunoWash 1575, Bio-Rad, Hercules, CA, USA). The plate was then blocked with 150 µL of 1 % bovine serum albumin in 0.1 M $NaHCO_3$, pH 9.5 for 30 minutes at 37°C on an orbital shaker. The blocking is done to prevent the antibody to attach to any possible unbound sites. The plate was washed three times with PBS-T and 100 µL of the samples, usually diluted in PBS-T, was added to the wells in duplicate or triplicate. The plate was incubated for one to two hours at room temperature on an orbital shaker and was washed three times with PBS-T. Then 100 µL of labeled detection antibody, often rabbit anti-chicken IgY-HRP diluted in PBS-T, was added to the plate and the plate was incubated for 1 hour at room temperature on an orbital shaker. The plate was washed as previously described and 100 µL K-Blue, a horseradish peroxidase substrate (Neogen Corporation, Lexington. KY, USA) was added. The reaction was stopped after 10 minutes by addition of 50

μL of 1.8 M H_2SO_4 and the plate was read at 450 nanometers in a SpectraMax 250 (Molecular Devices, Sunnyvale, CA, USA).

3. RESULTS

Paper I

Larsson, A. Carlander, D. and Wilhelmson, M. (1998) Antibody response in laying hens with small amounts of antigen. Food and Agricultural Immunology, 10, 29-36.

In this paper the capability of the chicken to make antigens against small amounts of antigens was studied. In many circumstances there might be difficulties to get enough amounts of antigen to make an immunization. The reason could be scarce amounts found in nature, a difficult synthesis or maybe just a very expensive antigen. Another advantage by using small amounts of antigen is that it is often easier to obtain a pure fraction. In this experiment bovine serum albumin (BSA) was used as the model antigen representing an average seized serum protein and human insulin representing a conserved low molecular weight protein. The hens where immunized with 0.1 - 100 μg antigen with booster immunizations after 2, 4 and 6 weeks. An ELISA measured antibody response in serum and yolk. With 0.1 μg of BSA, one of three hens gave a good antibody response. If the amount of BSA was increased to 1 μg a better immune response was found. Further increase of the BSA amount did not improve the response. To obtain an antibody response with insulin, 10 μg was needed. The response was also weaker compared to the BSA response. The response in the yolk showed the same picture as in the serum but was somewhat delayed. The study show that is possible to obtain a good immune response with less amounts of antigen than usually recommended to immunize hens or rabbits.

Paper II

Carlander, D., Wilhelmson, M. and Larsson, A. (2001). Limited day to day variation of IgY levels in eggs from individual laying hens. Food and Agricultural Immunology, 13, 87-92.

In this experiment the day-to-day variation of IgY levels in yolk was studied. A hen weights about 1.5 kg and lays about 3-6 eggs per week. The amount of antibody in an egg yolk (100 to 400 mg) corresponds to the amount found in 30 ml of blood. The amount of IgY transported to the yolk during one week supersedes the total amount of IgY in the blood circulation. Eggs where collected from 10 non-immunized hens over a 30-day period. The yolks where diluted with PEG and centrifuged to obtain a clear solution that was analyzed by nephelometry. We did not see a decreased transport of IgY to the yolk when the hen broke the egg laying to set herself back in phase or a higher concentration in the first egg after the break. Further we did not see any correlation between IgY concentration and egg laying. The study shows that the IgY concentration in the yolk is independent of the egg production and that suggests that by genetic selection it would be possible to increase the IgY content.

Paper III

Carlander, D., Wilhelmson, M. and Larsson, A. (2000). Immunoglobulin Y levels in egg yolk from three chicken genotypes. Manuscript in preparation 2002.

The purpose of this study was to investigate the variation of IgY levels in egg yolk. We have compared IgY concentrations in egg yolks from two lines, selected for egg production traits at the Swedish University for Agricultural Sciences. Single Comb White Leghorn (84 animals) and Rhode Island Red (87 animals) and a cross between the two lines, SLU-1392 (41 animals). The yolks where processed as in paper II and the IgY content was measured by nephelometry. Single Comb White Leghorns have the highest mean concentration of yolk IgY, 2.21 mg/ml compared to SLU-1392 1.95 mg/ml and Rhode Island Red 1.68 mg/ml. The cross had an intermediate IgY concentration in relation two the two other lines. There were large differences between individual animals within each line. Our results indicate that it should be possible to increase yolk antibody production by using a high producing chicken line and

by genetic selection within the line. We found three individuals with very low yolk IgY concentrations among the Rhode Island Red hens. Newly hatched chickens with limited amounts of IgY from the hen may be more susceptible to infections.

Paper IV

Carlander, D. and Larsson, A Avian antibodies can eliminate interference due to complement activation in ELISA.. Accepted 2001.

Chicken antibodies do not activate the human complement system and is sometimes a more suitable choice in designing solid-phase immunometric assays than mammalian antibodies. In this work goat and chicken antibodies where compared regarding the influences of an active complement system.

The material often recommended for immunological assays is serum samples. In the clinical situation a rapid answer is often of interest. A freshly drawn serum contains an active complement system, which is inactivated during storage. Mammalian antibodies used in most immunological assays may activate the human complement system. Microtiterplates where coated with affinity purified goat or chicken anti-BSA antibody. To the wells where added human serum with active or inactivated complement. After incubation and washing biotinylated BSA was added and bound BSA was detected with streptavidin-HRP. Activated complement components will bind to the antibodies, thereby partly block the antibody binding epitopes. We showed that an active complement system in undiluted samples reduce the absorbance values by approximately 50% when using goat antibodies compared to chicken antibodies. However if the samples where diluted 1:10 this effect was eliminated. The difference in serum complement activation will cause erroneous test results that will vary depending on the handling and degree of complement activation in the samples. Chicken antibodies can be used to eliminate this interference problem in undiluted samples.

Paper V

Carlander, D., Kollberg, H. and Larsson, A. (2001) Duration of specific yolk IgY in the human oral cavity. Submitted 2002

We have studied the presence of IgY antibodies in the saliva after a mouth rinse performed in the evening. The model IgY used was directed against the bacteria *P. aeruginosa.* The reason for giving a dose of oral antibodies in the evening, after the toothbrush, is to avoid the mechanical rinse of the mouth cavity by foodstuffs and drinks. The saliva production is also decreased during night, which implies that the antibodies will be present for a longer time. Healthy research subjects performed a mouth rinse for one or two minutes and the presence of antibodies was tested in an ELISA. The next morning there were still active antibodies detected in the saliva from almost all subject. After 24 hours, active antibodies could be detected in saliva from only a few of the subjects. A two-minute mouth rinse resulted in higher mean ELISA activity than a one-minute rinse. The presence of active antibodies in the mouth cavity indicates that the antibodies can bind to and probably prevent adhesion of bacteria, thereby giving protection to infection.

Paper VI

Carlander, D., Olesen H., Kollberg, H., Johanesson M., Wejåker, P.-E. and Larsson, A. (2000) Oral IgY antibodies prevents colonization of *P. aeruginosa* in cystic fibrosis. Submitted 2001.

Respiratory infections are a major problem for Cystic Fibrosis (CF) patients. Chronic *P. aeruginosa* infection ultimately occurs in virtually all patients and is regarded as one of the major causes of morbidity and mortality in CF patients. It is impossible to permanently eradicate *P. aeruginosa* once the patient is chronically colonized. Immunotherapy with specific antibodies may be an alternative to antibiotics in preventing *P. aeruginosa* infections.

In this paper we have used IgY-antibody preparations, purified from eggs of hens immunized with *P. aeruginosa* bacteria, to prevent colonization in CF patients.
The trial was performed as an open study and the patients where followed by sputum cultures and lung function tests. The duration of the study was 380 patient treatment months (range 17-73) for 10 CF-patients (March 2001). There have been nine sputum cultures positive for *P. aeruginosa* out of 210 collected cultures. This is a reduction over time (2,4 % vs. 13,7 %) in comparison with a retrospectively collected control group of 21 patients. The time to the next positive colonization was increased by six months in the IgY treated group to 12 months compared to six months in the control group. During the study period, none of the patients have become chronically colonized with *P. aeruginosa* compared to five patients in the control group.

4. DISCUSSION

The study of IgY and its use *in vitro* and *in vivo* is interesting in several aspects. Not only due to the biochemical differences that IgY displays compared to mammalian antibodies, but also its use in therapy as an alternative, or in combination with antibiotics. The production of IgY, from the low amounts of antigen needed for immunization, the simple collection of eggs and straightforward purification and labeling techniques, to the better performances in immunological assays makes the use of IgY interesting. The potential use of IgY in therapy, both in animals and in humans opens up a vast field of interesting and useful applications. Replacing antibiotics as additives to mammalian feed will decrease the development of resistant microorganisms and still growth rate is not affected in a negative way. To reduce the risk of antibiotic resistance is becoming a more and more important task for society. Also, with IgY technology it would be possible to produce antibodies against antibiotic resistant bacteria and get a defense for hard to treat infections that are becoming a threat to compromised patients treated at hospitals.

To be able to immunize with small amounts of antigen and still get a good antibody response is advantageous in several aspects. It is often easier to get a pure antigen in small amounts, thereby produce antibodies with the correct structures as there will be less contaminating molecules in the preparation. When looking at the small, conserved protein hormone insulin, only one amino acid differs between human and pig insulin while there are seven amino acids that differ between human insulin and chicken insulin. In paper I there were some animals that produced antibodies with low titers. This implies the possibility of selecting animals with higher immuneresponse that would increase the antibody efficiency. To undertake such a selection it would be of interest to further study the IgY concentration in yolks from individual chickens and to investigate the appearance of IgY over time in the yolk (paper II).

By studying ten randomly chosen individuals we found that there were a more than twofold difference between individuals with high and low yolk IgY concentration (paper II). The same amount of antibodies was transported to each consecutive egg. This indicates that the hen gives the same amount of protective antibodies to her offspring, thereby giving her progeny the same chance of survival. Individuals that transport a high amount of antibodies to the yolk must replace the loss, which is costly in terms of increased metabolism. The increased metabolism might signify that other resources are depleted and as such, the animal can be more sensitive to infections and as a consequence transport less antibodies to the yolk. However, in paper II we did not see a correlation with yolk IgY concentration and egg production. This might be due to the unlimited supply of food and a hygienic production environment that can balance the protein loss. In a situation with limited food supply the reproductive effort would be achieved at the expense of other resources [125].

When looking at two genotypes and their cross (Paper III), it was found that there are differences in the mean IgY yolk concentrations. The cross had an IgY concentration in-between the genotypes which indicates a strong genetic correlation for IgY traits. For a commercial production of antibodies it is important to have a high IgY concentration, regardless of the antigen used. There were a few animals that during a prolonged time, three weeks, consistently showed low IgY yolk concentrations. These individuals probably have an increased risk of infection. After being infected, one might speculate that these individuals might spread the infection throughout the flock. It is also possibly that vaccination of such hens will give a poor protection and not be successful.

Works by several groups have demonstrated that antibody response to foreign antigens is genetically controlled. It has been possible to breed chickens that are high and low antibody responders to sheep red blood cells via either intramuscular [126], [127] or intravenous [128], [129] injections. In these studies there was a negative correlation between antibody production and body weight

[130]. Sexual maturity is delayed in chicken selected for high antibody response [129]. There is also a genetic association between the ability to produce antibodies and MHC. MHC haplotype B21 was is associated with higher antibody titers to sheep red blood cells while haplotype B13 was associated with lower antibody titers [131]. This is not the only MHC gene that influences immune response and other MHC genes have also been suggested to influence the immune response [129].

A short turnaround time (less than 2 hours) is of interest in clinical laboratories to deliver results as fast as possible back to the physician. As a result of this rapid processing, samples contain various degrees of activated complement while the standards and controls do not. This might cause erroneous results in assays based on immunological techniques due to cross reactivity. Cross reactivity can occur when mammalian antibodies from specie one interact with immunological effector mechanisms in another specie. In paper IV, by using IgY antibodies instead of goat antibodies the effect of complement activation can be decreased. In a model, by using the immunoglobulin as a capture antibody, complement activation blocked up to 50 % of the antigen binding when goat antibody was used. This may cause erroneous results by a factor of two. However, by diluting the samples more than 1:10 this is avoided. A disadvantage by diluting samples is that the sensitivity of the assay is reduced. Therefore, it is important to consider the complement activation when developing assays intended for freshly drawn blood samples. The use of IgY is one way to avoid this problem.

The average consumption in Sweden is approximately 0.5 eggs/day, which is similar to the IgY dose used in paper V and VI. Oral delivered antibodies are found to maintain their activities in saliva after administration. An active recombinant plant secretory antibody was found three days post oral administration, compared to 24 hours for an IgG antibody [132]. The recombinant antibody was an IgA like antibody with presence of the secretory component that probably protects the molecule from proteolytic degradation.

This result, as well as the IgY delivered to healthy subjects in paper V, suggests that the recovered antibodies from the oral cavity remain intact and immunologically active. Also, there were no signs that the plant antibodies were immunogenic when given orally, as concluded from absence of serum IgG, IgA or IgM samples from the volunteers binding to the purified antibodies [132].

In the study with CF patients IgY prevent infection of *P. aeruginosa* in the lungs (paper VI). The IgY presumably obstructs the adhesion of the bacteria to the mucosal membrane thereby hampering the bacteria to enter the airways. When IgY is given as a mouth rinse and then swallowed the main site of action is the oral cavity, the nasopharynx and the lymphatic ring. This means that the IgY antibodies will coat the mucosal membrane and make a protective barrier for bacteria entering both from the nose and mouth. In the mouth there is a low action of proteolytic enzymes and there is no acid present to destroy the IgY activity. The normal treatment for *P.aeruginosa* in CF patients is an aggressive antibiotic regime [133], [134], that has drawbacks such as the risk of developing allergy to antibiotics and oto- and nephrotoxicity [135], [136]. Since the route for infections to be established in the lungs is mainly either from the nose or from the mouth, specific IgY might be able to prevent the majority of airway infections and maybe cure some of them, such as tuberculosis, infections secondary to immunodeficiencies and to HIV, streptococci, common colds and other upper airway diseases.

5. CONCLUSION AND FUTURE PERSPECTIVES

The full beneficial use of IgY in therapy is yet to be explored. IgY could be a complement to antibiotics and as prophylaxis in several patient groups sensitive to various microbes. The IgY technology can be applied on humans, as well as on animals. Regarding CF there are two more pathogenic bacteria that would be interesting to study, *Burkholderia cepacia* and *Stenotrophomonas maltophilia*, both that are detrimental to CF patients. Other indications where IgY might be of use are in chemotherapy treated patients who often get colonized with the fungi *Candida albicans*. In animals, IgY against *E. coli* can be used to prevent diarrhea in piglets, thereby increase growth without the use of antibiotic feed additives. The IgY technology has probably not yet become as used as one might have expected in diagnostics and in research.

From the work presented in this thesis the following concluding remarks can be made:

- Small amounts of antigen can elicit an immune response in chickens
- There are small intra-individual differences regarding the antibody concentration in the yolk over time.
- Two breeds and their cross where studied and it showed that the IgY in the cross where in-between the two breeds indicating a genetic correlation of IgY concentrations.
- IgY does not activate the human complement system. By using IgY in immunological assays the antigen binding capacity of capture antibodies can be increased up to 50 %.
- Active IgY is present after 8 hours in the mouth cavity after a mouth rinse in the evening, indicating a protective effect.
- IgY can decrease colonization of *P. aeruginosa* in CF patients and delay the time until chronic infection.
- The use of prophylactic IgY decreases the need for antibiotic treatment, hence diminishing the risk of development of antibiotic resistant organisms.

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