From phage display to plant expression:

Fulfilling prerequisites for chicken oral immunotherapy against coccidiosis

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W.H. Wieland

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Voor mijn ouders,

die mij leerden dat je ook met kleine stapjes een berg kan bedwingen

ABSTRACT

The frequency and spectrum of infections with pathogens harbouring resistance to antibiotics and other drugs has dramatically increased over the last years. One of the main causes is the extensive use of antibiotics and other drugs in human and veterinary medicine. Parasites, such as *Eimeria* causing coccidiosis in chicken and pathogenic bacteria like *Salmonellae* and *Campylobacter* are examples of pathogens that acquired resistance. Furthermore, continuous use of drugs in diets of animals kept for human consumption increases the risk of residues in food, that possibly affect human health. These drawbacks of antimicrobial drugs have led to a demand for alternative treatments. In this thesis an alternative approach for prevention of coccidiosis in chicken is described, based on immune intervention by passively administered, plant produced, secretory IgA.

As a first step, *Eimeria* binding IgA fragments were selected using the phage display technique. The phage display system was adapted to be used for the display of chicken Fab fragments. A newly constructed vector, named pChick3, allows straightforward cloning of chicken variable antibody domains in frame with the constant domains of the chicken light chain and the first constant domain of the IgA heavy chain. In a following step, new plant expression vectors were designed and constructed. Ten antibodies, selected from the chicken phage antibody library were then transferred to this vector system and subsequently expressed *in planta* as full size IgA. Upon expression of the ten selected anti-*Eimeria* antibodies, differences up to 500-fold in yield were observed. Several factors on translational or protein level could cause the observed differences: e.g. processing, stability, assembly and silencing. Two were tested (silencing, chain compatibility i.e. assembly) and both have an influence on the levels of expression. An explanation may be found in the combination of several factors. These observations lead to the conclusion that an extra *in planta* selection step is inevitable for successful integration of phage display and plant expression systems.

Finally, the structure of the chicken polymeric immunoglobulin receptor was elucidated. In a fashion similar to its mammalian counterpart, this receptor transports IgA to the gut lumen forming secretory IgA. This complex is highly stable, and IgA is protected against degradation by proteases or pH-fluctuation, which makes secretory IgA the most suitable form for passive immunization. Interestingly, the chicken SC comprises only four immunoglobulin-like domains compared to five found in mammals.

Thus, an integrated system for both selection and expression of immunoglobulins was developed and with the final achievement of the production of *Eimeria*-specific secretory IgA in plants, the prerequisites for chicken passive immune therapy were fulfilled.

VOORWOORD

Elke AIO zal kunnen bevestigen dat een promotie onderzoek van vier jaar een continue race tegen de klok is- deze promotie vormt daarop geen uitzondering. Als ik terugkijk op de afgelopen 4 jaar is de tijd omgevlogen. Gelukkig heb ik me vanaf het begin thuis gevoeld binnen het "kippenproject": een project waarbij fundamenteel onderzoekswerk direct gekoppeld wordt aan een mogelijke toepassing voor maatschappelijk relevante problemen. Door te vertellen over mijn werk heb ik toch een paar zielen voor de biotechnologie gewonnen en ik ben er ook van overtuigd dat de productie van pharmaceutica in planten op den duur het hart van de consument voor genetiche modificatie zal winnen (dit, en natuurlijk de lagere prijzen van biotech-producten).

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Chapter 1

General Introduction

From phage display to plant expression:

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The use of antibiotics in animal production needs to be reconsidered. Antimicrobial agents have played an important role for decades in human and veterinary medicine because of their short-term efficiency as therapeutics against pathogens. The livestock industry is a hotspot for antibiotics, since they are applied for prevention and treatment of infections, as well as for their growth accelerating characteristics. Accumulating reports show development of resistance against antibiotics and other drugs in various pathogens, for example in parasites like *Eimeria* that cause coccidiosis or in pathogenic bacteria like *Salmonellae* and *Campylobacter* spp. Treatment of infections caused by resistant strains is severely impeded or even impossible, because they cease to respond to conventional therapeutics. The threat of emerging zoonoses is aggravated by resistance development. An additional effect of continuous use of drugs in diets of animals kept for human consumption is the risk of presence of residues in food, which could affect human health.

The poultry industry faces all the problems associated with antibiotics. To confine these problems, the use of many drugs will be restricted in Europe in the next ten years. Since active vaccination is not always an option, especially for short-living broilers, alternative treatments need to become available. In this thesis, an alternative approach is described which aims at intervention of the chicken mucosal immune system by oral administration of immunoglobulins

effective against pathogens. As a model for gastro-intestinal diseases caused by parasites in chicken coccidiosis was chosen.

Passive oral immune therapy for chicken requires a number of research advancements: First, development of a platform for the selection of pathogen-specific chicken IgA fragments. Second, adaptation of this platform for the production of full size immunoglobulins in a cost-effective and safe production system. And third, further characterization of the chicken secretory Ig complex, in order to determine the optimal form in which the antibodies should be applied.

Drawbacks of antibiotics & other drugs

Emergence of resistance

In the veterinary field big losses are ascribed to decreased effectivity of therapeutic drugs. Chicken are threatened by protozoan parasites belonging to the subclass *Coccidia* and family *Eimeriidae*, which can cause coccidiosis in poultry. This disease is characterized by severe diarrhea in chicken and has great economic impact on poultry production. Economic costs are estimated at $1.5 billion worldwide (Williams, 1999; Lillehoj et al., 2000). In spite of various attempts to prevent development of resistance against in-feed medication, resistance has been described for most anticoccidial drugs (Chapman, 1997). Chemotherapy against other parasitic infection faces similar problems: helminthic parasites causing diseases in production and companion animals which become incurable due to emergence of resistance (Geary et al., 1999), as well as *Giardia*, a protozoan parasite causing diarrhea (Upcroft et al., 2001; Sangster et al., 2002).

Several reports detect a direct link between agricultural antibiotic use in animals raised for food and subsequent emergence of antibiotic-resistant pathogens threatening humans (reviewed by Cohen, 1992, and Shea, 2003). This can be exemplified by the emergence of antibiotic resistant *Salmonella*, one of the primary causes of gastro-enteritis. Resistant bacteria can be transmitted to humans by consumption of contaminated poultry, beef, pork, eggs, and milk. When infection with *Salmonella* spreads beyond the intestinal tract, which mainly occurs in children, elderly or immuno-compromised persons, appropriate antimicrobial therapy can be lifesaving. Treatment with drugs cannot succeed when resistant strains are causing the infection. In a study conducted in the Washington D.C. area, *Salmonella* was isolated from 20% of the meat samples collected. Resistance to at least one antibiotic was found in 84% of the isolates, and 53% were resistant to at least three antibiotics. Sixteen percent of the isolates were resistant to ceftriaxone, the drug of choice for treating salmonellosis in children (White et al., 2001). Similar results have been found concerning *Campylobacter jejuni*, another food-borne pathogenic microbe, which causes

bacterial gastroenteritis and is a frequent cause of traveler's diarrhea. In the Netherlands, the prevalence of strains resistant against quinolone, an antibiotic used against *Campylobacter*, isolated from poultry products increased from 0% to 14% between 1982 and 1989, coinciding with the increasing use of this antibiotic in veterinary medicine. During the same period the prevalence of resistant strains in man increased from 0% to 11%. Taken together with the unique transmission route of *Campylobacter* from chicken to man, the data suggest that resistance observed in humans is mainly due to antibiotics used in the poultry industry (Endtz et al., 1991). In parallel, antibiotic resistant pathogens from food animals may facilitate the development of resistance in human commensal bacteria, which colonize healthy human intestinal tracts. Only in rare cases, these bacteria can cause opportunistic infections such as wound or bloodstream infections. Acquirement of resistance decreases the possibility to treat infections caused by these bacteria dramatically.

Exposure to residues

Another problem associated with continuous exposure to antimicrobial agents in food is the influence on human intestinal flora. This is an essential component of human physiology by acting as a barrier against colonization of the gastrointestinal tract with pathogenic bacteria. Furthermore, the endogenous flora plays an important role in the digestion of food and the metabolism of drugs, xenobiotics and nutrients. Disturbance of this complex, yet relatively stable ecological community of commensal bacteria, will make the organism more susceptible to infections (Cerniglia et al., 1999). Other concerns are focused on the possibility of a hypersensitive reaction of individuals to antibiotic residues. So far, no evidence has been found that any individual has become sensitized, presumably because the oral route is much less sensitizing than e.g. exposure through the skin and due to the low dose in which they occur. However, individuals have been reported with high sensitivity to certain antibiotics and exposure to even low dosages could cause major reactions (Dewdney et al., 1991). To predict and limit risks for consumers, various *in vitro* and *in vivo* model systems have been developed such as a variety of culture systems to test inhibitory effects of compounds found in faecal extracts, simulated gut models to determine for example acceptable daily intakes, or experimental set-ups with laboratory animals or human volunteers to determine resistance emergence and colonization barrier effects *in vivo* (Cerniglia et al., 1999). However, especially risks of low dosage exposure remain unpredictable. Complete and correct residue analysis of antibiotics and other drugs in food remains problematic due to their complicated extraction from animal tissue or products necessary for analysis (Di Corcia et al., 2002).

Zoonosis

These major drawbacks of the use of chemical treatment against pathogenic agents raise the demand for alternative treatments. This becomes even clearer in the light of zoonoses. Zoonosis occurs when a pathogen uses more than one species as a host, when it has the ability to "jump" from one species (e.g. chicken) to another (e.g. human). The microbial infection caused by *Salmonella* and *Campylobacter* as described before are examples of zoonosis. Zoonosis can be especially threatening when microbes have acquired resistance to therapeutic drugs.

Recently, zoonosis of viral nature have been a major concern to human healthcare: birds represent a zoonotic pool of viruses. Cases of humans infected with influenza A originating from birds or swine are accumulating (Claas et al., 1998; Shortridge et al., 2000; Lin et al., 2001). Human beings can act as "mixing vessels", exchange between human and avian influenza virus strains can lead to altered strains that are able to mislead the human immune system. Infections with these viruses represent an enormous threat to human welfare, especially when the virus is able to spread from man-to-man. Live-animal markets have been distinguished as high risk places because of a close contact between avian species and humans (Webster, 2004) and the same holds true for the livestock industry.

Case study: Coccidiosis in poultry

The problems described above are all applicable to birds in the livestock industry. Chicken are kept as production animals in high density worldwide, thus increasing chances of resistance and zoonosis development. To minimize these risks, accurate and safe protection of birds in livestock industry is inevitable for animal welfare as well as for human health.

One of the main threats for chicken is coccidiosis, a diarrhoeal disease caused by apicomplexan parasites (*Eimeria* spp.). Development for new treatment devices for coccidiosis is necessary for several reasons. *Eimeria* spp. have developed resistance against most (if not all) anticoccidial compounds (Chapman, 1997). The development of new drugs is costly, while new resistance is likely to occur sooner or later. The use of effective anticoccidial drugs at optimal levels hinders the development of a protective immune response which may be necessary in the withdrawal period, which lasts at least 10 days before slaughter, wherein these drugs cannot be used. Coccidiosis occurring in this period results in damage that cannot be compensated for. In the European union, coccidiostats will be banned in 2007 (Regulation (EC) No 1831/2003). Several alternative methods have been developed to induce protective immune response in chicken. Vaccination can be successfully achieved by administration of either virulent (wild-type) parasites or attenuated (modified, less virulent) strains. Problems can

arise however with progeny of the vaccine strains, which will circulate within the chicken population and might cause new infections. Furthermore, cellular based immune responses evoked by vaccines can in rare cases be accompanied by tissue-damaging inflammatory responses. The latter has potential negative effects on metabolism and growth of the birds and can lead to enhancement of microbial translocation eventuating in infections with pathogenic bacteria such as S*almonella* or C*ampylobacter*. Also, antigenic variation between *Eimeria* species present in the field and the vaccination strains might result in unprotected or only partially protected chicken (Vermeulen et al., 2001; Chapman et al., 2002). Immunization with recombinant antigens represents another vaccination strategy. However so far only partial protection has been accomplished following this strategy (Bhogal et al., 1992; Jenkins, 2001).

Alternative treatment by mucosal intervention

Passively provided protection against epithelial infection can prevent or diminish the need of cellular based immune responses evoked by vaccines to *Eimeria*. A natural mechanism of passively provided protection is maternal immunization. For bird species, immunization of the mother hen leads to specific antibodies in the egg yolk (Wallach et al., 1995; Witcombe et al., 2004). Protection of the young chick lasts three weeks at maximum. Coccidiosis occurring afterwards leads to negative effects of production that cannot be compensated for before the end of the grow-out in broilers. Efficacy of passively administered antibodies against *Eimeria* spp. has been demonstrated with specific antibodies from bovine colostrum (Fayer et al., 1992) and with monoclonal antibodies against antigens from different parasitic stages (Wallach et al., 1990; Karim et al., 1996). The role of secretory IgA in anti-coccidial immunity in chicken has been reported for long (Davis et al., 1978; Davis et al., 1979). Renewed interest in this strategy led to findings indicating that polyvalent neutralizing antibody formulations targeting epitopes on defined antigens may provide optimal passive immunization against cryptosporidiosis, a diarrhoeal disease caused by another apicomplexan parasite, *Cryptosporidium parvum*, which infects humans (Schaefer et al., 2000; Riggs et al., 2002).

Development of a successful and sustainable vaccination strategy for coccidiosis has not been achieved yet, partly because knowledge both on the parasite and on the immune system of birds remains limited. For the development of successful and sustainable passive immune therapy in avian species, basic knowledge on presence and nature of (secretory) immunoglobulins in the chicken gut is inevitable.

Chicken secretory IgA

In avian species as in mammals, IgA is the main isotype present in external secretions like bile, lacrimal fluid and faeces. IgM can be found as well, but this is more abundant in the serum, where also IgY (a homologue of mammalian IgG) is found. In mammalian species, IgA plays an important role in the first line defence against invading pathogens. As shown in the schematic overview of figure 1, specific dimeric IgA is transported from the internal to the external side of epithelial cell layers by the polymeric immunoglobulin receptor (Mostov et al., 1999). After transport the extracellular part of this receptor, called the secretory component (SC), is cleaved of and remains bound to the IgA. The secretory component exhibits several functions in mammalian species. Not only does it protect IgA from degrading proteases, which are very abundant in the gut, and against pH fluctuations, it also enhances its affinity for pathogens. A recent finding is that the secretory component, which is highly glycosylated, positions the IgA complex by binding to the glycocalyx layer lining the epithelial cells, providing optimal protection at the most important location (Phalipon et al., 2003).

In mammalian species, the secretory complex is formed by two IgA molecules, the secretory component, and the J-chain. In chicken, the J-chain has been described as a putative protein of 158 amino acid residues (including the signal peptide) with a high degree of homology with J-chain of human, cow, rabbit, mouse, frog and earth worm (Takahashi et al., 2000). Phylogenetic analysis of the entire J-chain group shows high homology between *X. laevis* and rabbit, whereas human, cow and mouse cluster and earthworm shows no strong similarities to any of the other animals, thus forming an outgroup. Together with reported high levels of J-chain mRNA expression found in spleen, intestine, Harderian gland, and bursa of Fabricius, these data suggested a similar role of J-chain in chicken as in mammalian species and thus presence and functionality of secretory complex.

The molecular structure of the polymeric immunoglobulin receptor in chicken was not available. However, presence of secretory immunoglobulins similar to mammalian sIgA in chicken was already suggested in 1972 by biochemical studies (Lebacq-Verheyden, 1972). Also, the existence of a transport mechanism of serum derived polymeric IgA across hepatocytes to bile was demonstrated by recovering i.v. injected, radio-labelled monoclonal dimeric human IgA in bile (Rose et al., 1981). As reviewed by Underdown and Schiff (Underdown et al., 1986) the chicken transport mechanism shows many similarities to the mechanism observed in rodents. In humans however, no pIgR expression has been found in hepatocytes and no IgA clearance from serum to bile occurs in the liver (Underdown et al., 1986).

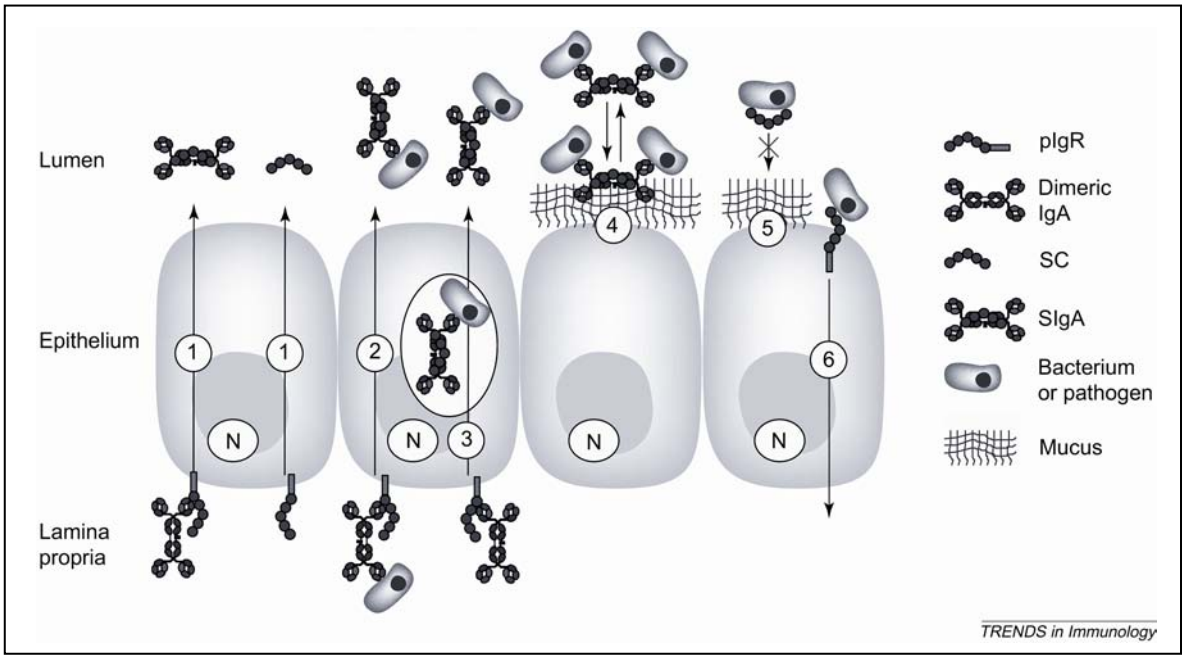

Figure 1

Multi-faceted functions of the polymeric Ig receptor (pIgR) and secretory component (SC) obtained in various *in vitro* and *in vivo* experimental models. Route (1): pIgR ensures transcytosis of polymeric IgA (pIgA) across the epithelial monolayer, leading to the local production of secretory IgA (SIgA). Route (1′): transcytosis of empty pIgR and release of free SC occurs similarly to route (1), yet with a lesser efficiency. Route (2): pIgR is able to transcytose pIgA loaded with protein present in the lamina propria suggesting excretion of pathogen-SIgA complexes. Route (3): on their way to lumenal secretion, pIgA can neutralize invading pathogens by the process of intracellular neutralization. Route (4): immune exclusion implies neutralization within the lumen, with epithelial anchoring of SIgA mediated by bound SC. Route (5): free SC might exhibit scavenger function by preventing epithelial cell-pathogen interaction. Route (6): apical-to-lumenal pIgR-mediated translocation of bacteria has been reported in one case.
This figure was adapted from Phalipon et al. (2003), with permission from Elsevier.

For mammalian species, a considerable amount of information is available on passively administered immunoglobulins. IgA has been suggested to be the optimal isotype of choice for passive immune therapy, since it shows both prophylactic and therapeutic efficacy in prevention or reduction of infections of the intestinal tract. Furthermore, immunoglobulins of the alpha isotype are the least likely to initiate complement or inflammatory responses (case studies by Offit et al., 1985; Winner et al., 1991; Czinn et al., 1993 and Enriquez et al., 1998 and reviews by Lamm, 1997; Corthesy et al., 1999 and Zeitlin et al., 2000). Under natural conditions, infections tend to be initiated by relatively small inocula of a pathogen, whether inhaled into the respiratory tract or ingested. Therefore, even small amounts of pre-existing secretory IgA antibody can be effective in preventing disease (Lamm (1997) and references therein). Assuming parallels in the mucosal immune system of chicken and mammalian species, (secretory) IgA

would be the isotype to choose for oral passive immune therapy. The possibility to combine passive and active immunization by using secretory IgA as a mucosal vaccine carrier represents an additional advantage of the use of IgA in passive immune therapy. Corthesy et al. (1996) inserted a foreign epitope into the secretory component. The modified secretory component was reassociated with dimeric IgA forming secretory complexes. These complexes were able to evoke immune responses against the foreign epitope, resulting in production of specific systemic and mucosal antibodies. The course of events was clarified recently: In the lumen of the gut, secretory IgA adheres selectively to IgA receptors at the apical surfaces of M cells. Subsequently, the sIgA is transported through the epithelial layer and presented to underlying gut-associated organized lymphoid tissue, where it boosts the systemic and secretory immune response (Mantis et al., 2002; Rey et al., 2004).

Production platforms for therapeutic antibodies

Since 1986 the US Food and Drug Administration (FDA) has approved 11 monoclonal antibodies for therapeutic application, for example for treatment of transplant rejection, non-Hodgkin lymphoma, rheumatic conditions and leukaemia. At least 400 other antibodies are in clinical trial worldwide (Gura, 2002). Especially the technological achievement to display antibody fragments on the surface of bacteriophages has had a major impact on isolation of therapeutic antibodies. This technique allows rapid screening of enormous (patient-derived) antibody repertoires for binders with optimal characteristics (Hoogenboom et al., 1998). To date, in addition to selection of human antibodies, phage display has been applied to studies with rabbits (Ridder et al., 1995; Foti et al., 1998), sheep (Charlton et al., 2000; Li et al., 2000), bovine and porcine species (O'Brien et al., 1999; Li et al., 2003), camels (Ghahroudi et al., 1997) and chicken (Davies et al., 1995; Yamanaka et al., 1996; Andris-Widhopf et al., 2000), allowing antibody discovery for veterinary purposes.

Despite the therapeutic successes of antibody-based therapy, large-scale application remains limited due to financial costs. Although antibodies require relatively low investment in initial research and development compared to conventional drugs, they are hugely expensive to manufacture. The first generation monoclonal antibodies produced artificially were murine mAbs derived from mouse B-cell hybridoma's was awarded with the Nobel Prize (Köhler et al., 1975). Since then, several platforms for large-scale production have been developed including bacteria (Simmons et al., 2002), yeast, insect and mammalian cell cultures (Verma et al., 1998, and references therein) and more recently plants (Hiatt et al., 1989; Düring et al., 1990; Fischer et al., 2003; Ma et al., 2003). For therapeutic application, antibodies produced in bacterial systems are no option since *E. coli* is not capable of glycosylating proteins. Furthermore,

the transcription and translation machinery of prokaryotes is not able to assemble complex molecules like antibodies. All other systems are based on eukaryotic cell expression and are more capable of producing correctly assembled and glycosylated molecules. Comparing different systems for recombinant protein production reveals that plants represent –by far– the most cost-effective system (Hood et al., 2002; Schillberg et al., 2003; Twyman et al., 2003). Furthermore, pharmaceuticals produced in plants limit risks of contamination with endotoxins or human pathogens. Moreover, vaccines or antibodies can be expressed in edible plant organs, which makes laborious and purification steps before administration unnecessary.

Outline of this thesis

The aim of the research described in this thesis was to develop an alternative for the use of antibiotics in agriculture. As a model system coccidiosis was chosen, a diarrheal disease in chicken that is caused by apicomplexan parasites of the genus *Eimeria*: the disease causes major economic losses worldwide and resistance against antibiotics has emerged. This approach comprises the integration of two biotechnological techniques developed in recent years: phage display-based antibody selection and plant-based production of recombinant proteins. The separate steps that were undertaken are described in the following chapters, an overview is given in figure 2.

In **chapter 2**, the adjustment of the phage display system is described to allow display of chicken antibodies. A phage display vector was adjusted to display chicken IgA Fab fragments. With this vector, named pChick3, an *Eimeria*-specific library assembled from cDNA obtained from bursal tissue, cecal tonsils and spleen of infected chicken was constructed. Applying phage display of antibody fragments allowed us to specifically select IgA Fab fragments with binding to different parasitic stages of the *Eimeria* parasite.

After selection of anti-*Eimeria* antibody fragments, new cassettes were constructed for the transfer and expression of phage display-selected antibodies (IgA) in plants using *Agrobacterium tumefaciens*. Surprisingly, differences in expression levels up to 500-fold were found among the different antibodies. In **chapter 3**, the plant expression system and the experiments conducted in order to understand the rules governing plant expression levels are described in detail.

To provide protection by passive immune therapy, we tried to mimic the natural mucosal Ig complex as close as possible. Thus, the aim was to produce the entire secretory complex in plants for subsequent delivery to chicken. Since no detailed information on the chicken secretory component was available, the cloning and characterization of this molecule is described in **chapter 4**. Using a homology-based PCR approach, the chicken *pIgR* gene was elucidated. This was the first avian *pIgR* to be reported. Its nucleotide sequence revealed a striking difference with their mammalian counterparts: one of the mammalian extracellular, immunoglobulin-like domains did not have homologous domain in the chicken *pIgR* gene. Still, the chicken pIgR was found to be functional and to play similar role in the secretory immune system as mammalian pIgRs. In **chapter 5**, the chicken pIgR expression pattern is described in more detail. An antiserum against the chicken SC was raised and used in immunohistochemical studies. This revealed an expression pattern parallel to IgA expression and very similar to mammalian physiology. These data were confirmed by a Real Time PCR approach with cDNA isolated from chicks of different ages.

In a final step, the several constructs comprising the secretory complex (i.e. IgA heavy and light chains, the J-chain and the secretory component) were co-infiltrated in plants. The results of this study are given in the **chapter 6**. Different infiltrations led to formation of complexes with different sizes: co-infiltration of IgA lead to a smaller protein complex than IgA with J-chain (forming dimeric IgA). Co-infiltration with all constructs lead to expression of the largest protein complex, suggesting formation of full secretory IgA *in planta*.

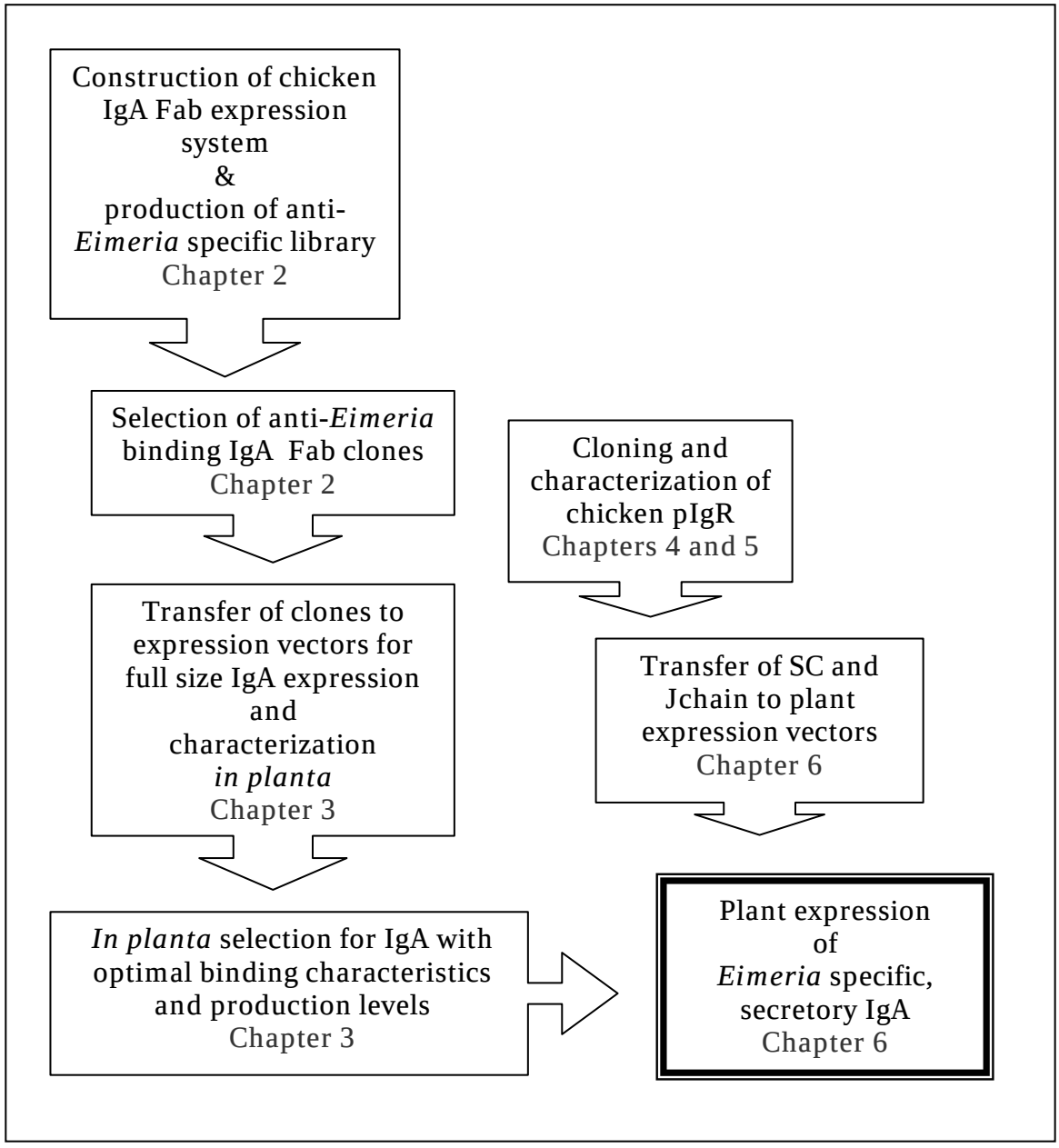

Figure 2
Overview of the different experimental steps described in this thesis, which led to the expression of *Eimeria*-specific, secretory IgA produced in tobacco plants.

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Chapter 2

Display and selection of chicken IgA Fab fragments

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SUMMARY

Passive immune therapy has regained interest to prevent and cure infective diseases both in human and veterinary medicine. Therefore, systems are required that enable efficient targeted selection of antibodies originating from virtually any animal species. Here, such a system for the selection of chicken IgA is described. It includes the possibility to rapidly transfer selected antibodies to systems capable of expressing full-length antibodies. A novel phagemid vector for the display and selection of chicken IgA antibodies in Fab format was developed. The vector pChick3 allows, in a versatile fashion, the incorporation of genes coding for chicken variable antibody domains in frame with constant domains of chicken IgA Fab fragments. The functionality of pChick3 was demonstrated by the construction of an *Eimeria acervulina* specific antibody library. Three polyclonal phage populations binding to different developmental stages of the parasite were selected. From these phage pools, ten different IgA fragments with specific binding to either the *Eimeria* antigen mix, the sporozoites or oocyst fractions were selected. These results demonstrate the efficiency and versatility of the pChick3 vector system that can readily be applied to construct libraries and subsequently select antibodies of the alpha isotype against a wide variety of pathogens and parasites.

INTRODUCTION

Until today preventive and curative drugs are effectively used to treat infectious diseases of poultry. The development of resistant pathogens, the possible presence of residues in meat and eggs destined for human consumption as well as the increasing concern of zoonosis demand development of alternatives. These are offered through various vaccination strategies. Active vaccination with live virulent or attenuated vaccines has been envisaged as a promising strategy. Insufficient immunogenicity, high cost of production, antigenic variability between species and danger of transfer of genetic material to wild-type strains represent major drawbacks that have hindered large scale application. Prevention or reduction of infections of the intestinal tract can also be achieved through passive vaccination by orally administering immunoglobulins. This strategy shows both prophylactic and therapeutic efficacy against viral, bacterial and protozoan infections (Offit et al., 1985; Winner et al., 1991; Czinn et al., 1993; Enriquez et al., 1998).

Secretory immunoglobulins form the first line of defence of mucosal surfaces. The ability of immunoglobulins to protect mucosal surfaces is mainly based on immune exclusion, where binding to pathogens prevents attachment and colonization. Several observations suggest a role for chicken IgA in the protection of mucosal surfaces similar to mammalian IgA (Klipper et al., 2000; Muir et al., 2000; Wieland et al., 2004). For therapeutic applications, IgA might be the most desirable isotype because of its stability in the secretory IgA complex and as IgA is less likely to initiate complement or pro-inflammatory responses (Lamm, 1997; Corthesy et al., 1999; Zeitlin et al., 2000). Furthermore, (secretory) IgA interacts with specialized epithelial M cells in the gut and a role in antigen sampling and processing to induce secretory immune response has been suggested (Neutra, 1998; Heystek et al., 2002; Rey et al., 2004). Passive immunotherapy with orally administrated IgA can therefore be a promising alternative for chemical treatments to protect poultry against parasitic, viral and bacterial infections and in parallel induce specific immune responses. Increased avian mucosal immunity is relevant both for animal welfare as well as for human health. The chances for zoonosis through bacteria such as *Salmonellae* and *Campylobacter* and viruses such as the influenza virus and the possible presence of drug residues in meat or eggs for human consumption will be reduced.

One of the most abundant infective diseases in chicken is coccidiosis, an intestinal disorder caused by intracellular protozoan parasites belonging to the genus *Eimeria*. These parasites use the oral route of infection and cause diarrhea, morbidity and mortality in poultry (Rothwell et al., 1995; Lillehoj et al., 1996). Since coccidiostats will be banned in the EU (Regulation (EC) No 1831/2003), alternatives are needed. The role of secretory IgA in anti-coccidial immunity in chicken has been described for long (Davis et al., 1978; Davis et al., 1979), but

more attention has been paid to vaccination strategies (Jenkins, 2001; Vermeulen et al., 2001; Chapman et al., 2002). Alternatively, passive immunoprophylaxis by orally administered IgA could have major benefits especially for young broilers because of undelayed effectivity, even when exposure to the pathogen precedes immunoglobulin application.

An enormous reservoir of antibodies with possible therapeutic potential is present in the body. Using combinatorial phage display libraries, such antibodies can be selected by binding to a specific antigen (Hoogenboom et al., 1998; Benhar, 2001). These phage libraries have originally been described for murine and human antibody repertoires (McCafferty et al., 1990), and subsequently extended to other mammalian organisms like bovine and porcine species (O'Brien et al., 1999; Li et al., 2003). Only recently the technology has been adapted to non-mammalian species like chicken (Andris-Widhopf et al., 2000). These species-specific combinatorial libraries allow selection of recombinant antibodies for therapeutic application in other animal species, including pets and livestock. Following selection and characterization, the antibodies have to be produced on a large scale. Recently, novel methods for production of full size antibodies have been developed (Andersen et al., 2002; Ma et al., 2003; Twyman et al., 2003). These production systems overcome disadvantages like the risk of transfer of pathogens and high costs of production, thus allowing fast and cost-effective production of any desired antibody for therapeutic applications.

For chicken, the application of oral passive immunotherapy using recombinant IgA requires the development of new biotechnological tools specifically designed for birds. Here, we present a new method for the display of chicken antibodies in Fab format. The system contains two novelties: First, it incorporates the possibility to clone chicken V-genes in frame with chicken constant regions. Second, instead of the traditional gamma isotype, the constant heavy chain domain corresponds to the alpha isotype, facilitating the subsequent conversion into full-length IgA antibodies for oral immunotherapy. The applicability of our system has been demonstrated with the generation of an anti-*Eimeria acervulina* phage display library. This combinatorial IgA library was generated from *E. acervulina* infected chicken. Subsequently, specific antibodies were selected which bound to an *Eimeria* antigen mixture, or sporozoite and oocyst preparations. Following this strategy single *Eimeria*-binding IgA Fab fragments in an anti-*Eimeria* enriched polyclonal library were obtained.

RESULTS

Display of chicken IgA antibodies in Fab format

For the display and selection of chicken IgA antibodies in Fab format a new system based on the pChick3 phagemid, derived from pHEN2 (Hoogenboom et al., 1991), was designed (Fig. 1). By introducing a new ribosomal binding site in the pHEN2 vector, the monocistronic operon directed by the LacZ promoter was converted into a dicistronic operon. This strategy allows the coordinate expression of two genes encoding heavy and light chains under the control of a single promoter. The first gene in pChick3 contains the pelB secretion signal and is intended for introduction of chicken IgL. The second open reading frame uses the secretion signal of the gene III capsid protein (pIII) of M13, and incorporates the Cα1 domain of chicken IgA (Mansikka, 1992) fused to a penta-His tag, a c-myc tag and to the M13 gene III capsid protein. An amber stop codon separates Cα-1/pentaHis/c-myc from pIII, allowing the expression of the recombinant Fab fragments either or not fused to pIII. Additionally, the second ORF contains a cloning site for the introduction of chicken VH.

Since no data were available on structure and stability of chicken IgA Fab fragments and their behaviour in phage display, we first tested pChick3. Chicken variable heavy and light chain repertoires were amplified with specific oligonucleotides from cDNA samples from chicken lymphoid tissues and cloned into pChick3. The resulting phagemids were transformed into *E. coli* and the production of recombinant phages was induced with IPTG. The expression of IgL and VH-Cα1 either or not fused to pIII was assessed in a Western blotting experiment (Fig. 2A). Both IgL and VH-Cα1-PIII were found when expressed in TG1 cells (Fig. 2A, “S” lanes). A band of approximately 25kDa was found next to the VH-Cα1-PIII band. This band is likely free VH-Cα1 as it corresponds to the band in the N-lanes wherein the ability to express free Fab fragments was shown in SupA$^-$ *E. coli* strain. *In vivo* association between chicken light chain and VH-Cα1-pIII fusions was tested in a sandwich ELISA assay. As shown in figure 2B, phage preparations gave positive signals in ELISA when sandwiched between anti-IgA and anti-IgL antibodies, demonstrating formation of stable Fab fragments resulting from the association of light chains with VH-Cα1-pIII.

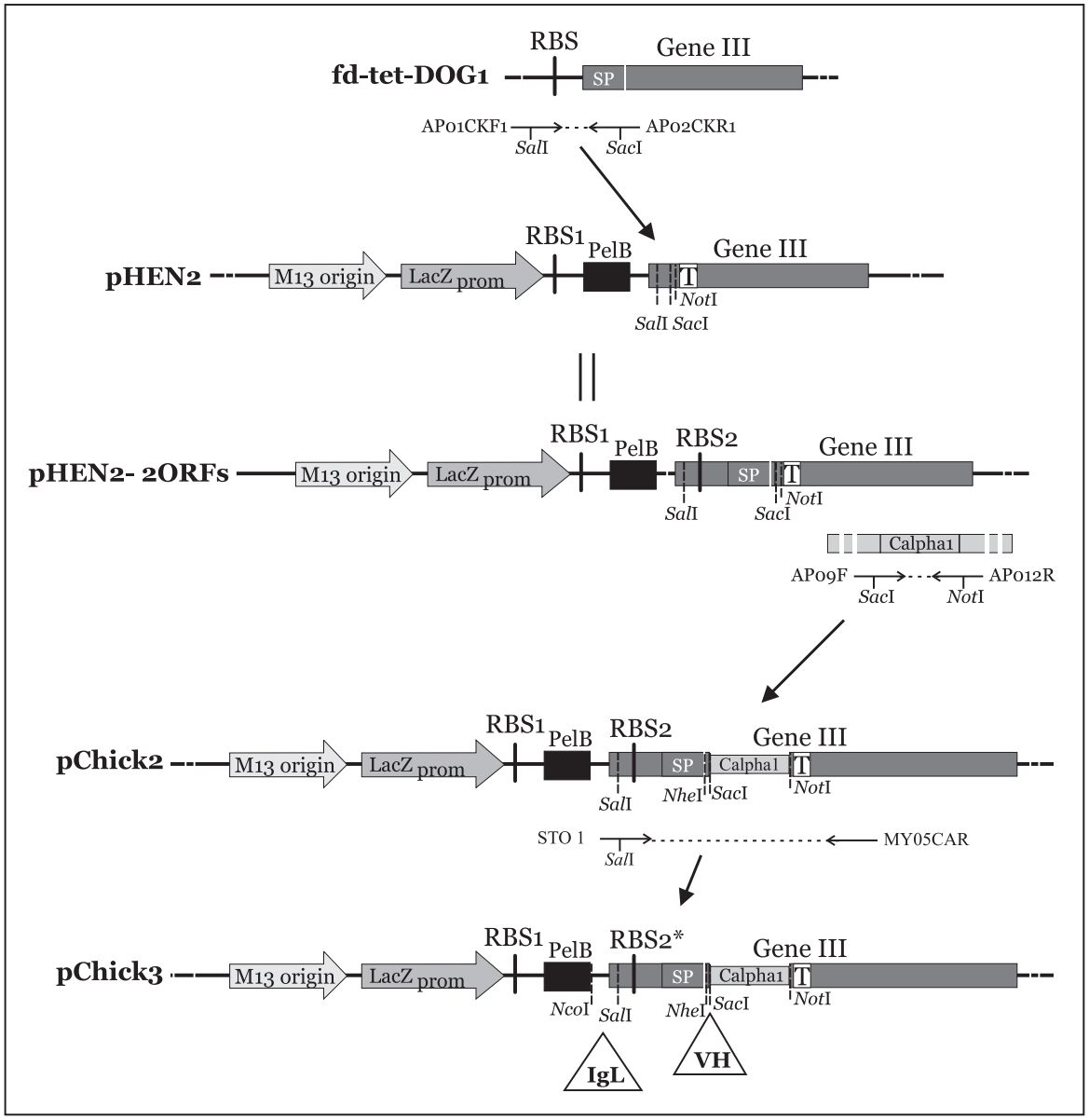
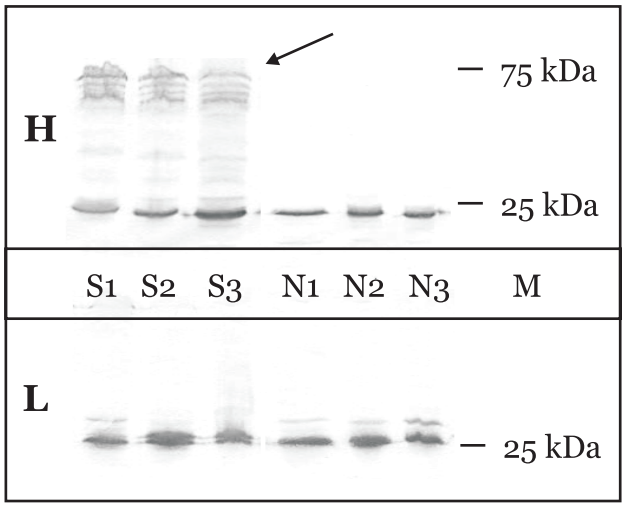


Figure 1 Schematic representation of the pChick3 phagemid vector construction for the display of chicken IgA Fab fragments. The LacZ promoter ($LacZ_{prom}$), directs the expression of a bicistronic operon. The two ribosomal binding sites are indicated as RBS1 and RBS2. The first cistron is originally present in the vector pHEN2 (Hoogenboom et al., 1991) and comprises the PelB signal sequence for secretion of the chicken light chain. The second cistron was constructed by insertion of the RBS (RBS2) and secretion signal sequence of the pIII protein (SP) as derived from fd-tet-DOG2, modified by adding the first constant domain of the chicken alpha heavy chain (Calpha1). In a final step, RBS2 with the initial start codon were modified by directed mutagenis (depicted by an asterisk). The final VH and Calpha1 construct is fused to two tag sequences, c-myc and His6 (T), followed by an Amber stop codon and the pIII coat protein of M13 phages.

A



B

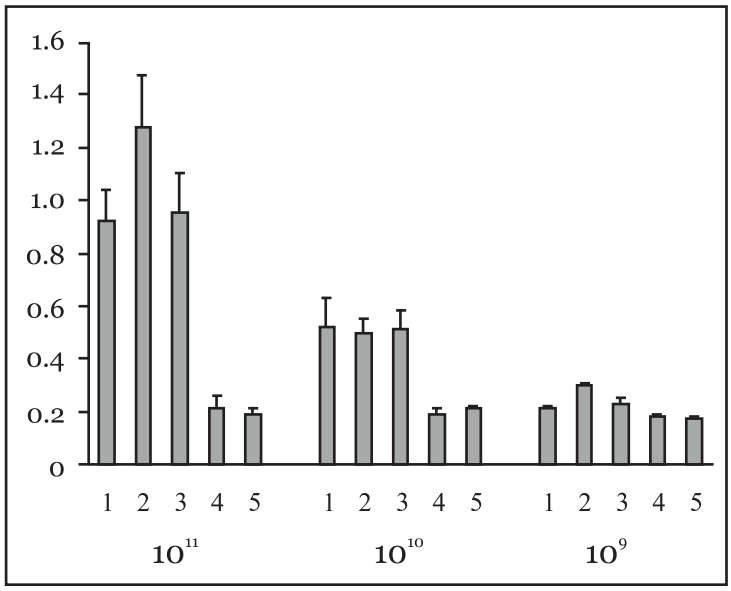


Figure 2 Demonstration of the ability of pChick3 vector to produce recombinant chicken antibody fragments. **(A)** Western blot analysis showing expression of the VH-αCH_1 fragment (Panel H) and the light chain (panel L). Numbers (1-3) represent randomly chosen bacterial colonies. "S" lanes correspond to SupA+, TG1 host cells, expressing the VH-αCH_1-pIII fusions (see arrow in panel A); "N" lanes correspond to SupA- TOP10 cells expressing only the free VH-αCH_1 form. **(B)** Sandwich ELISA showing functional association of heavy and light chains. Recombinant phage preparations were captured with anti-chicken IgA and detected with anti-chicken IgY antibodies fused to peroxidase (recognizing the chicken light chain). Numbers represent individual colonies expressing pChick-transformed, randomly selected colonies (1, 2 and 3) and unrelated scFv recombinant phages (4, 5). Serial dilutions of phage-containing supernatant are indicated.

Construction of anti-*Eimeria* Fab library

To obtain IgA fragments with specificity for *Eimeria* parasites, an antibody library from lymphocytes isolated from *E. acervulina* infected chicken was generated. To determine the optimal time point for collection of antibody-coding mRNA, the serum antibody response following *Eimeria* infection was monitored.
After primary infection, parasite specific antibody levels were low (data not shown). Three days after secondary infection an increase of both oocyst and sporozoite specific antibodies was found (Fig. 3). Maximum levels of specific antibodies were found one week after secondary infection. The germinal centers of lymphoid organs contain the largest repertoire two days before serum immunoglobulin levels reach their maximum, therefore bursa, spleen and cecal tonsils were isolated three and five days after secondary infection. To synthesize cDNA, mRNA was isolated from spleen, bursa and cecal tonsils. Following this approach, the library comprised antibodies induced systemical in spleen and mucosal in bursa and cecal tonsils. Amplified IgL and VH cDNA fragments were incorporated into pGEMT and pChick3 respectively. In this fashion, two primary libraries were constructed: pGEMT-IgL library containing 2.0 x 10^6 clones, 95% of them containing an insert; pChick3-VH library consisting of 4.4 x 10^6 clones, with a percentage of insert-containing colonies of 97%. Both libraries were combined into the pChick3 frame, generating a secondary library named Eckα1. The Eckα1-library comprises 1.5 x 10^7 independent clones, 82% of them showing both IgL and VH inserts. Diversity of the library was analysed by PCR and fingerprinting of the immunoglobulin variable regions and showed high diversity of among the clones (> 80% different clones with *Bst*NI digestion, data not shown).

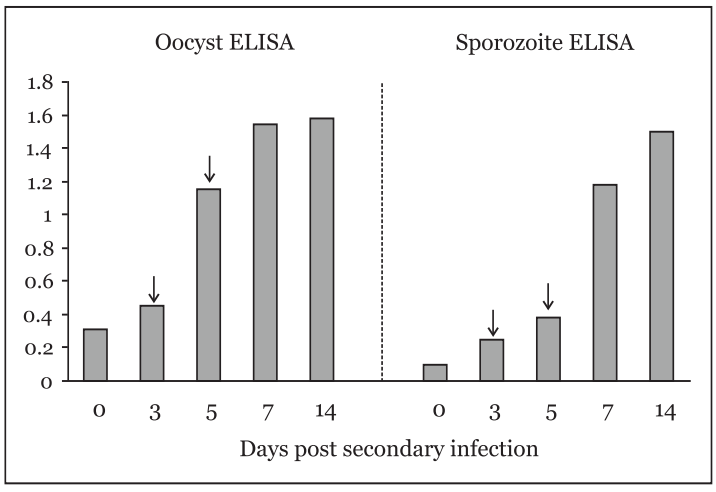


Figure 3
Eimeria specific antibody levels (serum dilution 1/200) from chicken infection experiments detected with anti chicken Ig, conjugated to peroxidase. Chickens used for library construction were sacrificed on day 3 and 5 after the secondary infection (depicted with arrow), just before maximum serum levels of specific antibodies were reached.

Selection of *Eimeria* binding IgA Fab fragments from Eckα1

To demonstrate the applicability of the library, Fab fragments specific for antigens of different parasitic stages were selected. Three approaches were undertaken: selection against a suspension containing all *Eimeria* antigens (oocyst- and sporocyst-walls and sporozoites), a sporozoite and an oocyst antigen suspension. The antigens were immobilized in microtiter plates and incubated with phage preparations derived from Eckα1. Prior to selection, little binding to *E. acervulina* antigens could be found (results not shown). After 4 selection rounds, a gradual rise in *Eimeria*-specific binding was observed in the selection against sporozoites and oocysts (figure 4B and C), whereas only weak increase in absorbance was found when binding of phages to BSA was tested. Panning against the suspension containing all *Eimeria antigens* showed the highest increase in binding already after the second panning round (figure 4A). The rise in polyclonal binding affinity by specific selection procedures shows the feasibility to enrich the Eckα1-library within two or four selection rounds for specific *Eimeria* binding Fab fragments.

Further characterization of five clones selected on a mix of *Eimeria* antigens (2; 8; 10; 11; 14; second selection round), three on sporozoites (A2; C3; C4; fourth selection round) and two on oocyst walls (E4 and G5; fourth selection round) was performed. These ten clones were isolated and the presence of both VH and IgL inserts was confirmed by PCR. DNA fingerprinting of the isolated inserts showed a high variation between the selected clones (data not shown), a confirmation of the polyclonal nature of the selection. ELISA results show significant anti-*Eimeria* specific binding activity (see figure 5A and B).

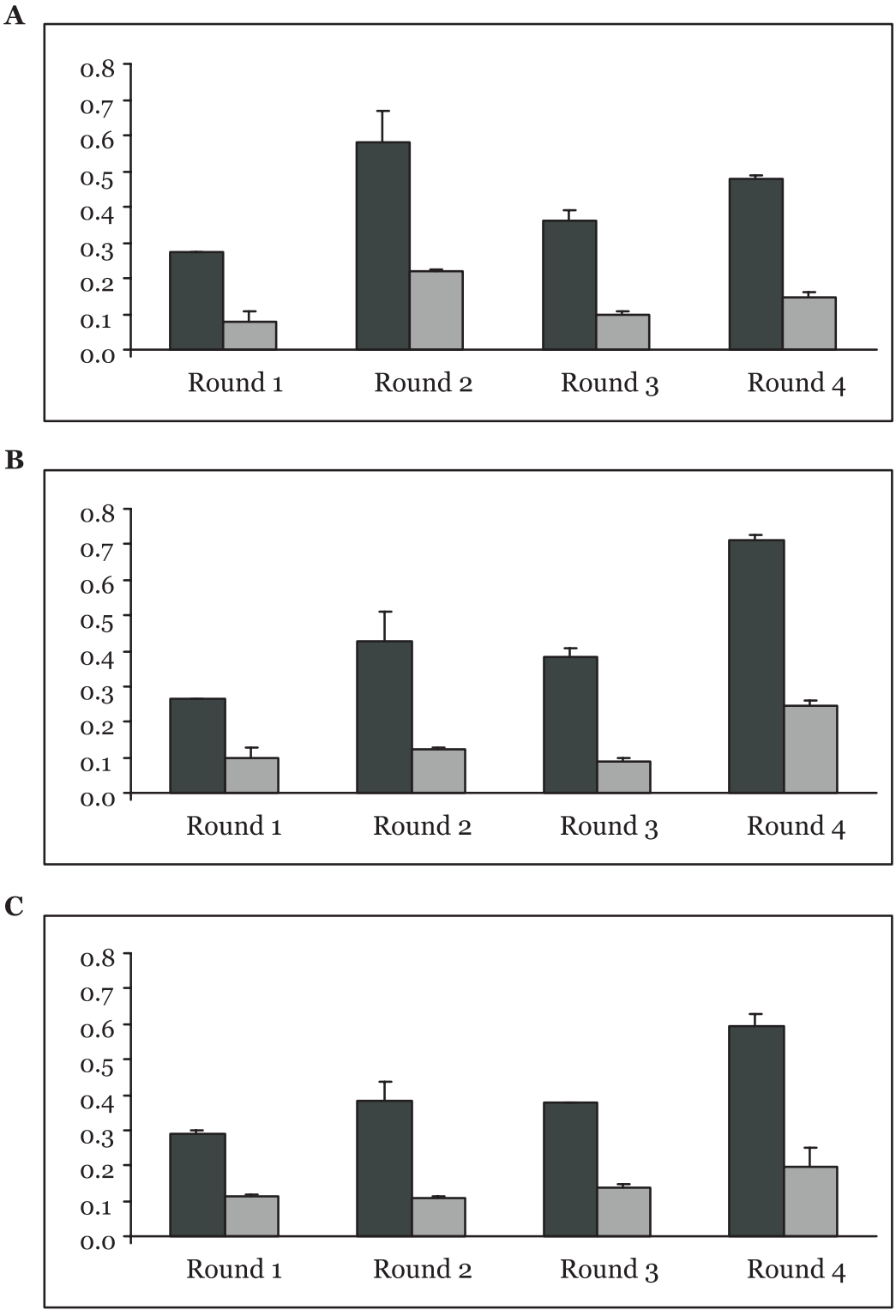


Figure 4 Binding of selected and unselected polyclonal phage suspensions panned against **(A)** *Eimeria* whole antigen suspensions, **(B)** sporozoites and **(C)** oocysts. Dark bars represent specific ELISA, light bars show reactivity against BSA. The unselected library showed little binding to the three antigen preparations shown by ODs lower than those of phage preparations obtained in the various selection rounds.

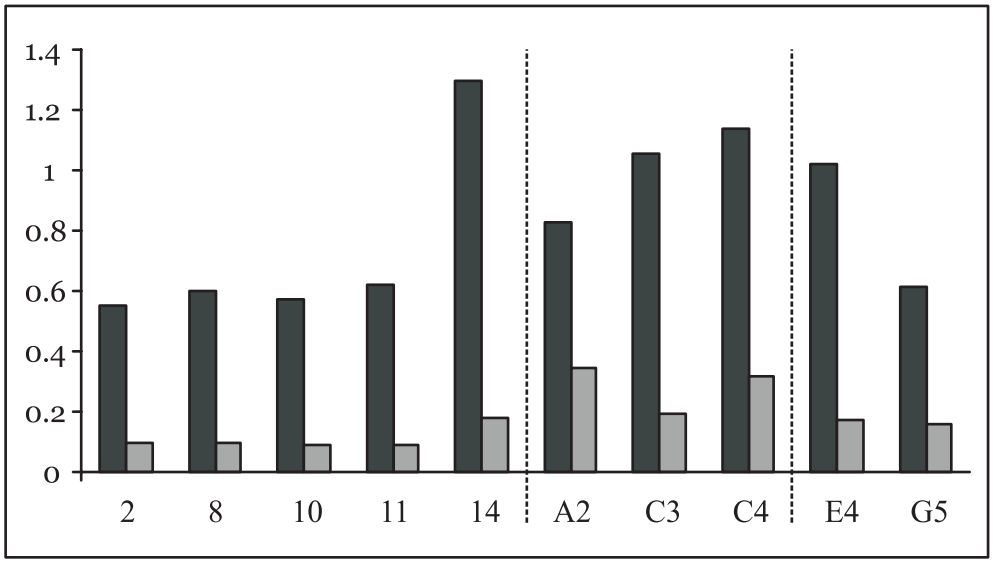


Figure 5 Phage preparations derived from individual colonies after selection were tested in an ELISA against crude *E. acervulina* antigen preparations, sporozoites or oocysts as depicted (dark bars) using BSA coated wells as a control for unspecific binding (light bars). Five phage clones (2, 8, 10, 11, 14) were chosen from the second selection round panning against the whole antigen preparation, three respectively two clones were picked from selection against sporozoites A2; C3; C4) or oocysts (E4 and G5) after four selection rounds.

The nucleotide sequences of the variable regions of the light and heavy chains originating from the ten binders were determined. The ten clones were found to be very different with variation mainly located in the CDR regions, as expected. Alignment of the CDRs of the deduced protein sequences of the variable domains is shown in figure 6A and B. It is worth noting, that amino acid sequences of individual CDRs of several different clones are identical. For example, CDR1 of the light chains of clones 11 and G5 are identical, whereas CDR2 of clone 11 matches CDR2 of clone 8, and CDR2 of clone G5 matches clone 14. Most variation in amino acid content, caused by changes of nucleotide sequences such as nucleotide substitutions, insertions and deletions, was found in CDR3 of both light and heavy chain sequences. Variation in the heavy chains is higher than in the light chains.

A

	→---CDR 1--←	→----CDR 2----←	→-------CDR 3------------←
10	SGYNMQWVRQAP	QISSTGRYTEYGAAVK	CAKASGSG.CS....GYGCYG.YTASID
C4	-S-ALG------	S--N--SG-W----M-	---TTY--.--...SV---G..VAG---
A2	-SFY-F------	G-NAANT--G--P---	---SAAGFS-A....NGWC.D.-AGQ--
14	-S-D-L------	G-HC--NSKY-AP--R	----ARGY.-GW..T.....PYT-DI--
11	-S-G-N------	G-GN-----G--S---	---SAV-D.--...S-.....WRAG---
8	-S-A-N------	G-NPA-S--A------	---GATGYS.............-DGN--
G5	-S-A-N------	A---D-SS-A------	---NA.................-A-N--
E4	-S-G-H------	G-Y-GSS-MY-AP---	---DD-IT.............PDAGE--
C3	G--I-H------	G-GN-----G--S--R	---SAYG-S.W...SY.....TTA-F--
2	-S---G------	A--ND-SW-G-AP---	---TT-N-Y-AWWASPL--G-YTIGT--

B

	→----CDR 1----←	→CDR 2←	→CDR 3←
10	SG..GSYS.....YGW	SNNNRPSD	SSYV..GI
C4	--..----.....---	--DK---N	G-ST...A
8	--..D-DY........	N-------	--ST..AA
11	--..D-SY........	N-------	--ST..D-
G5	--..D-SY........	A-T----N	--NT...A
14	--..VAAMMEVIT---	A-T----N	-TDA...A
2	--..D-SW........	D-DK---N	--SA.A--
C3	LR..---T........	N-DK---N	--SA.A--
E4	--GGS-SY....----	DSTST---	--GTYG-M
A2	R-..--S.....---	N--K----	--G....-

Figure 6 Alignment of the deduced amino acid sequences (shown using the single letter code) of the three CDRs of VL **(A)** and VH **(B)** of 10 *Eimeria* binding clones. Sequence gaps are indicated by dots, identical residues are depicted as dashes. Clone numbers correspond to those shown in figure 5.

DISCUSSION AND CONCLUSIONS

A new system for the expression and selection of chicken IgA Fab fragments was developed. Applying this technique, antibody fragments of the alpha isotype with specificity against parasites can be isolated and easily transferred to suitable expression systems for future use in immune therapeutic applications. The use of antibodies in prevention and treatment of infective diseases has regained interest (Dunman et al., 2003). This renewed interest is a consequence of the development of antibiotic resistance of pathogens demanding development of alternative immunotherapeutic strategies. Antibody-based therapies can be useful, for example, to prevent and cure *Eimeria* infections, where antibodies are known to play a role in protection against the parasite. Shortly after infection B cells found in the intestinal tract of mature chicken begin producing parasite-specific antibodies and biliary sIgA antibodies are detected within one week after oral infection and correlate with low parasite numbers (Lillehoj et al., 2000).

In this study, an increase of oocyst-specific serum antibodies at day 5 after secondary infection with *Eimeria acervulina* was found, with a maximum after the first week. For sporozoite-specific antibodies, a significant elevation was found after one week, with a maximum after two weeks. These data correlate with the presence of the different stages in the chicken gut. Preceding the increase of parasite specific serum immunoglobulins, chickens generate germinal centres in their lymphoid tissues where secondary diversification of the antibody repertoire takes place. The observations justify the time point of sacrificing chickens at three and five days after secondary infection to generate an optimal *Eimeria* specific antibody library. Including cDNA from the main mucosal lymphoid sites, the bursa of Fabricius, spleen and cecal tonsils ensures coverage of both the systemic and mucosal anti-*Eimeria* immunoglobulin repertoire.

The applicability of the Eckα1 library is demonstrated by the selection of polyclonal and monoclonal antibody fragments directed against either a mix of *Eimeria* antigens, or against more specific antigens like oocyst- or sporozoite-fragments. The selected clones revealed high variability with more homogeneity in the light chains than in the heavy chains. This is in contrast to the findings by Andris-Widhopf et al. (2000). In this study, generally a combination of a homogeneous set of heavy chains in conjunction with a largely heterogeneous set of light chains was found, thus suggesting a more important role for the heavy chain in determining antigen specificity of the antibody. For mice with a single heavy chain transcript it has been shown that combinatorial light chain variability alone is able to build up a sufficiently complex B cell repertoire to mount protective immunoglobulin responses against a variety of pathogens (Senn et al., 2003). Diversification of chicken heavy chains takes place during bursal ontogeny through hyperconversion of V_H pseudogenes acting as donors of VD sequences.

The heavy chain locus contains a single J_H segment, a unique functional VH gene and approximately 16 D elements and about 80 heavy chain pseudogenes (Reynaud et al., 1989). The chicken IgL locus contains a single functional VL gene, which undergoes rearrangement in all B cells to a JL sequence. Upstream of the VJL complex is a family of ~25 pseudo-V region genes that act as donors for the replacement of homologous sequence within the rearranged VJL complex (Reynaud et al., 1987). The discrepancy of heavy and light chain diversity of the two studies can be accredited to the complexity of the antigen mix used in the selection. This might also explain the fact that among the selected phages described in this study, relatively intermediate binders predominate. Further investigation on the effectivity of the clones for prophylactic purposes is needed.

Parasite-specific antibodies in the intestinal tract may serve an indirect role in immunity by reducing infectivity of the pathogens. The mode of action can be a consequence of parasite agglutination, neutralization, steric hindrance, reduced motility or a combination of these effects. Several monoclonal antibodies have been characterized showing such modes of action (Lillehoj et al., 2000). In order to enhance effects and simultaneously avoid antigenic shifts of the parasite a polyclonal approach targeting multiple antigenic variants could be advantageous. Antigenic variation between *Eimeria* species limits commercial application of live parasite vaccination. Oral passive immunization using polyclonal antibodies directed against conserved epitopes could represent a promising alternative. By oral delivery of a protective antibody mix, the natural mucosal immune response is mimiced and protection against several *Eimeria* strains can be provided. The Eckα1 library presented here can be used to isolate binding antibodies against conserved epitopes of several *Eimeria* species. A comparable strategy has been described recently, indicating that polyvalent neutralizing antibody formulations targeting epitopes on defined antigens may provide optimal prevention of cryptosporidiosis, a diarrhoeal disease caused by the apicomplexan parasite *Cryptosporidium parvum* (Schaefer et al., 2000; Riggs et al., 2002).

This phagemid vector in Fab format incorporates the combined use of constant domains of the chicken alpha isotype and demonstrates that phage display technology can be extended to chicken IgA antibodies. The incorporation of the Cα1 fragment into the phagemid constitutes an additional novelty with potential benefits. On the one hand, when IgA is the final goal, it could be beneficial to perform the selection steps in a format that matches closely the final product. In addition, it facilitates mass transfer of binders into IgA full size antibodies intended for polyclonal oral protection. In this sense, pChick3 represents the first step towards extending oral protection to chicken and could, for instance, contribute to decreasing the risks of zoonoses. In this report, we also show the functionality of the vector for potential coccidiosis prophylaxis: an *Eimeria acervulina* specific library was constructed from which both polyclonal

and monoclonal phage preparations were selected which recognize different stages of the parasite.

This design responds to our global objective to extend passive oral immunotherapy to poultry, which requires the expression of specific polymeric IgA complexes. In order to be therapeutically active in oral passive immunization approaches, chicken IgAs need to be produced as full length antibodies, together with their partners in the sIgA complex, the J-chain and the secretory component (Ma et al., 1998; Corthesy et al., 1999). The next step in our approach will consist of the construction of a system for the transfer from Fab to full-size antibodies in animal feed plants and further evaluation of the selected clones for their *in vitro* and *in vivo* parasite neutralizing activities.

BIOLOGICAL SAMPLES, MATERIAL AND METHODS

Parasites and antigen preparations

The Houghton strain (Wheybridge, UK) of *E. acervulina* was maintained and sporulated using standard techniques (Long, 1976). Oocysts were disrupted using glass beads (diameter 0.5 mm). Oocyst wall fragments were prepared as previously described (Karim et al., 1996). Excystation of sporozoites was evoked by treatment with trypsin and bile salts (Sigma). For collection of all *Eimeria* antigens, the suspension with oocyst and sporocyst walls and sporozoites, was washed in RPMI to remove trypsin and bile salts. The pellet was resuspended in RPMI containing protease inhibitors (Roche) and sonicated. Sporozoites were purified over a DE-52 column as previously described (Shirley, 1995). Antigen preparations were frozen at -70 °C.

Chicken Infections

Isa Brown (Warren et al., 1998) layer pullets were purchased for infection (28 days of age). Birds were orally infected with 2 x 10^5 sporulated oocysts of *E. acervulina*. On day 36 after the primary infection the birds obtained a second oral dose of 2 x 10^5 *E. acervulina* oocysts. On day 3 and 5 after second infection two chickens were sacrificed and serum, bursa of Fabricius, cecal tonsils and spleen were collected. Organs were immediately frozen in liquid nitrogen and stored at – 70 °C for future RNA extractions.

Construction of pChick3 phagemid vector

Previously described pHEN2 and fd-tet-DOG1 vectors (Hoogenboom et al., 1991) were used as basic frames for the construction of the pChick3 vector following a similar approach as described by O'Brien (O'Brien et al., 1999). A DNA fragment containing a ribosomal binding site (RBS) and the signal sequence of gene III from M13 bacteriophage was amplified from the fd-tet-DOG1 vector using oligonucleotides AP01CKF1 and APO2CKR1 (Table 1), and subsequently cloned *Sal*I/*Sac*I into the pHEN2 frame. In this way, a new open reading frame (ORF2) under the control of the lacZ promoter was generated. This ORF2 was modified with the introduction of a DNA fragment encoding the Cα1 region of chicken IgA. For this purpose, the Cα1 fragment was amplified from chicken cDNA with oligonucleotides APO9F and APO12R (Table 1). The resulting fragment, which incorporated flanking *Sac*I and *Not*I sites, was subsequently cloned *Sac*I/*Not*I into the vector frame for the generation of pChick2, an intermediate vector. Further improvements consisted of the introduction of a methionine at the initial position of ORF2 and the modification of the second RBS to give higher expression levels. For this purpose, oligonucleotides STO1 and MYO5CAR were used in a directed mutagenesis strategy. Briefly, 15 cycles PCR reaction were conducted in the presence of STO1 and MYO5CAR (Table 1) using pChick2 as a template, the resulting fragment was cloned *Nhe*I/*Sal*I into pChick2 generating the final version of the chicken IgA Fab phagemid vector pChick3.

Table 1 Sequence of the oligonucleotides used for **(A)** the construction of pChick3 and **(B)** the amplification of chicken IgL and VL repertoire. Restriction enzyme sites used for cloning are underlined.

A

Name	Sequence
AP01CKF1	5'-GATACAGTCGACGGCTCCTTTTGGAGCCTT-3'
AP02CKR1	5'- CCTTTCTATTCTCACAGTGCTAGCGTCCAAGAGCTCGAGCT-3'
APO9F	5'-GTCATCGTGAGCTCCGCCTCCGCCAGCCGCCCGACC-3'
APO12R	5'-GCTCAGG GCGGCCGCTTTGGAGGTGAATATGGGGC-3'
STO1	5'-TGGAGCCGTCGACTAAGGAGGAAATCAACATGAAAAAATTATTATTCGC-3'
MYO5CAR1	5'-GTTTTGTCGTCTTTCCAGACG-3'

B

Name	Sequence
01Ap03IgLF1	5'-CCTCAGGTTCCCTGGCCATGGCAGCGCTGACTCAGCCGKCCTC-3'
01Ap04IgLR1	5'-GAAGAGGTCCGAGTGCTAATAGTCGACCTGGGGATGCAATGT-3'
01Ap02VHF1	5'-CTGGCCGCCCTGCCAGCTAGCATGGCGGCCGTGACGTTGGAC-3'
01Ap01VHR1	5'-CCACGGGACCGAAGTCATCGTGAGCTCCGCCTCCGCCACCCG-3'

Eckα1 library construction

PolyA+ RNA preparations were isolated from the bursa of Fabricius, cecal tonsils and spleen samples of *Eimeria acervulina*-infected chicken three and five days after infection using standard techniques and used to synthesize cDNA (cDNA-synthesis kit; Life technologies, Breda, The Netherlands). First strand cDNA was pooled and the variable heavy chain (VH) and the complete light chain (IgL) of the immunized antibody repertoires were amplified. A two-step strategy as described (McCafferty et al., 1996) was applied for the construction of Eckα1 library. In a primary step, IgL and VH individual libraries were constructed. IgL was amplified by primers 01Ap03IgLF1 and 01Ap04IgLR1 (Table 1). Bands of correct size were purified from agarose gels, pooled, and digested *Nco*I/*Sal*I. One microgram of digested and purified IgL was co-incubated with 500 ng of *Nco*I/*Sal*I-digested pGEM-T vector in a 50µl ligation reaction. Aliquots of 2 µl were used for electroporation into TG1 epicurean competent cells (Stratagene). Cultures were incubated for 1h at 37°C and grown overnight on 2xYT solid medium containing ampicillin. Serial dilutions were also plated for estimation of library sizes. On the next day, ampicillin-resistant cells were collected in 2xYT medium containing 15% glycerol and kept at -80°C for further processing (primary IgL library). In a similar manner, VH bands were amplified using 01Ap02VHF1 and 01Ap01VHR1 (Table 1), purified, pooled, digested with *Nhe*I and *Sst*I, cloned into pChick3 and transformed into TG1 cells for the generation of pChick3-VH primary library. For combination of both repertoires, representative aliquots of both VH and IgL primary libraries (1 ml each) were grown for plasmid extraction during 6 hours in 50 ml 2xYT medium. Ten micrograms of pGEM-IgL plasmid preparation was digested *Nco*I/*Sal*I and the released band was purified from agarose. In parallel, 10 µg of pChick3-VH plasmid was linearized with *Nco*I/*Sal*I and purified. The two libraries were combined in a 100 µl ligation and transformed into TG1 cells to generate the Eckα1 library. Serial dilutions of the pooled transformation cultures were plated separately for the estimation of the size of the library. Randomly selected, individual clones were used for testing the functionality of pChick3 vector in ELISA and Western blot assays.

Phage display and panning

An aliquot of viable cells from the library covering approximately ten times the number of primary transformants was grown and infected with M13-KO7 helper phages (Amersham Biosciences, Roosendaal, the Netherlands) as described (McCafferty et al., 1996). After overnight incubation, the phage-antibody particles were purified by polyethylene glycol/NaCl precipitation and resuspended in a total volume of 3 ml PBS. Selection for binding fragments was performed with overnight coating of *Eimeria* preparations (50 µg/ml for whole antigen suspension, 10^5 sporozoites/oocysts per well for specific antigen suspensions) in

microtiter plates at 4 °C. Wells were blocked with 2% BSA in PBS buffer containing 0.1% (v/v) Tween 20 (PBS-T) for 1 h at room temperature and subsequently incubated with 50 µl phage suspension and 50 µl 2% BSA/PBS for 1 hour at 37 °C. Bound phages were eluted by addition of 100 µl 200 mM glycine. For the amplification of binders, the phage suspension was neutralized with 200 µl 0.2 M $NaPO_4$ buffer (pH=7). *E. coli* TG1 cells were infected with 200 µl of the eluted phage suspension. After amplification and purification, phages were selected for three additional rounds using the same protocol. An aliquot of each of the polyclonal phages obtained after each round of selection was stored at 4 °C for further analyses.

Enzyme-linked immunosorbent assays (ELISA's)

To determine expression levels and specificity of phage produced IgA Fab fragments or *Eimeria* specific antibody levels in chicken serum, ELISA was used. Microtiter plates were coated o/n with *Eimeria* antigens (50 µg/ml for whole antigen ELISA, 10^5 sporozoites/ well for sporozoite ELISA) or goat anti chicken IgA (1:2000, Bethyl, Montgomery, USA) in carbonate buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6). Non-specific binding sites were blocked with 5% low fat milk powder in PBS-T.
Detection of the binding phage-antibody particles was determined with peroxidase-labelled anti M13 (1:2000; Amersham Biosciences, Roosendaal, the Netherlands). Functional association of the immunoglobulin heavy chain fragment with the light chain was determined using a sandwich ELISA. For this purpose, microtiter plates were coated with goat anti chicken IgA (1:2000; Bethyl, Montgomery, USA) to capture recombinant phages and blocked as described. As a second antibody, an anti IgY (peroxidase-conjugated; 1:5000; Bethyl, Montgomery, USA) recognizing the chicken lambda chain was used. ELISA reactions were developed with ABTS (Roche, Mannheim, Germany) and colour development was determined after 1h at room temperature.

Western blot analysis

For further characterization of IgA Fab expression of the phages, samples were mixed 1:1 with buffer containing 50 mM Tris·HCl (pH 6.8), 2% (w/v) SDS, 10% (w/v) glycerol, 0.01% bromophenolblue and DTT (40 mM). Samples were boiled for 5 minutes and subsequently cooled on ice. All samples were centrifuged for three minutes at 13,000 rpm. Supernatant was collected and resolved on 7.5% polyacrylamide gel. Total protein content of the samples was determined by staining of the gels by coomassie blue. Protein concentrations of the samples were equalized, run on SDS page gels and finally the proteins were blotted onto nitrocellulose membranes. After overnight incubation at 4 °C in PBS-T buffer

containing 5% ELK, blots were washed shortly in PBS-T. To detect chicken IgA, the blots were incubated at room temperature for 1h in PBS-T with 5% ELK containing goat anti-chicken IgA-AP (Bethyl, Montgomery, USA) or goat anti-chicken IgY-AP (sigma). The reaction was visualized by incubation of the blot in developing solution (1M diethanolamine-HCL (pH=9.8) with 4mM $MgCl_2$, 4 mg of 5-bromo-4-chloro-3-indolyl phosphate and 5 mg of Nitro Blue Tetrazolium) for 30 minutes.

Screening of individual clones

Individual clones from positive phage pools were grown in 5 ml TYE and infected with helper phages as described in 2.6. Antibody-phage particles were purified by polyethylene glycol/NaCl precipitation and resuspended in a total volume of 200 µl PBS. Serial dilutions of the phage solutions were tested for binding to *Eimeria* antigens (total antigen, sporozoite or oocyst fraction) and to BSA by ELISA. The variable regions of these clones were sequenced and subsequently analysed with Vector NTI Suite, version 8 (InforMax).

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Chapter 3

Avian antibodies produced in plants: A comparative study with antibodies selected from a chicken antibody library

Authors

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SUMMARY

Antibody expression in heterologous systems with subsequent successful application depends on the ability to obtain sufficiently high yields. Low expression levels, caused by a variety of intrinsic properties of an antibody, is an important bottleneck encountered in most eukaryotic expression systems. A reliable system for fast and efficient assessment of such bottlenecks is useful to anticipate failing, costly, and long lasting experiments. Here an integrated system is described wherein the *in planta* expression characteristics of antibodies selected from phage antibody libraries can rapidly be assessed, and used as an additional selection step. It is based on *Agrobacterium tumefaciens* mediated, transient expression in leaves of tobacco plants. Ten functional, full size IgA antibodies were tested. Among these antibodies, strong variations in expression levels were observed: (I) immunoglobulins showing no detectable expression; (II and III) intermediate groups (0.01-0.02% of total soluble protein (TSP) and 0.1-0.2% TSP) and (IV) antibodies expressing at high levels, which showed IgA concentrations up to 1.5% TSP. mRNA levels for immunoglobulin heavy or light chains of all antibodies were comparable and, thus did not correlate with protein yield. Evidence is provided for a co-dependence of heavy and light chain expression. Co-expression of silencing suppressing proteins cannot compensate for the observed differences. The results illustrate the usefulness of this extra *in planta* selection step when pursuing immunotherapeutic application of plant-derived antibodies from phage display libraries.

INTRODUCTION

Plants are a promising system for the production of antibodies due to their capacity to efficiently produce large proteins (Giddings et al., 2000; Ma et al., 2003; Twyman et al., 2003). Several reports show the possibilities to express correctly folded and assembled antibody molecules with high yields. A variety of functional antibodies in diverse formats has been produced, ranging from single chain molecules (scFv) to diabodies, Fab fragments, full size IgG and chimeric secretory IgA (Ma et al., 1994; Ma et al., 1995; Larrick et al., 2001; Sharp et al., 2001). The possibility to scale up production at low cost and the limited risk of contamination with human pathogens makes plants attractive for the production of antibodies with therapeutic applications (Fischer et al., 2003; Warzecha et al., 2003). In particular, the engineering of plant recombinant antibodies for oral immunotherapy is promising, as antibodies can be produced in edible parts of the plant, thus avoiding laborious purification processes before application (Larrick et al., 2001).

Most plant produced therapeutic antibodies described so far were initially selected using the hybridoma technique. Antibody technology has recently been boosted by the application of phage display. This technology allows the selection of specific binders from large combinatorial libraries (10^7-10^{11} individual transformants) displaying variable antibody fragments as fusion proteins at the surface of recombinant bacteriophages. For selection of specific antibody fragments a variety of more or less sophisticated binding assays has meanwhile been developed (Hoogenboom et al., 1998; Jefferies, 1998; Ghosh et al., 2001; Labrijn et al., 2002). In contrast to the hybridoma technique, phage display can be used to obtain specific antibodies from virtually any animal species (O'Brien et al., 2002). To date, studies have been reported with rabbit (Ridder et al., 1995; Foti et al., 1998), sheep (Charlton et al., 2000; Li et al., 2000), bovine and porcine species (O'Brien et al., 1999; Li et al., 2003), camel (Ghahroudi et al., 1997) and chicken (Davies et al., 1995; Yamanaka et al., 1996; Andris-Widhopf et al., 2000). Thus, the possibilities for immunotherapy can be extended to, for instance, livestock animals avoiding the dangers that accompany anti-isotype reactions.

For mass production of antibodies selected by phage display, appropriate expression systems ensuring proper folding, assembly and posttranslational processing are required. *E. coli* is a widely used production host for antibody fragments (Fab, scFv) in the preclinical phase (Hoogenboom et al., 1991). However, stability and solubility problems hinder large scale applications (Worn et al., 2001). Furthermore, antibody fragments lack the Fc domain, required for effector functions, which limits their use as therapeutic proteins (McCormick et al., 2003). Systems based on mammalian (Mahler et al., 1997; Persic et al., 1997) or insect cells (Liang et al., 2001) fulfill all prerequisites for large scale production. However, it is unlikely that the growing demand of therapeutic antibodies can be

met using production systems solely based on animal cells. It has been estimated that by the end of this decade a production capacity is required of more than 1.5 million liters cell culture when based on animal cell systems. This requires investments of several billion dollars, thus maintaining immense costs for producing therapeutic antibodies. It has been estimated that production of antibodies in mammalian cells are 3000 times as expensive as production in plants (Hood et al, 2002).

Next to production costs, the ability to rapidly assess characteristics determining production levels such as protein stability, is important as this can significantly shorten the research and development phase. The time between selection and production should be as short as possible. Thereto, vector systems are required that connect, in a versatile fashion, phage display and eukaryotic expression systems, resulting in data on protein stability. An important aspect in this respect is the different stability of antibody idiotypes when produced in mammalian cell culture systems (Bentley et al., 1998). Transient expression systems can be rapidly applied for these purposes, as they provide a highly convenient selection step for the discrimination of antibodies with respect to their intrinsic stability properties.

Although plants represent an attractive alternative to animals for the production of therapeutic antibodies, no system has been described that allows, in a combined fashion, a rapid and versatile transfer from phage selection to production in plants including assessment of idiotypic stability. Regarding the latter, the use of V-gene families in human immune responses has been taken into account in the construction of human phage antibody libraries (Ewert et al., 2003). Thus, this assessment procedure is of specific importance when antibodies of other than human or murine origin have to be expressed, since little is known of V-gene usage in for example avian species. We recently developed chicken phage libraries for the selection of antibodies against protozoa of the genus *Eimeria*, the causal agent of coccidiosis (see chapter 2 of this thesis). Coccidiosis is an intestinal disease that causes substantial economical losses in poultry industry (Williams, 1999). Feeding chicken with protective antibodies has been proposed as a prophylactic alternative to the profuse use of drugs against coccidiosis. To achieve this, we designed an integrated system for the rapid transfer of chicken antibodies obtained from a phage selection system to *in planta* expression systems including the possibility to assess idiotype stability. Thereto, a strategy based on agroinfiltration of tobacco leaves (Fischer et al., 2000) was chosen resulting in transient expression. Using this versatile system it was found that the expression levels of antibody idiotypes in tobacco cells differ even more than previously found in mammalian expression systems. This proves that the described *in planta* selection steps are crucial to rapidly assess specific parameters in the choice of promising therapeutic antibodies.

RESULTS

Design of plant expression vectors for chicken immunoglobulins derived from phage selection

The design of the pChick3 vector for generation of chicken antibody phage display libraries in Fab format has been described in detail in chapter 2 of this thesis. This phagemid vector incorporates in a bicistronic transcriptional unit the coding regions of chicken IgL and the variable region of IgH fused to the first constant domain of the chicken α heavy chain. A schematic representation of pChick3 is given in figure 1A.

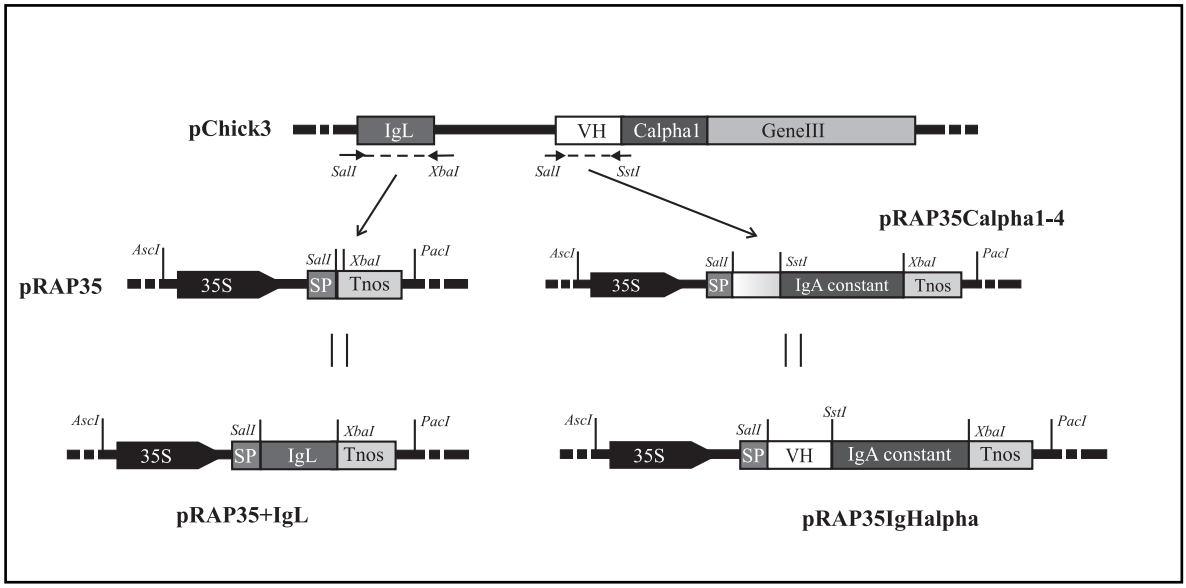
For the production of full size chicken IgA in plants, expression cassettes (pRAPIgL and pRAPIgH$_{alpha}$) were constructed (figure 1A). The vector pRAPIgL was designed to function as an expression cassette for pChick3-selected IgLs. It comprises a 35S promoter region followed by the signal peptide of the murine kappa light chain, a multiple cloning site, and the terminator of Nopaline synthase. In a similar way, pRAPIgH$_{alpha}$ incorporates the same promoter region and signal peptides, but also the four constant domains of chicken IgH$_{alpha}$.

The transfer of heavy and light chains from pChick3 is a two-step procedure. First, appropriate restriction sites are introduced into the IgL and VH regions by PCR using oligonucleotides 1 and 2 (table 3). In a second step immunoglobulin chains are cloned into the corresponding pRAP expression cassettes. In a further improvement of the transfer system, new restriction sites were engineered into the pRAP vector series allowing the direct cloning from pChick3 into pRAP cassettes without an intermediate PCR step. In a final step, the entire expression cassette is transferred to the pBIN+ vector for plant expression, using low frequency restriction enzymes *Pac*I and *Asc*I. The resulting binary vectors pBIN+IgL and pBIN+IgH$_{alpha}$ can be used either for stable transformation or used in agroinfiltration transient assays. Alternatively, pRAP-based expression cassettes are directly utilizable for transient or stable transformation using biolistic approaches.

To establish the functionality of the transfer system, we first assayed the expression *in planta* of a randomly chosen fragment (R1) whose expression in *E. coli* had previously been demonstrated. IgL and VH region of R1 were transferred to pRAP and subsequently pBIN+ for expression in *N. benthamiana*. Equal volumes of *Agrobacterium* cultures carrying, respectively, pBIN+IgLR1 and pBIN+IgH$_{alpha}$R1 constructs were mixed and infiltrated in *N. benthamiana* leaves. Four days after inoculation, antibody production in leaves was assessed by Western blotting. Under reducing conditions, both heavy (75 kDa) and light (26 kDa) chains were detectable, together with an intermediate band of 50 kDa which was considered the result of IgH degradation (Figure 1B, left panel). Under native conditions, the different reactive bands assemble to a high molecular weight

complex with a size comparable to that of a full size chicken IgA molecule (Figure 1B, right panel).

A



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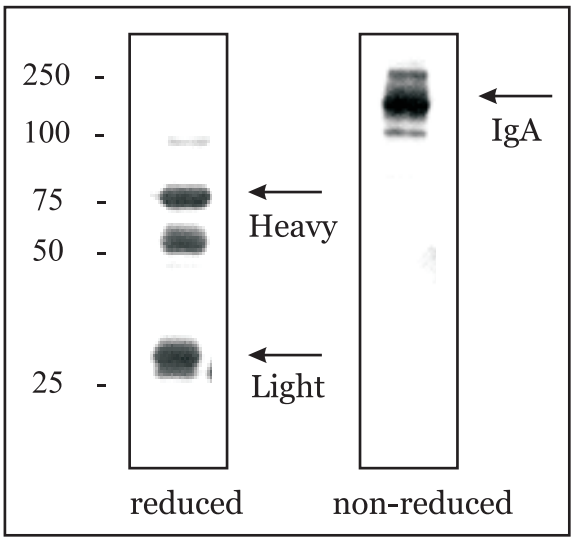


Figure 1

(A) Schematic overview for the of cloning of IgA light (IgL) and α-heavy chains (IgHalpha) in plant expression cassettes. SP, mouse κ signal peptide; 35S, 35S promoter; Tnos, nopaline synthase terminator.

(B) Western blot analysis of an in planta produced, randomly selected immunoglobulin under reducing and non-reducing conditions visualized using an anti IgA antibody.

Individual chicken antibodies show variable expression levels *in planta*

To investigate the stability of phage display-derived chicken antibodies upon *in planta* expression ten different antibodies were chosen. These antibody fragments were selected from the same anti-*Eimeria* library and show equal expression and *anti-Eimeria* binding activity in Fab format. For every fragment, pBIN+IgL and pBIN+IgH$_{alpha}$ constructs were generated and co-infiltrated in *N. Benthamiana* leaves. After four days, leaves were harvested and analyzed. The functionality of the full-size antibodies in crude plant extract was confirmed in an ELISA against *Eimeria* antigens (figure 2A). In parallel, plant-produced IgAs were quantified in a double antibody sandwich ELISA using chicken serum with known IgA concentration as a reference. Controls with either heavy or light chain alone rendered no significant signal. As can be deduced from figure 2B, large differences were found in the production levels of the different immunoglobulins. Expression levels were subdivided into four categories: (I) immunoglobulins showing no detectable expression; (II and III) intermediate expression (0.01-0.02% TSP and 0.1-0.2% TSP respectively) and (IV) high expression, (IgA concentrations up to 1.5% TSP). *In planta* IgA production levels were also found to be influenced by physiological and environmental conditions (leaf age, incubation time and temperature), but the relative accumulation levels of the different antibodies remained invariant independent of the conditions assayed (data not shown). Northern blot analysis of agroinfiltrated leaves clearly showed that IgA accumulation levels are not governed by the abundance of their respective mRNAs (figure 3).

Insight in the factors affecting relative expression levels of the different immunoglobulins was provided by Western blot analysis. Figure 4 shows the results of a representative infiltration experiment. Under reducing conditions, two major bands of approximately 75 and 50 kDa were detected by the anti chicken α-heavy chain antibody in most the infiltrations. In IgAs 14 and E4 only the 50 kDa band is visible. The anti chicken light chain antibody recognizes a single fragment in a limited number of constructs, 2, 10, 11, A2, C3 and C4. Comparing ELISA and Western results, we concluded that stable IgA expression (as quantified by double antibody sandwich ELISA) required coordinate expression of both heavy and light chain, as can be clearly seen in the expression levels of immunoglobulins of type III and IV. It is worth noting that all type I and II antibodies show low or undetectable IgL levels.

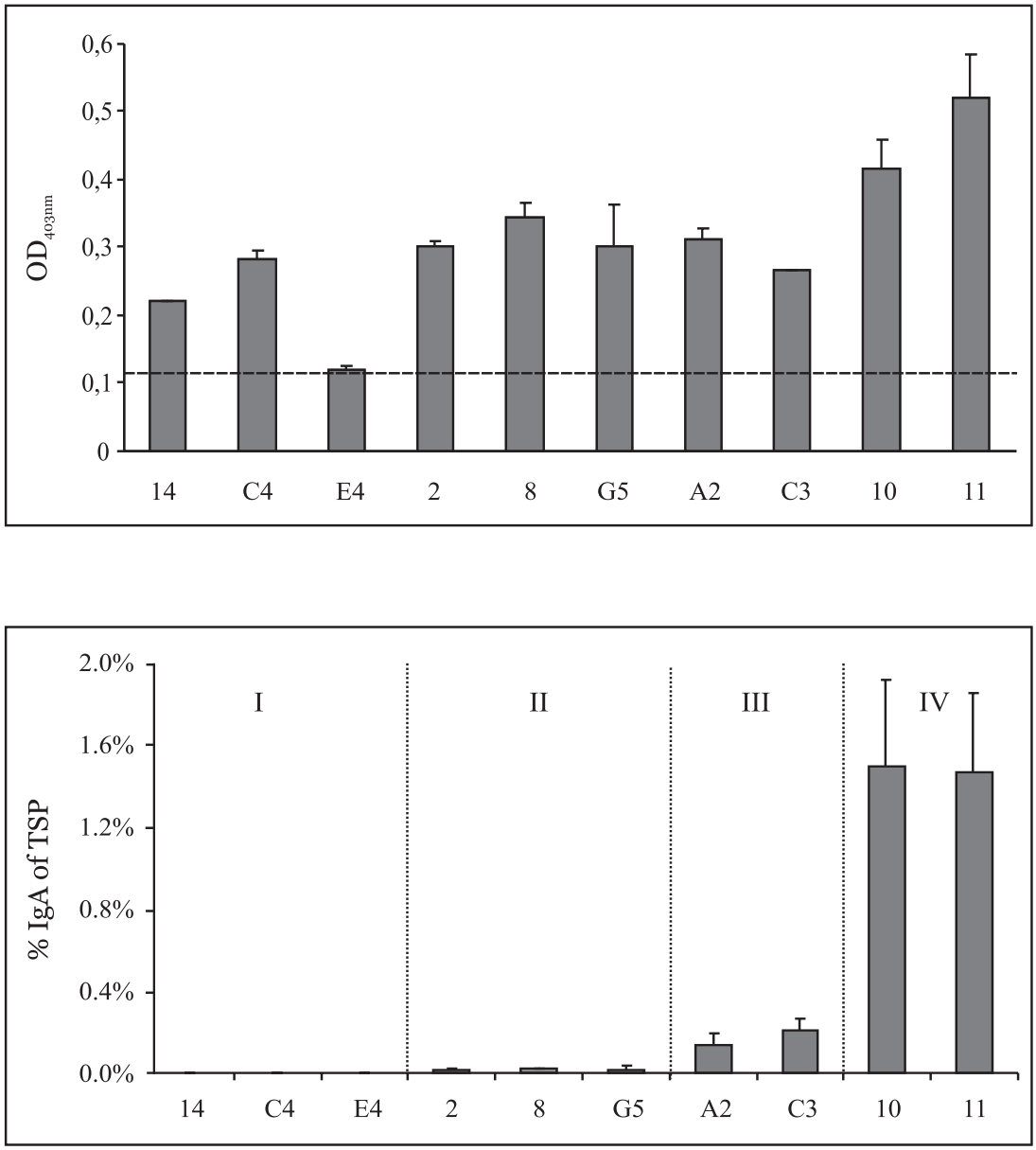


Figure 2 **(A)** Results of an ELISA to assess specific binding of chicken IgA in crude extracts of agro-infiltrated *N. benthamiana* leaves. IgA was bound to *Eimeria* antigens and detected with an anti-α heavy chain antibody conjugated to peroxidase. Antibody binding is expressed as measured OD_{403nm}, depicted on the Y axis. A-specific background binding of an extract from leaves expressing a non-related recombinant protein is shown by a striped line. **(B)** Results of an ELISA to quantify chicken IgA concentrations in crude extracts of agro-infiltrated *N. benthamiana* leaves. IgA was captured with anti-IgA antibody and detected with anti-light chain antibody conjugated to peroxidase to show assembly of heavy and light chains *in planta*. Antibody expression levels are calculated as percentage of total soluble protein (TSP) in the leaf extracts.

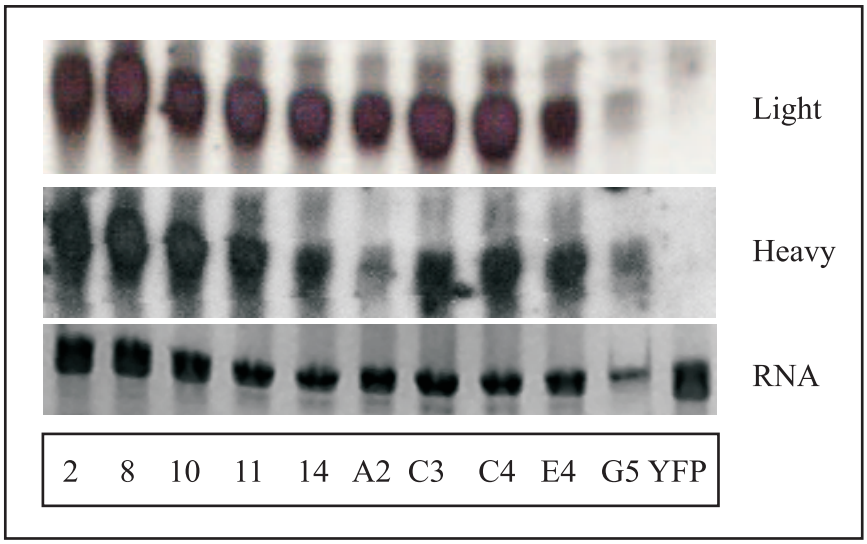


Figure 3 Northern blot for analysis of transcription levels of plant immunoglobulin mRNA. Approximately 10 µg total RNA was loaded per lane, blotted onto nitrocellulose membrane and probed with a [α-^{32}P]-labeled chicken immunoglobulin light (Light) or α-heavy chain (Heavy) DNA probe, located in the constant domains of the immunoglobulin chains; Detection of specifically bound probe was visualized by exposure to an X-ray film. Ethidium bromide staining of ribosomal RNA (RNA) shows loading of the samples. Numbers correlate with the ten antibody fragments that were selected from the Eckα1 library, YFP corresponds to plant infiltrated with a construct coding for yellow fluorescent protein.

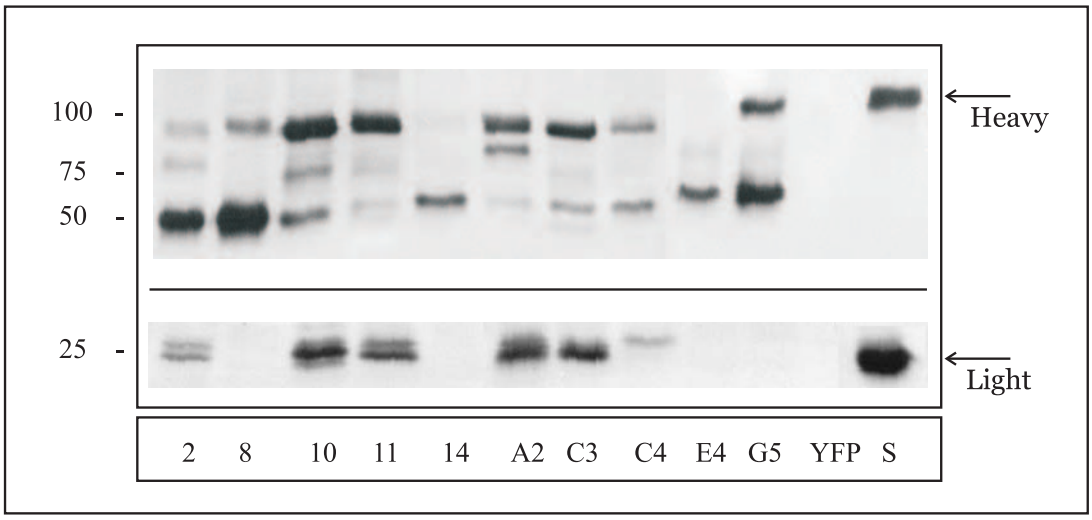


Figure 4 Western blot analysis of ten co-expressed heavy and light chain constructs in tobacco leaves. Heavy chains and light chains were detected separately in the same crude extracts under reducing conditions using anti- chicken α-heavy chain and light chain antibodies conjugated to alkaline phophatase. Putative degradation fragments are seen as 50 kDa bands. The detected heavy and light chains are of a similar molecular weight as serum-derived IgA (S). Plant extracts from a tobacco plant infiltrated with a construct coding for yellow fluorescent protein (YFP) was loaded to exclude a-specific binding of the detecting antibodies in both analyses.

Chain shuffling reveals role of balanced expression on IgA stability

To answer the question whether the Ig expression level is directly related to the primary sequence or whether expression levels are affected by stabilizing factors acting in *trans*, we first analyzed the sequences of the different Ig constructs. An interpretation of the results is presented in table 1. It was concluded that the amino-acid composition as such had no deleterious effect. Less favored codon use could explain the low expression levels found with IgH14, IgHE4 and IgL14. However, the abundant occurrence of less favored codons did not always predict the poor performance of a construct, as exemplified by IgL11. Conversely, poor performance of a given immunoglobulin is not always the consequence of poor codon use as can be deduced from the performance of IgLE4 or IgL8.

Table 1 Characteristic features of variable regions of the ten constructs selected for *in planta* expression. Table 1A represents features of the variable regions of the light chain constructs (in percentages); in table 1B characteristics of the variable regions of the heavy chain constructs are shown (in percentages). The relative levels of expression are based on observations in western analyses and ELISA's of original combinations and chain swapping experiments, where "-" represents no detectable expression and "+++" easily detectable expression in all experiments. Threshold for low abundant codom usages was set at <10%. As can be seen in the table, no single sequence characteristic determines *in planta* yields of the recombinant antibody chains.

A

Construct	Relative level of expression	Low abundant codon usage	Charged (RKHYCDE)	Acidic (DE)	Basic (KR)	Polar (NCQSTY)	Hydrophobic (AILFWV)
L 2	++	2.8	21.9	11.4	4.4	36.8	28.1
L 8	-	3.8	17.9	7.1	4.5	42.9	27.7
L 10	+++	4.2	18.4	6.1	4.4	43.0	27.2
L 11	+++	5.6	18.4	7.9	4.4	41.2	29.8
L 14	-	5.7	17.0	7.6	3.4	38.1	33.1
L A2	+++	5.2	23.4	6.3	9.9	37.8	26.1
L C3	+++	5.2	18.4	6.1	5.3	38.6	29.8
L C4	+	5.2	17.7	6.2	5.3	41.6	27.4
L E4	-	4.1	17.0	6.8	3.4	40.7	27.1
L G5	-	5.2	17.7	7.1	3.5	42.5	29.2

B

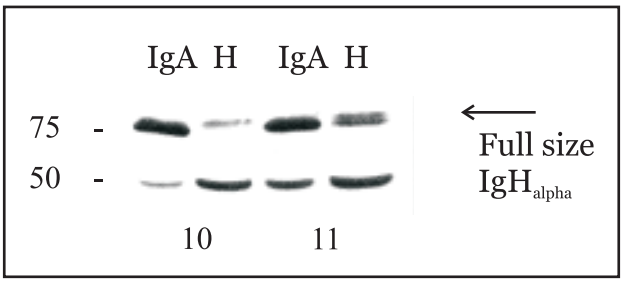
Con-struct	Relative level of expression	Low abundant codon usage	Charged (RKHYC DE)	Acidic (DE)	Basic (KR)	Polar (NCQST Y)	Hydro-phobic (AILFW V)
H 2	+	4.9	21.4	7.1	6.4	32.9	31.4
H 8	++	4.7	22.1	7.9	7.1	31.5	33.9
H 10	+++	5.1	24.6	7.5	7.5	35.8	29.9
H 11	+++	5.3	22.9	7.6	8.4	32.8	32.1
H 14	+	7.5	25.8	8.3	8.3	30.3	34.9
H A2	++	3.7	21.6	7.5	6.7	29.9	36.6
H C3	++	5.3	24.4	7.6	8.4	30.5	32.1
H C4	++	5.9	20.9	6.7	6.0	34.3	33.6
H E4	-	7.0	25.2	9.5	7.1	29.9	32.3
H G5	++	3.2	22.0	8.1	7.3	31.7	35.0

An important factor for efficient expression of full size murine immunoglobulin G *in planta*, previously reported by van Engelen et al. (1994), is the coordinate expression of both the heavy and light chain. Similar observations were done for chicken IgA. Upon expression of the heavy chain of Ig10 and Ig11, belonging to class IV expressers, in absence of their light chain, the 50 kDa product accumulated to significantly higher levels (figure 5A).

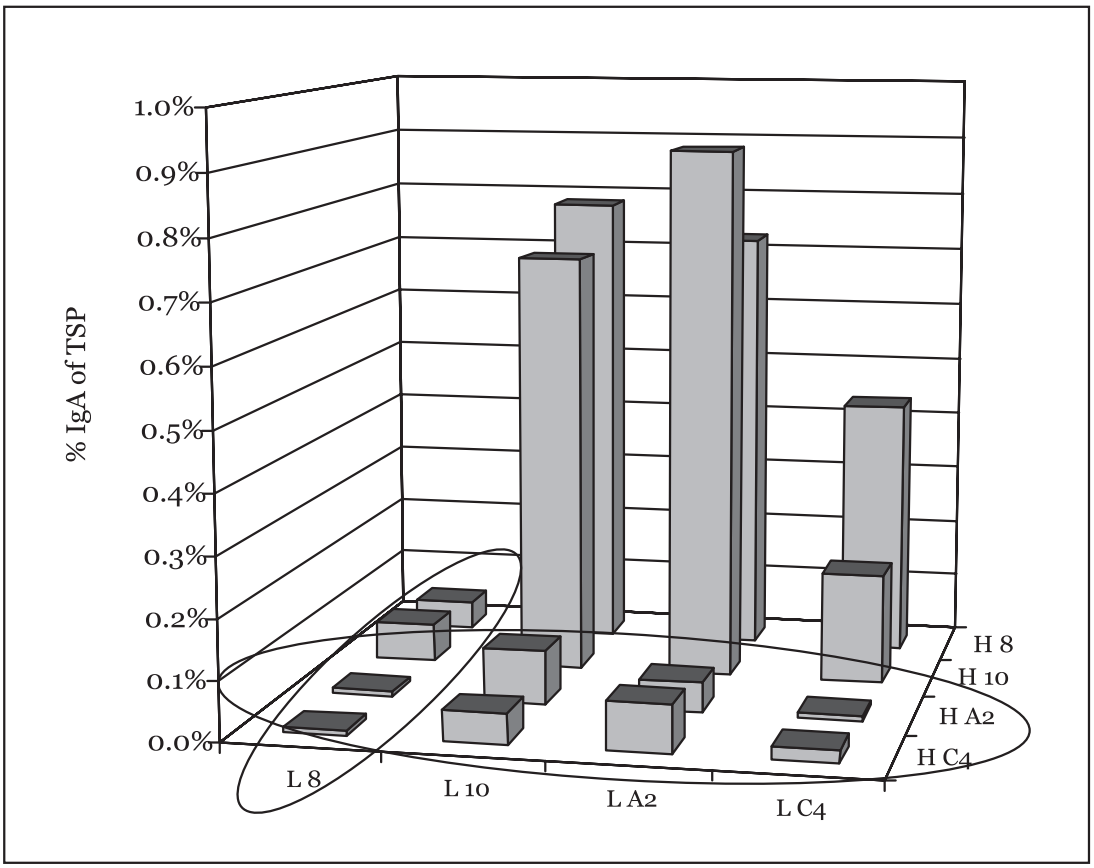
To gain further insight in the importance of coordinate expression and to obtain insight in the background of the low or almost absent accumulation of some of our antibodies, we conducted a chain shuffling experiment. One antibody of each of the four classes defined above was taken. In the experiment advantage was taken of the versatility of agroinfiltration, enabling analysis of all possible 16 combinations. Resulting expression levels of IgA were determined using a double antibody sandwich ELISA (Figure 5B). As can be observed, chain swapping greatly influences expression levels of immunoglobulin complexes. The most striking is the deleterious effect of IgL8 when expressed in combination with any of the four heavy chains used in the experiment. In contrast IgH8 results in class IV expression levels when combined with any of the three other light chains.

A similar result was observed with heavy chain combinations. Heavy chains A2 and C4 do not lead to significant accumulation of a full size antibody when combined with any of the four light chains. These results were confirmed in a Western blot experiment wherein the expression of either the heavy or the light chain of Ig10 was assessed in the presence of respectively the light or the heavy chain of each of the four antibodies used in this experiment (figure 5C). Taken together it can be concluded that in analyzing these four antibodies either the light or the heavy chain dictates expression, low expression of either of the chains cannot be compensated for by the other.

A


IgA H IgA H
75 -
50 -
Full size
IgH alpha
10 11

B


% IgA of TSP
1.0%
0.9%
0.8%
0.7%
0.6%
0.5%
0.4%
0.3%
0.2%
0.1%
0.0%
L 8
L 10
L A2
L C4
H 8
H 10
H A2
H C4

C

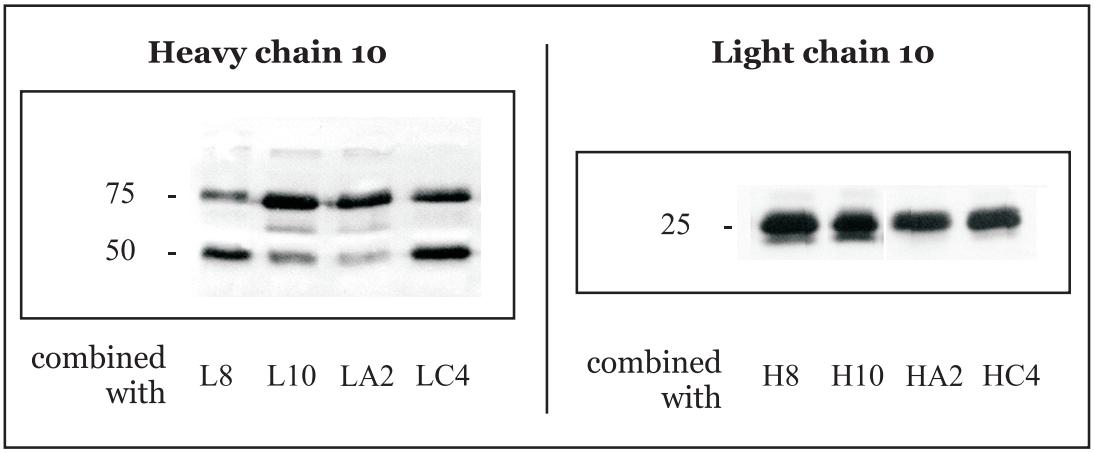


Figure 5 **(A)** Expression pattern of two representative heavy chain constructs with and without light chain in tobacco leaves (IgA and H respectively)) analyzed by Western blot detection under reducing conditions. **(B)** Quantification of antibody levels in chain swapping experiments. Starting with four individual immunoglobulins, sixteen possible heavy (H) and light chain (L) combinations were assayed in agroinfiltration experiments. Encircled bars highlight heavy or light chains dictating unequivocal low antibody accumulation levels independent of its partner chain. **(C)** Western blot analysis of heavy and light chain concentrations from eight selected combinations in the chain swapping experiments, heavy chain 10 combined with 4 different light chains and light chain 10 combined with 4 heavy chains (total protein concentrations per lane were equalized)

Silencing suppressors show variable effect on expression levels of different plantibodies

Silencing suppressors have been shown to enhance expression levels of foreign genes in plants. An experiment was set up to investigate whether differences in expression level could be equalized by using silencing suppressors of viral nature (2B, AC2, P1, P19 and P25; all silencing constructs obtained from D. Baulcombe, Sainsbury lab, UK). An overview of the constructs used is provided in table 2.

Table 2 Overview of the silencing suppressors used for boosting Ig expression levels in tobacco plants by *A. tumefaciens* mediated transient expression. miRNAs, microRNAs, ~21 nucleotides; siRNA, short interfering RNAs.

Name	Viral origin	Mode of action	Localization	References
2B	Cucumber mosaic virus (Cucomovirus)	Prevents initiation of gene silencing at growing points of plant & prevents translocation of the silencing signal through the phloem (interferes with systemic signal)	Nuclear	Brigneti et al., 1998; Lucy et al., 2000; Guo et al., 2002
AC2	African cassava mosaic virus (Geminivirus)	Blocks and reverses silencing	DNA binding (= nuclear)	Voinnet et al., 1999
P1	Rice yellow mottle virus (Sobemovirus)	Reverses silencing	?	Bonneau et al., 1998; Voinnet et al., 1999
P19	Tomato bushy stunt virus (Tombusvirus)	Suppressor of gene silencing/ operates in the vicinity of the vein tissue of new emerging leaves	siRNA and miRNA binding (=cytoplasmic)	Brigneti et al., 1998; Voinnet et al., 2003; Chapman et al., 2004
P25	Potato virus X (Potexvirus)	prevents accumulation and/or transport of the plant mobile silencing signal thus inhibiting systemic silencing	?	Voinnet et al., 2000

Leaves were co-infiltrated with heavy and light chain constructs in combination with one of the constructs or with a similar vector lacking an insert. Antibodies representing the four expression categories were tested. After quantification of IgA, the expression levels were related to the IgA yield found co-infiltrated with an empty vector, as shown in figure 6. The co-expression of silencing suppressors was shown to enhance expression levels in general, especially suppressors 2B, P1 and P19. However, this did not compensate for differences in expression levels between immunoglobulins. Interestingly, the combination of the different silencing suppressors with the four different IgAs revealed antibody dependent effectivity of each silencing construct. As shown in figure 6, silencing suppressor 2B for example caused a huge elevation of IgA yield with 10, whereas this silencing suppressor affected IgA levels of 8, C3 and C4 to a much lesser extent. AC2 showed no or hardly any effect on any of the four immunoglobulins. Yield of IgA C3 was not elevated by any of the silencing suppressors.

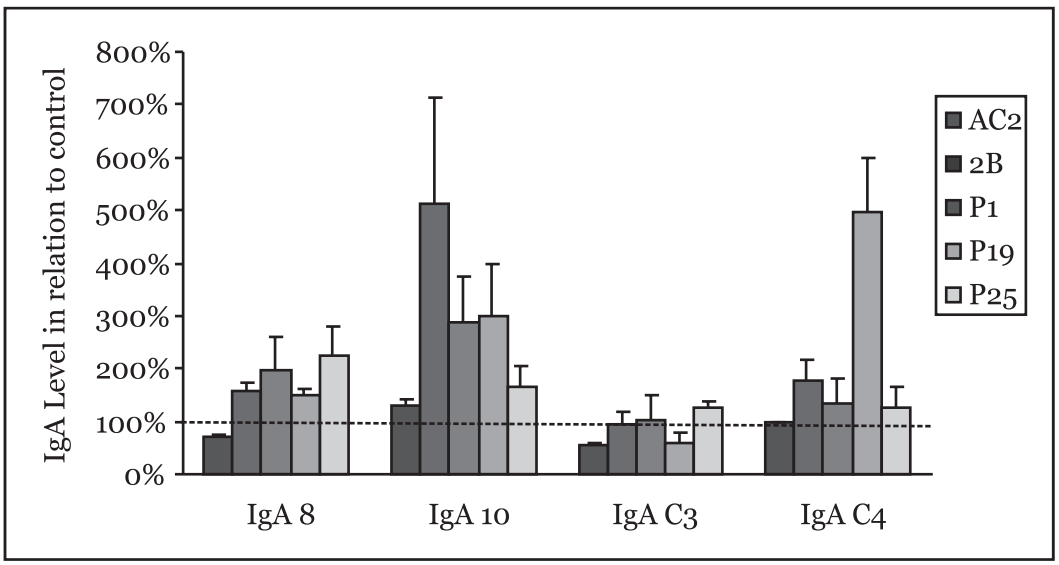


Figure 6 Quantification of the influence of 5 different silencing suppressors on antibody levels of four different immunoglobulins. Levels are represented as percentages of IgA expression with an empty vector, as indicated by the striped line. All yields above this line represent an increase in yield under influence of a silencing suppressor.

DISCUSSION

The step from antibody selection using phage libraries to efficient expression *in planta* needs an appropriate system for the transfer of antibody fragments to plant vectors. In addition a system is required to rapidly assess the influence of differences in the antibody idiotype on the expression level in the plant. In this paper we have described an efficient transfer system for antibody variable fragments as well as a system that allows the rapid assessment of antibody expression levels in the plant. High antibody expression levels *in planta* can now more easily be screened for and this provides the possibility to routinely produce antibodies in plants. Thus, large scale production is enabled and extension of immunotherapy approaches, also to non-human targets, is facilitated.

An application for this integrated approach in veterinary medicine is described for immune therapy of chicken coccidiosis, a diarrheal disease caused by the apicomplexan parasite *Eimeria*. Due to development of resistance to antibiotics and other drugs (Chapman, 1997) and increasing concerns of consumers on residues in food, there is a great demand for alternative prophylactic and therapeutic treatments. Oral administration of antibodies

represents a promising alternative. For example, hybridoma-derived monoclonal antibodies showed *in vivo* efficacy against cryptosporidiosis, a diarrheal disease caused by the apicomplexan parasite *Cryptosporidium parvum*, which could be increased significantly when two or three monoclonals were combined. As we have shown in chapter 2 of this thesis we were able to make an *Eimeria acervulina* specific Fab library. This library was enriched for parasite binding fragments. It now becomes possible to transfer the IgL and VH gene pairs to plant production vectors already containing the gene segments encoding for the constant antibody domains and produce full length, specific immunoglobulins in plants. In parallel to the recently described transfer of phage display–derived antibody fragments to mammalian cells (Chen et al., 2003), the integrated approach of selection and expression in plants enables fast screening and expression of antibodies for therapeutic purposes.

The described system combines two expression systems: first prokaryotic (*E.coli*) to select specific antibody fragments followed by eukaryotic cells (plants) for full size and upscaled production. Good expression of antibody fragments in *E. coli* however is no guarantee for appropriate expression in eukaryotic systems such as plants. Yields of scFv and Fab-fragments in bacterial systems seem to result from a compromise between intrinsic protein stability and *in vivo* folding efficiency (Worn et al., 2001), especially when scFvs are secreted. Ewert et al. (2003) were able to match human scFv properties such as yield and thermodynamic stability to consensus framework sequences derived from germline V_H and V_L families. A strong correlation between the properties of the isolated domains and their combination in a scFv fragment was demonstrated. No single features were found to attribute to the observations, but a number of minor characteristics. In eukaryotic systems the situation is more complicated than in bacteria, since complex interactions are taking place during the sorting process that brings the immunoglobulin chains through the secretion pathways (Vitale et al., 1999). Persic et al. (1997) compared expression levels of a hybridoma derived immunoglobulin and one selected by phage display in transient and stable eukaryotic transformations using mammalian cells. Expression levels were found to depend on the promoter, the cell line and on the construct itself. Thus, for large scale antibody production solutions are required that allow a rapid assessment of the intrinsic properties of antibodies that determine whether or not they are likely to be expressed successfully. Such solutions need to be flexible and adaptable to antibodies derived from any animal species. In this chapter we have evaluated a two step procedure. The first step involved grafting of variable domains onto species-specific constant domains. In the second step evaluation of the expression level of pairs of V_H and V_L genes is facilitated using a transient expression system that provides the required information in a time- and cost-effective manner. Selection in this assay system is made on the basis of antibody yield, but can also be made on the basis of the binding affinity and in a later stage on the abilities of *in vivo* protection. To meet the latter two requirements the same system can be

used with some minor modifications to improve antibody yield. The recent introduction of viral expression systems is particularly useful in this respect.

Another feature of the transient expression system described here is the possibility to study, in detail, antibody stability in plants. This is of equal importance since unpredictability in yields of a given molecule is a major issue in the molecular farming field. An important factor causing yield variability is the position of the inserted gene in the genome (Matzke et al., 1998). Using a transient assay, this can be discarded as a source of variation. This is reflected by the fact that relative accumulation levels of the individual antibodies remained constant regardless of the physiological and environmental conditions during the assay. Another feature is the construction of vectors carrying individual expression cassettes for the heavy and the light chain allowing comparative studies by exchanging one of both chains with that of a random other antibody. This possibility has been exploited here with the co-infiltration of 16 combinations of heavy and light chains from four antibodies each showing a different expression profile (figure 2). Our results show a co-dependence of the expressed heavy and light chains. When both chains are expressed well, hardly any products other than full length polypeptides are found in the plants. When the heavy chain is expressed alone, the majority of the proteins expressed is present as a protein of approximately 50 kDa. Similar effects have been reported for Ig-producing mammalian B-cells. Here, incorrectly folded heavy chains or heavy chains expressed in absence of a light chain remain associated with BiP, a molecular chaperone present in the endoplasmic reticulum, and directed towards the proteasome for degradation (Gething, 1999; Knarr et al., 2002). Binding of light chains to the BiP-heavy chain complex rescues the heavy chain (Vanhove et al., 2001). In plants, BiP plays a similar role (Vitale et al., 1999) and was shown to play a role in folding and assembly of IgG in transgenic plants (Nuttall et al., 2002). BiP concentrations were highest in transgenic plants with only heavy chain. Association of BiP with the heavy chain could be replaced by a light chain. In mammals, BiP also binds to the light chain in a similar fashion (Skowronek et al., 1998; Lee et al., 1999). The physical association between the light chain and BiP, which determines the half-life of the protein, was shown to take place in the variable domain, which is also important for the biological activity. However, other investigations claim only transient BiP-κ-light chain association (Lee et al., 1999). In our study, we show an increase of the level of expressed light chain when co-infiltrated with a weaker expressing heavy chain, although to a lesser extent than vice versa. When infiltrated alone, only full size light chain is found. BiP recognizes a conserved motif, that can be represented as Hy(W/X)HyXHyXHy, in which Hy represents hydrophobic residues and X any amino acid residue. This motif could not be found in the deduced amino acid sequences of the low expressing chains. This suggests that incorrect folding of the heavy chains could be responsible for the higher level of breakdown. As shown in our analysis of expression levels of ten heavy and ten light chain constructs, no general rule can

be deduced solely by regarding features of the primary structure of the variable regions. Although analysis of codon usage can give an indication of the potential expression levels of a construct, other intrinsic sequence characteristics should also be taken into account.

Using viral silencing suppressors under optimal conditions, we were able to boost the expression level up to 3.5% TSP in a similar fashion as has been demonstrated by Voinnet et al. (2003), who used the system to boost yields of a range of recombinant proteins. Our observations suggest that the effectivity silencing suppressing is dependent both on the antibody idiotype and on the silencing suppressor. The observed differences among the antibody constructs could reflect specific bottlenecks in the translational process depending on biochemical properties of the constructs. Silencing suppressors have been proposed to act by distinct mechanisms on several stages of the (post-) transcriptional pathway (table 2), thus affecting different proteins and possibly even different antibodies to a variable extent. For example silencing suppressor AC2 does not affect any of the immunoglobulins used in this study, although Voinnet et al (Voinnet et al., 1999) find unvarying silencing suppressing activity of GFP expression in tobacco.

A conclusion that can be drawn from our findings is that the observed differences between individual chains are not due to silencing effects or physiological conditions, but rather depend, among other factors, on co-ordinate expression of both heavy and light chains. Thus, molecular farming strategies aimed at the production of antibodies in plants would benefit from an integral design in which the constraints imposed by the eukaryotic production system (plants or mammalian cells) are taken into account from antibody selection onwards. In such a design, the incorporation of a fast transfer system as the one described here, which enables the implementation of a final step of selection *in planta*, will return its investment in terms of antibody yield. As long as specific plant expressions levels remain unpredictable, an extra *in planta* selection step is inevitable.

EXPERIMENTAL PROCEDURES

Phage display vectors and antibody selection

The display and selection of chicken antibodies using a phage display approach is described in chapter 2 of this thesis. Briefly, a new phagemid vector, pChick3, was constructed by incorporating chicken specific sequences into a bi-cistronic operon. pChick3 displays chicken antibodies as α-Fab fragments with the first constant domain of the α-heavy chain fused to the capsid protein pIII of M13 phage. A combinatorial antibody library was constructed by cloning antibody cDNA isolated from lymphoid tissues of *Eimeria* infected chickens into pChick3, to obtain a library with $1.2*10^7$ individual transformants. Successive selection steps against complex *Eimeria* antigens enriched the library in anti-*Eimeria* binding antibody fragments. Ten of those antibody fragments, showing detectable anti-*Eimeria* binding activity, were selected for full length expression and characterisation in planta.

Construction of pRAPIg expression cassettes and transfer of Ig sequences

The pRAP-Ig vectors were derived from pUCAP (van Engelen et al., 1995) with added mouse kappa light chain signal sequence for secretion (van Engelen et al., 1994). The basic vector structure (pRAPIgL1), comprised a double 35S promotor, a signal peptide, a polylinker, and the nopaline synthase terminator. In the pRAPIgL1 version, phage display derived chicken IgL sequences were incorporated as *Sal*I/*Xba*I fragments into the vector frame. For the construction of pRAPIgH1, a DNA fragment (Igα1-4) encoding the four constant domains of chicken αIgH was amplified by PCR using cDNA from chicken bursal tissue as a template. The oligonucleotides used in the amplification (1 and 2) incorporated *Sst*I and *Xba*I sites for cloning into a pRAPIgL1-derivative containing a stuffer fragment with a unique *Sst*I site.
For the cloning of phage display-derived chicken sequences into pRAP-Ig vectors, pChick3 selected fragments were selected from Eimeria-specific library Eckα1, as described in chapter 2 of this thesis. The purified phagemid was then used as a template in two separate PCR reactions of 10 cycles. In the amplification of IgL flanking *Sal*I/*Xba*I restriction sites were incorporated using oligonucleotides 3 and 4. Amplifications with oligonucleotides 5 and 6 incorporated *Sal*I/*Sst*I sites in chicken VH fragments for their introduction into pRAPIgH1 vector. PCR-amplified bands were purified from gel, digested and ligated into pRAP vectors following standard procedures. The sequences of all PCR-derived constructs were verified by two-strand sequencing to discard PCR-born errors.

A second generation of pRAPIg vectors was designed for the direct cloning of pChick3-derived sequences without intermediate PCR step. Vector pRAPIgL2 is a modification of pRAPIgL1 that incorporates *Nco*I/*Sal*I sites for the direct cloning of *Nco*I/*Sal*I released IgL fragments from pChick3. The modifications are introduced by oligonucleotides 7 and 8 using a PCR-based directed mutagenesis strategy. In a similar manner, PCR-based mutagenesis with oligonucleotides 9 and 6 generate pRAPIgH2, a vector that incorporates VHs directly as *Nhe*I/*Sst*I -released fragments from pChick3. A schematic overview of the cloning procedure is provided in figure 1. For *Agrobacterium* mediated expression, cassettes containing IgA heavy and light chain sequences were digested with *Asc*I/*Pac*I and ligated into pBIN+ vector (van Engelen et al., 1995).

Table 3 Primers used for plant expression

Name	Sequence
1	5'-CCGAGCTCCGCCTCCGCCAGCCGCC-3'
2	5'-CGCCGACGTCACCTGTTATTAATCTAGAGG-3'
3	5'-GGGTCGACGGTTCCCTGGTGCAGGCAGCGCTGACTCAGCC-3'
4	5'-CCTGAAGAGGTCCGAGTGCTAATAGTCTAGACC-3'
5	5'-CCGTCGACGGCGTGACGTTGGACGAGTCC-3'
6	5'-CCACGGGACCGAAGTCATCGTGAGCTCCGCCTCCGCCACCCG-3'
7	5'-CAAATACTTCCATCATGAGCATCAAGATGG-3'
8	5'-GGGGGTCTAGAGTCGACCGCTGCCATGGCCAGGGAACCCTCGACACC-3'
9	5'-GTGTCGACGGCGCTAGCTTGGACGAGTCCGGGG-3'

Agroinfiltration experiments

For plant expression of chicken immunoglobulins pBIN-derived vectors were transferred to *Agrobacterium tumefaciens* LB404 strain and grown as described (Kapila et al., 1997). Cultures were infiltrated into *Nicotiana benthamiana leaves* by forcing the solution with little pressure with a 3ml syringe into the leaf. Infiltrated areas were marked and harvested after 4 days. Agroinfiltration of full length IgA constructs was achieved by mixing equal volumes of 1.0 OD_{600nm} IgL and IgH *A. tumefaciens* cultures. For comparison with full length IgA expression, single IgL or IgH infiltrations were always combined with equal volumes of empty vector-carrying bacteria. Infiltrations with silencing suppressors, or with an empty vector (provided by D. Baulcombe, Sainsbury lab, UK) were mixed 1:1 with IgA combinations to a final OD_{600nm} of 1.0 as described by Voinnet et al. (Voinnet et al., 2003). Upon harvesting, leaf material was immediately frozen in liquid nitrogen. Soluble protein was extracted by grinding the frozen leaf material using a mortar and pestle after addition of PBS containing anti 1 mM Pefablock (Boehringer Mannheim, Germany), to avoid proteolytic degradation, and 0.05% tween20 (PBS-T). Cell debris was removed by centrifugation in an Eppendorf centrifuge (5 min/13,000g/ 4ºC). Supernatant was stored at -80ºC for further analyses.

Western blotting and ELISA

For the detection of individual antibody chains or assembled IgA complexes, protein extracts of plant samples were mixed 1:1 with sample buffer (50 mM Tris·HCl pH 6.8; 2% (w/v) SDS; 10% (w/v) glycerol; 0.01% bromophenolblue, with or without addition of 40 mM DTT). SDS-PAGE was carried out using 7.5% polyacrylamide gels. After electrophoresis, the proteins were transferred to nitrocellulose membranes (Millipore, the Netherlands) by semi-dry blotting according to standard procedures. The membranes were blocked with PBS containing 0.1% Tween-20 and 5% low fat milk powder. The immunoglobulin heavy and light chains were visualized by incubating the blots with goat anti-chicken IgA-peroxidase antibodies or with peroxidase-labelled goat anti chicken light chain antibodies (both 1:7,500; Bethyl, Montgomery, Texas). The reaction with ECL substrate (Amersham Biosciences, The Netherlands) was visualized by exposure to X-ray film.

The concentration of plant-produced IgA was determined by sandwich ELISA. Microtitre plates were coated o/n with anti chicken IgA (1:2,000; Bethyl, Montgomery, Texas,) in carbonate buffer (1:5; 15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). Non-specific binding sites were blocked with 5% low fat milk powder in PBS-T. For determination of IgA concentration, standard chicken serum with a known IgA concentration was used (Bethyl, Montgomery, Texas). Dilutions of crude plant extracts and reference serum were made in PBS-T. After incubation for 1 hour at room temperature, the amount of plant-produced antibodies was determined with peroxidase-labelled goat anti chicken light chain antibodies (1:5,000; Bethyl, Montgomery, Texas) and developed with ABTS (Amersham). Reactions were carried out for 30 minutes at room temperature. Specific binding of plant-produced immunoglobluns was detected following a similar protocol. Plates were coated with *Eimeria* antigens and incubated with crude plant extracts for 1 hour at 37°C, ABTS development was monitored after 3 hours. Concentrations of IgA in the plant extracts were calculated by nonlinear regression using the GraphPad PRISM 4.0 algorithm.

Northern blotting

RNA was isolated from co-infiltrated leaves 4 days post-infiltration. Total RNA was isolated using the RNeasy Plant Mini kit including an on-column DNAse digestion with RNAse-free DNAse (both obtained from Qiagen, Leusden, the Netherlands). RNA concentrations were equalized by OD_{260nm} measurements. Ten micrograms of RNA per sample was blotted onto nylon membranes (HybondN+; Amersham Biosciences, Roosendaal, The Netherlands) and hybridised with radio-labelled probes (^{32}P-dCTP) according to standard procedures. Both heavy and light chain probes were located in the constant domains of the immunoglobulin chains. DNA for the probes was obtained by digestion of pRAPIgH vector with *Kpn*I and *Not*I, releasing an insert of 543 bp and digestion of pRAPIgL with *AVR*II and *Xba*I, resulting in a 261 bp insert. Labelling of the chicken heavy and light chain specific restriction fragments with [α-^{32}P] dCTP for use as hybridization probes was performed with ready-to-go DNA labelling beads (Amersham Biosciences, Roosendaal, The Netherlands).

Sequence analyses of individual constructs

DNA sequences and subsequent analyses like translations and multiple alignments of V_H and V_L were performed with Vector NTI Suite, version 8 (InforMax). Codon usage analyses of the sequences were done with tools provided at http://gcua.schoedl.d/sequential.html.

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Chapter 4

A functional polymeric immunoglobulin receptor in chicken (*Gallus gallus*) indicates ancient role of secretory IgA in mucosal immunity

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SUMMARY

Animals are continuously threatened by pathogens entering the body through natural openings. Here we show that in chicken secretory immunoglobulin A (sIgA) protects the epithelia lining these natural cavities. A gene encoding a polymeric immunoglobulin receptor (*GG-pIgR*), a key component of sIgA, was identified and shown to be expressed in the liver, intestine and bursa of Fabricius. All motifs involved in pIgR function are present, with a highly conserved Ig binding motif in the first Ig-like domain. Physical association of GG-pIgR with pIgA in bile and intestine demonstrates that this protein is a functional receptor. Thus, as shown for mammals, this receptor interacts with J-chain-containing polymeric immunoglobulin A (pIgA) at the basolateral epithelial cell surface resulting in transcytosis and subsequent cleavage of the pIgR, releasing sIgA in the mucosal lumen. Interestingly, the extracellular portion of GG-pIgR protein comprises only four Ig-like domains, in contrast to the five domain structure found in mammalian pIgR genes. The second Ig-like domain of mammalian pIgR does not have an orthologous domain in the chicken gene. The presence of pIgR in chicken suggests that this gene has evolved before the divergence of birds and reptiles, indicating that secretory immunoglobulins may have a prominent role in first line defence in various non-mammalian species.

INTRODUCTION

Most pathogens invade the body of their host through mucosal surfaces, especially those lining the gastrointestinal, respiratory and genito-urinary tracts. Despite their phylogenetic distance, both mammals and bird species use polymeric IgA (pIgA) produced by plasma cells in the lamina propria underlying the mucosal epithelia (Mostov et al., 1999) in the primary immunological defense against such infections.

For mammals it has been shown that mucosal epithelial cells synthesize the polymeric Ig receptor (pIgR). This is an integral membrane glycoprotein binding pIgA at the basolateral epithelial cell surface. The binding of pIgA to pIgR stimulates transcytosis via a signal transduction pathway that is dependent on a protein tyrosine kinase (Luton et al., 1999). Receptor Ig complexes are endocytosed and subsequently transcytosed to the apical surface where secretory immunoglobulin A (sIgA) is released into the mucosal lumen by proteolytic cleavage of the receptor ectodomain (Tomasi et al., 1968; Crago et al., 1978). This cleaved fragment is called secretory component (SC). The structure of mammalian pIgRs is highly conserved, comprising an N-terminal extracellular region with five immunoglobulin like domains (ILDs), an acidic transmembrane region and a C-terminal cytoplasmic region. Initially, pIgR ILD 1 binds polymeric IgA non-covalently (Frutiger, 1986; Bakos et al., 1993). Subsequently, covalent bonds are formed between conserved Cys residues in pIgR ILD 5 and IgA Cα2 domains (Fallgreen-Gebauer et al., 1993). The J-chain, regulating the polymerization of IgA, is also important in the formation of secretory IgA, as its C-terminus forms a docking site on pIgA for pIgR (Johansen et al., 1999).

In contrast to the detailed information available on the structure and function of mammalian pIgRs, little is known about equivalent structures in birds. In chicken particularly, several reports have shown the presence of antigenic determinants associated with high molecular weight IgA complexes (Lebacq-Verheyden, 1972; Karaca et al., 1997). Studies on the transport mechanisms of chicken immunoglobulins across epithelial layers also suggested the presence of a pIgR-related carrier (Rose et al., 1981; Peppard et al., 1986). However, genes encoding the avian pIgR/SC counterparts remain to be identified.

Increased insight in avian mucosal immunity is important as it can aid in preventing colonization and, eventually, infection of mucosal epithelia. This is relevant both for animal welfare as well as for human health. Bacteria such as *Salmonellae* and *Campylobacter* and viruses such as the influenza virus can cause zoonosis and thus form a serious threat for humans. Understanding mucosal immunity can aid in improving vaccination of poultry and in developing and evaluating other means, for instance based on probiotics or prebiotics such as

oligosaccharides (Macfarlane et al., 1999), to enhance immune responses. Consequently the chances for zoonosis can be reduced.

Here, we report the cloning and characterization of the chicken pIgR (*GG-pIgR*) gene, the first non-mammalian pIgR orthologue. Its association with pIgA, yielding high molecular weight complexes, shows that *GG-pIgR* is the functional orthologue of mammalian pIgRs. As a distinctive feature, this pIgR was found to comprise only four immunoglobulin like domains in its extracellular region, in contrast to all mammalian pIgR proteins consisting of five ILDs. The phylogenetic relationship between the ILDs of GG-pIgR and mammalian pIgR proteins was determined and revealed that the GG-pIgR ILDs are the most distantly related and do not group with any mammalian pIgR-ILD.

RESULTS

The chicken pIgR has four immunoglobulin-like domains

In mammals, the polymeric immunoglobulin receptor is expressed in mucosal epithelia. For cloning the avian counterpart we therefore synthesized cDNA using mRNA isolated from the bursa of Fabricius, duodenum and jejunum tissue. Using degenerate oligonucleotides, based on the most conserved region of mammalian pIgR sequences, a PCR fragment was amplified showing significant similarity with pIgR sequences. Using a RACE approach the full-length cDNA clone was obtained henceforth referred to as *GG-pIgR*. In BLASTp homology searches this clone revealed high levels of identity with mammalian pIgR sequences. Alignment of amino acid sequences (see figure 1) showed that the motifs involved in pIgR functions are conserved in chicken.

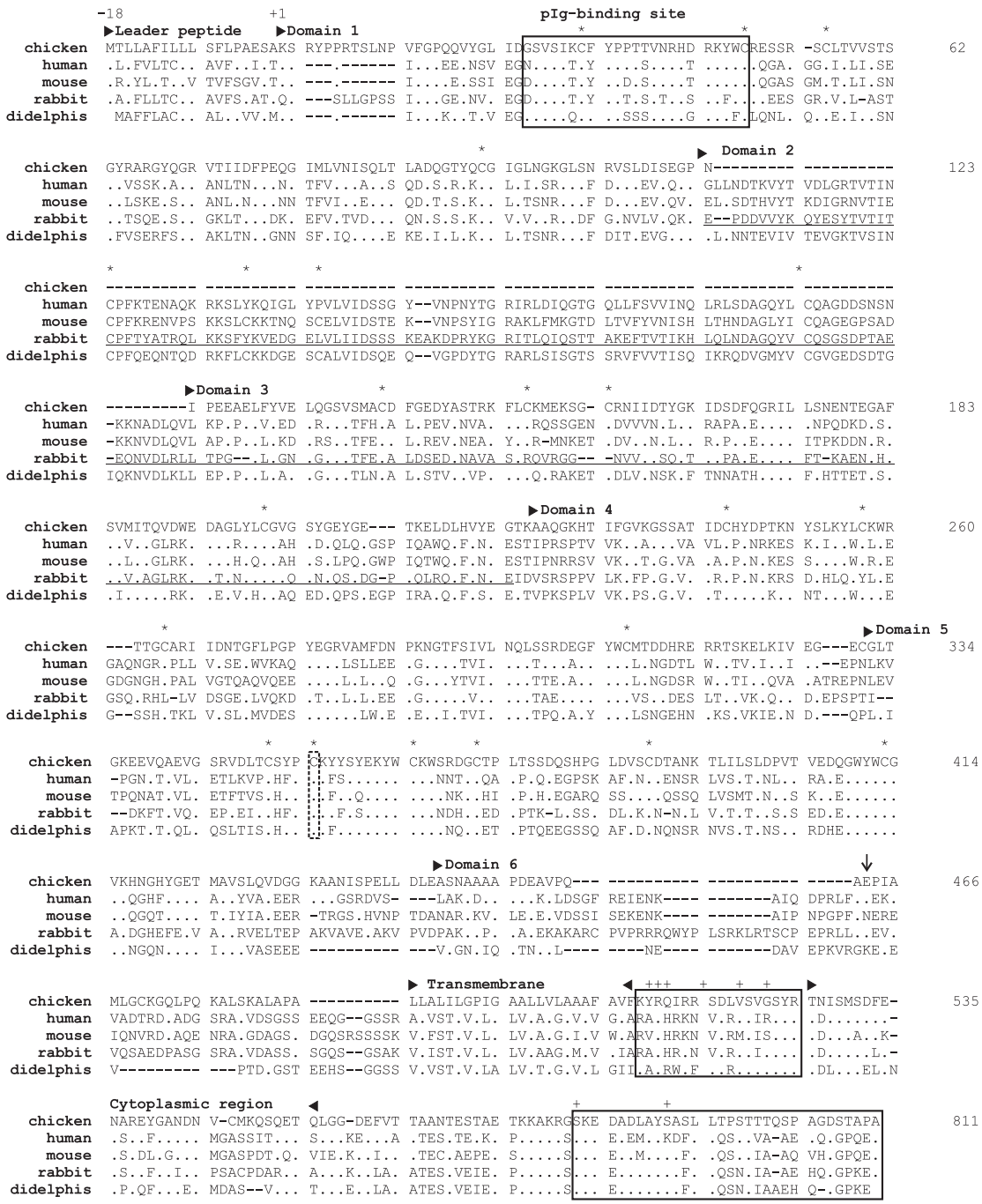

Figure 1 Interspecies alignment of pIgR amino acid sequences. The sequences of human, mouse and rabbit pIgRs were aligned as described in Piskurich et al. (1995), including sequences of GG-pIgR and *Didelphis* pIgR. Numbering is based on the chicken pIgR sequence beginning with the N-terminal Lys of the mature protein. Dashes indicate identity with the chicken pIgR sequence, and lines indicate gaps in the sequence homology. The noncovalent immunoglobulin binding site in domain I is boxed, the boxed residues in the cytoplasmic region denote sequences shown to be required for transepithelial trafficking of rabbit pIgR. Other residues found to be critical for pIgR trafficking are indicated with plus signs. Asterisks point out perfectly conserved cysteine residues (Fallgreen-Gebauer et al., 1993); the cysteine residue in the dotted box within domain 5 is responsible for covalent IgA binding. Underlined part of the rabbit sequence indicates the two domains that are spliced out in the short form of pIgR.

These include the calmodulin binding motif, the signal for rapid endocytosis, the initial non-covalent binding site for IgA and the cysteine residues involved in the formation of intramolecular disulfide bridges. In contrast, the transmembrane region and the putative cleavage site of SC showed low levels of similarity. The open reading frame of the putative chicken pIgR was approximately 100 amino acids shorter than all known pIgR proteins. To investigate this discrepancy in length, the domain-structure of the putative chicken pIgR was analysed using the SMART algorithm (Schultz et al., 1998). It was found to comprise an N-terminal signal peptide for secretion, four Ig-like domains (ILDs), a transmembrane domain and a cytoplasmic C-terminal region. Since all pIgR proteins described so far contain five ILDs, the shorter size of GG-pIgR was attributed to the lack of one ILD.
To investigate which domain was lacking, the levels of sequence identity between individual chicken and mammalian ILDs were compared (see table 1).

Table 1 Interdomain comparison of chicken, human, rabbit and didelphis immunoglobulin-like domains. The amino acid sequences of individual pIgR immunoglobulin like domains were compared by pairwise alignment. Percentage identity was calculated using the formula: 100* ((# identical AA- residues)/(total length of alignment-gaps)). N.s. = no significant alignment possible.

ILD	Species	Chicken ILD a	Chicken ILD b	Chicken ILD c	Chicken ILD d
1	Human	51.8	34.4	36.6	35.4
	Mouse	50.5	33.8	34.5	34.4
	Rabbit	49.1	34.2	34.1	34.0
	Didelphis	47.3	34.0	32.1	25.0
2	Human	34.1	33.7	33.3	n.s.
	Mouse	29.0	29.8	30.4	n.s.
	Rabbit	n.s.	32.2	n.s.	n.s.
	Didelphis	33.3	36.9	23.1	25.5
3	Human	36.7	44.7	29.6	n.s.
	Mouse	31.5	48.9	32.4	n.s.
	Rabbit	28.7	40.9	33.3	n.s.
	Didelphis	26.7	46.2	31.3	27.6
4	Human	36.3	30.1	44.7	34.7
	Mouse	32.7	32.4	43.3	35.8
	Rabbit	38.8	30.0	42.6	34.1
	Didelphis	39.2	33.3	46.4	34.8
5	Human	22.8	25.3	35.2	48.3
	Mouse	25.0	28.6	44.9	48.4
	Rabbit	28.6	31.7	35.8	46.1
	Didelphis	23.4	27.7	45.2	45.8

Mammalian domains 1, 3, 4 and 5 matched significantly with, respectively, chicken ILDs a, b, c and d (for overall sequence alignment of several pIgRs see figure 1 and figure 2 for details about the nomenclature used for chicken Ig-like domains). In contrast, the mammalian domains 2 showed relatively low levels of identity with chicken sequences, suggesting that this particular part is missing in bird species.

The genomic organization confirms four ILDs in *GG-pIgR*

Alternative splicing has been reported for bovine and rabbit pIgR (Deitcher et al., 1986; Kulseth et al., 1995). The finding of a chicken pIgR cDNA lacking one ILD prompted us to consider the occurrence of alternative splicing in the chicken pIgR transcript. To clarify this, a BAC clone containing the *pIgR* gene (AU bw 061 B21) was selected from a chicken BAC library. The sequence of a 10.4 Kb genomic DNA fragment containing the complete coding sequence (CDS) of *GG-pIgR* was determined. The GENESCAN algorithm predicted a CDS consistent with the cDNA. No regions showing Ig-like homology could be found in the predicted introns. It was concluded that *GG-pIgR*, unlike the mammalian pIgR genes, comprises only four ILDs.

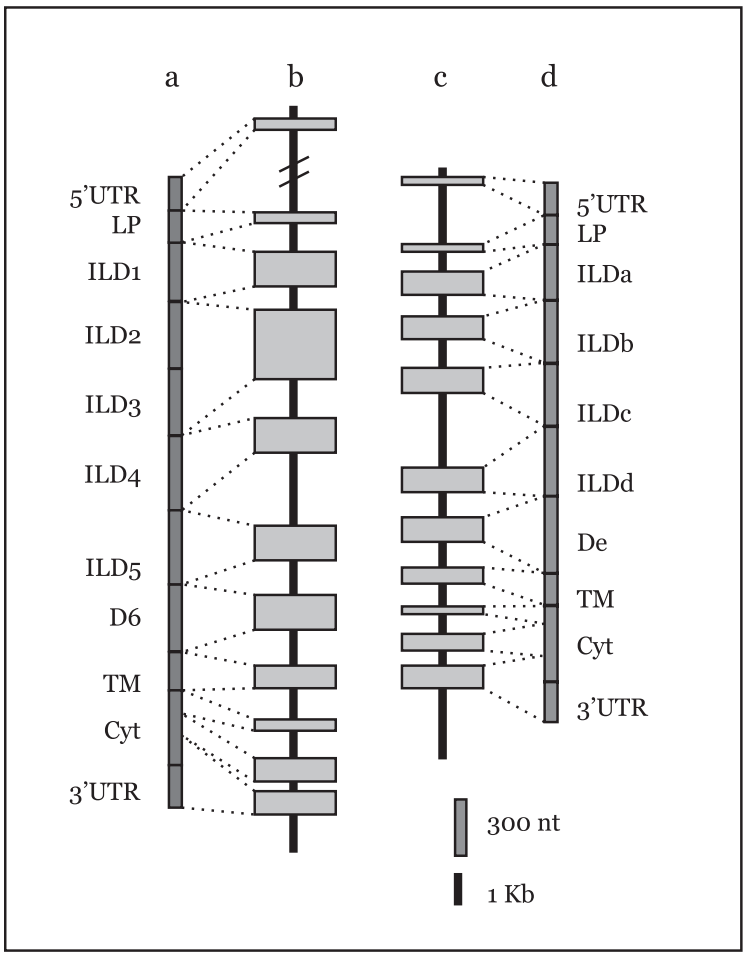

Figure 2
Schematic representation of human and chicken pIgR genes and corresponding mRNAs.
(a) Domain-structure of human mRNA; (b) Intron-exon distribution in human pIgR gene; (c) Intron-exon distribution in chicken pIgR gene; (d) Domain-structure of chicken mRNA.
LP: leader peptide; ILD: Immunoglobulin-like domain; D: extracellular domain with SC cleavage site; TM: transmembrane region; Cyt: Cytoplasmic domain; Light grey boxes represent exons, dark grey boxes represent domains of the protein. Neither the human nor the chicken 3' terminus of the exon coding the 3'-untranslated (UTR) region of pIgR mRNA has been mapped so far. Human data from Krajci et al. (1992).

The predicted genomic structure of *GG-pIgR* in comparison with its human counterpart is depicted in figure 1. Exons 1 and 2 encode the 5' untranslated region. The initiator AUG codon and the N-terminal part of the leader peptide is also encoded by exon 2. The four ILDs are encoded by exons 3, 4, 5 and 6, respectively. It is worth noticing that in human and mouse *pIgR* exon 4 encodes two ILDs.

Phylogenetic relationship between different pIgR domains.

To gain insight in the phylogenetic relationships among the different Ig-like domains in polymeric immunoglobulin receptors, individual Ig-like domains from GG-pIgR and seven different mammalian pIgRs were used in the generation of similarity matrices. In addition, the derived amino acid sequences of two *Xenopus leavis* ESTs, having significant homology with pIgR ILDs 1 and 4, were included in these analyses. The exact boundaries of every ILD were determined using available structural or genomic information. The resulting unrooted phylogenetic trees are presented in figure 3. The chicken ILDs do form a separate branch in the trees for domains 1, 3, 4 and 5. This also applies to the *Xenopus* ESTs in the trees for domains 1 and 4. Within each branch formed by the mammalian ILDs, marsupial and murine ILDs always form separate subgroups. It is worth noticing that rabbit ILDs do not cluster together with other mammalian species.

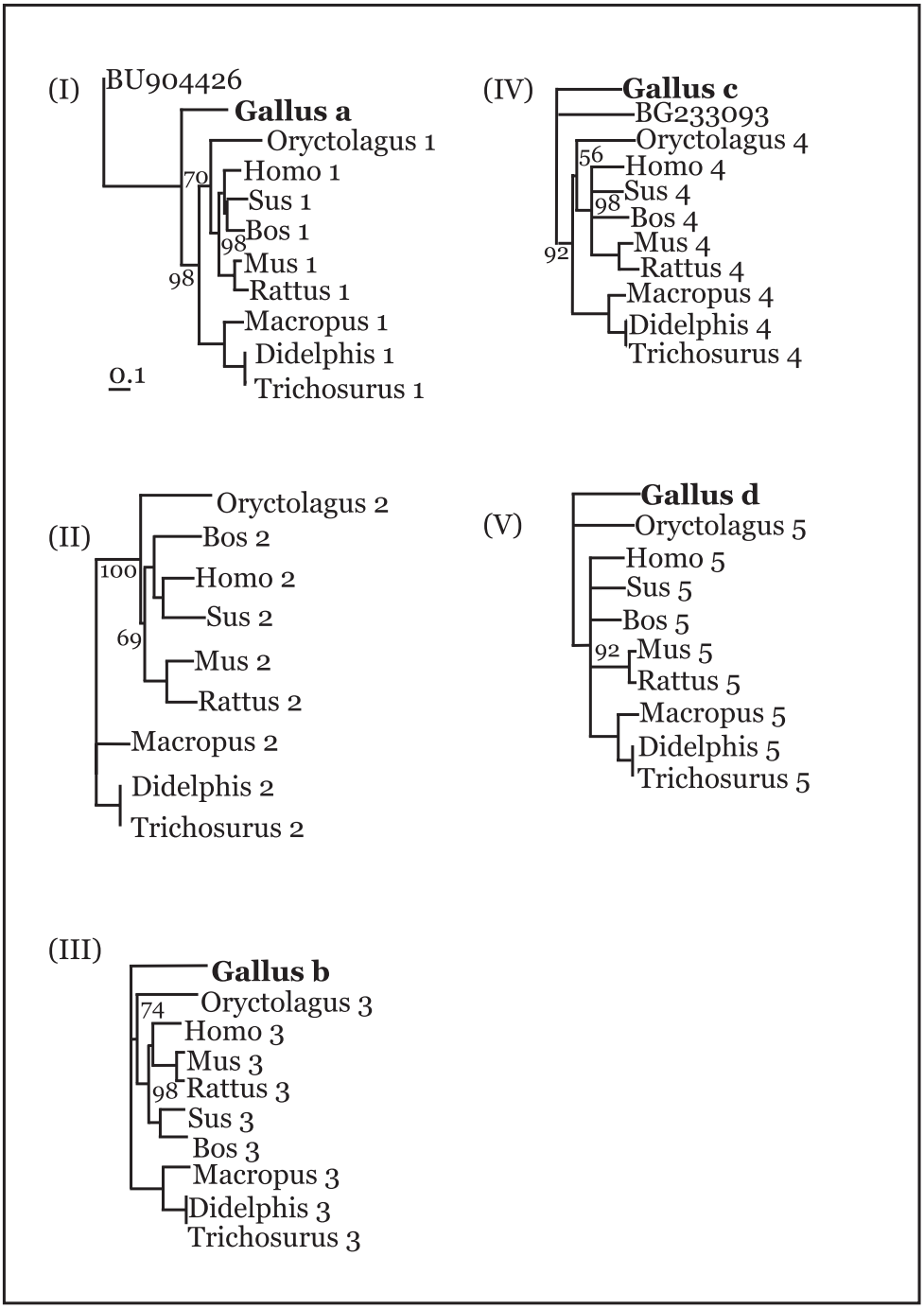

Figure 3 Domain-based phylogenetic trees comparing ILDs from eight pIgRs and two *Xenopus leavis* translated ESTs. Analysis was done with Neighbor Joining distance matrixes for the generation of unrooted trees. The trees are numbered I, II, III, IV and V for ILDs 1-5. Numbers at branch nodes represent confidence level of 1000 bootstrap replications; bar indicates the number of amino acid changes per site. Arabic numbers and letters represent domain identity as depicted in figure 1 for mammalian and chicken pIgRs. (*Homo* (human); *Mus* (mouse); *Rattus* (rat); *Oryctolagus* (rabbit); *Bos* (cow); *Sus* (pig); *Didelphis* (opossum); *Macropus* (wallaby); *Trichosurus* (possum)).

GG-pIgR is expressed in jejunum, bursa of Fabricius, liver and thymus

RNA samples from different chicken organs were isolated and used in Northern blot analysis with a gene-specific probe to determine which organs express *GG-pIgR*. As can be seen in figure 4, it is expressed as a single transcript in jejunum, bursa of Fabricius, liver and thymus, but not in heart, caeca, caecal tonsils and spleen. The highest abundance of transcript was found in thymus and liver.

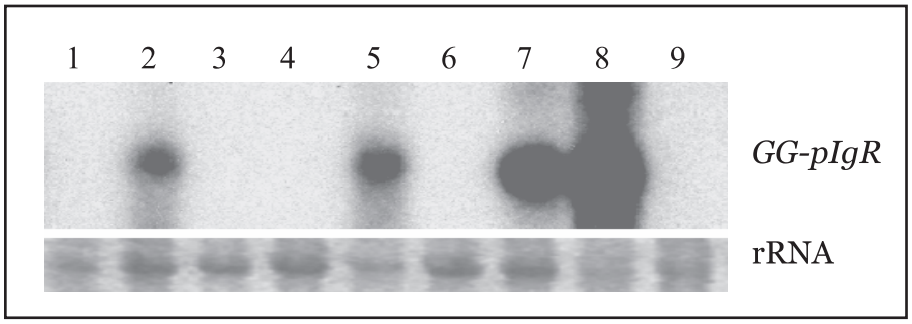

Figure 4 Expression profiling of chicken polymeric immunoglobulin receptor (*GG-pIgR*). **(A)** Northern blot analysis of *GG-pIgR* mRNA in different organs: bone marrow (1), jejunum (2), caecal tonsils (3), caeca (4), bursa of Fabricius (5), heart (6), liver (7), thymus (8) and spleen (9). **(B)** Ethidium bromide staining of ribosomal RNA (rRNA) shows equal loading of the samples.

Polymeric IgA associates with GG-pIgR

Upon transcytosis to the basolateral surface of the epithelium, the extracellular, ligand-binding domains of the pIgR are cleaved off. The resulting soluble form, known as SC, is found predominantly associated with polymeric immunoglobulins (pIgA and pIgM), known as secretory immunoglobulins. To investigate the association of chicken SC with polymeric IgA to sIgA a rabbit antiserum (α-SC) was raised against the ILDd of GG-pIgR expressed in *E. coli*. The presence of sIgA in serum, bile and fecal extracts was analysed by SDS-PAGE followed by immunoblotting using α-SC and α-IgA antibodies.

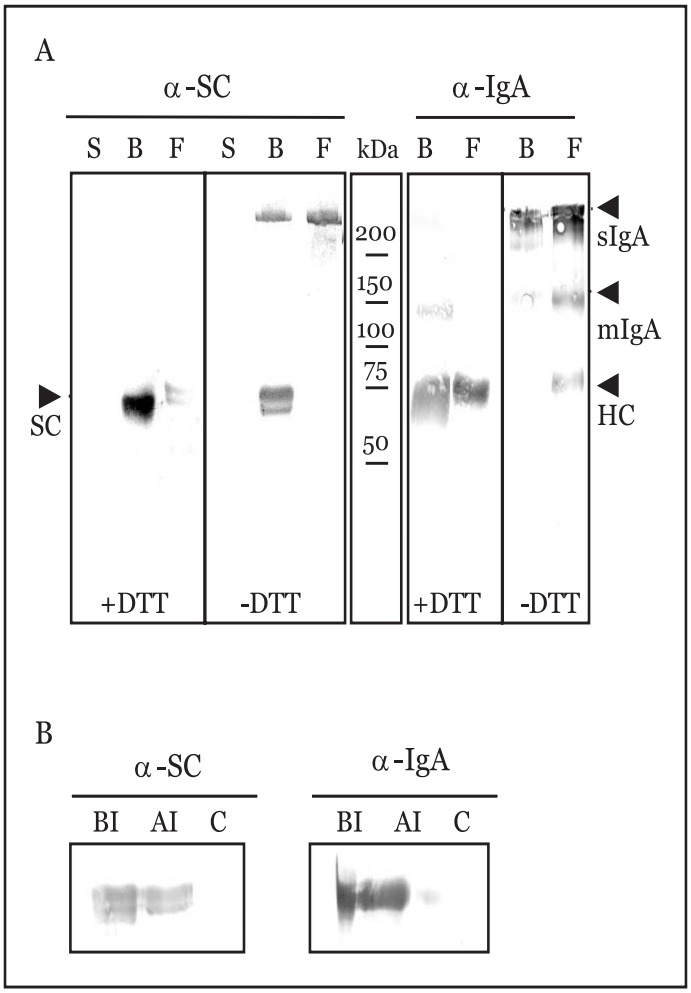

Figure 5
Association of the chicken secretory component ($_{GG}$SC) and polymeric IgA. **(A)** Western blot analysis of serum (S) and bile samples (B) and fecal extracts (F) under reducing (+DTT) and non-reducing (-DTT) conditions; sIgA: secretory IgA; mIgA: monomeric IgA; HC: IgA heavy chain; SC: secretory component.
(B) Western blot of co-immunoprecipitation of SC and IgA from bile with anti IgA antiserum under reducing conditions (BI: before immunoprecepi-tation; AI: after immuno-precipitation; C: control. Blots were developed with a rabbit serum raised against GG-SC (α-SC) or with an anti-chicken IgA antiserum (α-IgA).

Under non-reducing conditions, high molecular weight products were found in bile and fecal extracts upon blotting with either antisera (figure 5A). These bands presumably correspond to chicken secretory IgA, with an estimated size of 400-500 kDa. In bile we also found substantial amounts of free chicken SC, while in faeces mainly proteins corresponding in size to monomeric IgA and the α heavy chain were found. The physical interaction between SC and chicken IgA was confirmed in a co-immunoprecipitation experiment. Bile samples were subjected to immunoprecipitation with α-IgA coupled to sepharose beads followed by immunoblotting. As shown in figure 5B, SC co-immunoprecipitated with chicken IgA, confirming that the secretory component assembles with polymeric IgA into sIgA complexes in chicken.

DISCUSSION

Cloning of the avian pIgR orthologue (*GG-pIgR*) revealed a protein with four immunoglobulin like domains (ILDs) that assembles with chicken IgA to form a secretory immunoglobulin complex. The motifs involved in pIgR functions are conserved. It was concluded that the equivalent of mammalian ILD 2 is absent. This does not compromise the ability of chicken pIgR to bind IgA, which is not surprising, provided that differentially spliced forms of mammalian pIgRs lacking ILD 2 and 3 are known to retain IgA binding capacity (Frutiger, 1986). Also in other studies it was demonstrated that ILDs 2 and 3 do not play a critical role in the assembly with pIgA. Crottet and Corthésy (1999) found that the insertion of recombinant epitopes in murine ILD2-3 did not affect non-covalent binding of pIgA. In addition, both full-length human and the short version of rabbit pIgR translocate dimeric IgA with comparable efficiency (Tamer et al., 1995). We have not been able to show that chicken IgM associates with GG-SC. In concordance with the situation in mice and rats (Underdown et al., 1992), no or only poor binding is expected because GG-pIgR is expressed in the liver. Binding of IgM by GG-pIgR in this organ and subsequent transport into bile, would compromise the protective role of IgM in the systemic circulation. In addition, none of the residues in the motif involved in IgM binding, amino acid residues 61-64 (figure 1), is conserved in chicken when compared to its mammalian counterparts.

The overall organization of the chicken pIgR gene is comparable to that of human and murine pIgR genes. All three genes have their CDS interrupted by 11 introns. The chicken gene spans, excluding the promoter region, 10.4 kb, thus being considerably shorter than the human and mouse pIgR genes (19 kb and 32 kb respectively; Krajci et al., 1992; Martin et al., 1997). This shorter size was to be expected, as the length of introns in avian species is generally shorter than in mammals (Hughes et al., 1995). In chicken, the four extracellular ILDs are encoded by exons 3, 4, 5 and 6, thus complying with the one domain/one exon rule characteristic for the Ig superfamily (Williams et al., 1988). In contrast, the exceptionally large exon 4 in mammalian pIgR genes encodes two ILDs. The mammalian exon 4 seems in this sense unique. Interestingly, Bruce et al. (1999) reported that an 84 nt deletion in the murine 654 nt pIgR exon 4, activated cryptic 5'and 3' splice sites within this exon. As a consequence, two smaller exons of 158 and 334 nt were generated, separated by a 78 nt intron. The activated cryptic 3' splice site was located at the N-terminal end of ILD2, which is exactly the location of intron 4 in the chicken pIgR gene. The occurrence of cryptic splice sites in mouse exon 4 could be interpreted as relics of ancient active splice sites, which may have become inactive as a consequence of duplication events. The finding of a four-domain structure in chicken pIgR reinforces this hypothesis. It seems reasonable to assume that the unusually large exon 4 found in mammals appeared as a result of one or more duplications within a primitive, single-domain exon. The lack of a homologue of ILD 2 in chicken could be explained assuming that ILD

2-3 duplication resulting in the extant four domain structure of GG-pIgR either never took place in the bird lineage or was rapidly reverted. As previously mentioned, in some mammalian species the large exon 4 is entirely spliced out as a consequence of differential splicing, giving rise to a shorter version of pIgR encoding only ILD 1-4-5 (Deitcher et al., 1986; Frutiger, 1986).

The cloning of *GG-pIgR*, the first non-mammalian pIgR, allows studying phylogenetic relationships. It is worth noting in this respect that a phylogenetic relationship between the two immunoglobulin families binding Fc fragments, pIgR and Fcα/μ receptors (Shibuya et al., 2000) has been suggested. This hypothesis is based both on sequence similarities and on their topological proximity within the human genome (Shibuya et al., 2000; Shimizu et al., 2001). The unique Ig-like domain of Fcα/μ receptors is related to the N-terminal ILD of all known pIgR proteins. This conservation, likely maintained by strong selection pressure, is most evident in the CDR1-like loop of the first ILD, where the initial binding site of IgA/M is located. The equivalent motif in GG-pIgR, between residues 25 and 46 (see figure 1), is highly conserved (77% identity with human pIgR), suggesting a similar role in pIg binding.

From the domain-based sequence comparison of the pIgRs it became apparent that the GG-pIgR ILDs form a separate branch in the unrooted phylogenetic trees. A similar result was obtained for the two ESTs from *Xenopus leavis* showing significant homology to the mammalian and chicken ILDs 1 and 4 of pIgR. This is not surprising considering the phylogenetic distance existing between mammals and, respectively, birds and amphibians. The fact that *Xenopus* ESTs do show homology may be a first indication for the presence of pIgR-like proteins in amphibians. This is relevant in view of the fact that in *Xenopus,* an IgA analogue, IgX (Mussmann et al., 1996), has been described, but no transport system has been identified so far. The same applies to a hypothetical protein from the *Fugu rubripes* genome project (Scaffold_687_1) showing both sequence and structural similarities with pIg receptors (data not shown). The GENESCAN algorithm predicted a 763 amino-acid protein containing a signal peptide, four ILDs, one transmembrane domain and a cytoplasmic domain. The ILDs in the *Fugu* hypothetical protein are highly self-similar and seem to have arisen from recent duplication events. Although no conclusions can be drawn from these findings regarding the presence of pIgR-like molecules in poikilotherm vertebrates, they are at least indicative of the versatility of pIgR-related ILD combinations. Our observations point at an ancient origin of pIgR and suggest that this gene is present in clades that have evolved before the divergence of birds and reptiles (Sugden et al., 2003).

An explanation for a smaller but still functional chicken pIgR may be found in the protective role of SC against IgA degradation. With three constant domains and a hinge region, mammalian IgA differs structurally from chicken IgA having four constant domains and no hinge region (Mansikka, 1992). Crottet and Corthésy (1998) showed that SC exerts its protective role by delaying cleavage in the hinge/Fc region of the α-chain. Since chicken IgA has no hinge region, the absence of an ILD could be related to a less demanding role for the secretory component in IgA protection.

We have shown that upon translocation polymeric IgA associates with GG-pIgR, forming high molecular weight sIgA complexes, which can be detected in bile and fecal samples. Additionally, free GG-SC was found in bile. This has also been described for mammals (Phalipon et al., 2002) and a role as microbial scavenger has been suggested. As expected from a receptor involved in transcytosis, GG-pIgR was found to be mainly expressed in epithelia associated with lymphoid organs. Some peculiarities in *GG-pIgR* expression and accumulation patterns were found when compared to the mammalian counterparts (Mostov et al., 1999, and references therein). The low level of GG-SC in faeces contrasts the abundance in bile (Fig 5A), but is in agreement with the mRNA expression patterns of intestine and liver tissues respectively (Fig 4A). This could be indicative for a distinct balance in the roles of chicken pIgR in hepatobiliar transport and gut protection. The expression of *GG-pIgR* in bursa of Fabricius, the central lymphoid organ for B-cell lymphopoiesis in birds, is difficult to explain considering the known functions of this organ. A role of pIgR in antigen sampling should be considered, as it is known that antigen presentation takes place in bursal epithelia during lymphopoiesis (Ratcliffe et al., 1996). Similarly, the expression in the thymus is hard to explain and additional research is required to clarify the roles played by GG-pIgR both in thymus and bursa of Fabricius.

In conclusion, *GG-pIgR* is the functional homologue of mammalian pIgRs, the first non-mammalian polymeric Ig receptor described, and provides additional insight in the functioning of the avian mucosal immune system. Conservation of motifs for the various pIgR functions indicates that assembly to secretory immunoglobulins and transcytosis take place in a similar fashion as in mammals. The indications that this gene has evolved before the divergence of birds and reptiles lead to the hypothesis that secretory immunoglobulins have a prominent role in the defence of mucosal epithelia in other jawed vertebrates.

BIOLOGICAL SAMPLES, MATERIALS AND METHODS

Biological samples

For cloning of the chicken pIgR cDNA 3-week-old SPF chickens from Cobb broiler breeder parent stock (bred with Hybro parent stock) were used. Northern blots were carried out with tissues from a 10 week old hen.

Cloning strategy

The bursa of Fabricius and parts of the intestinal tract (duodenum and jejunum) were collected and directly frozen in liquid nitrogen. Total RNA extraction was performed using TRIzol ®LS Reagent (Life Technologies) according to the manufacturer's recommendations and cDNA was synthesized. By aligning all published pIgR sequences a conserved region was identified and used to design two degenerate oligonucleotide primers (sense primer 5'-AGCTACGARAARTAYTGGTG-3', corresponding to the amino acid sequence Ser-Tyr-Glu-Lys-Tyr-Trp-Cys (SYEKYWC; human pIgR residues 475-481) and antisense primer 5'-GTAGGCCATRTCXGCYTCYTCYTT-3', corresponding to the amino acid sequence Lys-Glu-Glu-Ala-Glu-Met-Ala-Tyr (KEEAEMAY; human pIgR residue 126-133). A PCR reaction was carried out under standard conditions with bursa of Fabricius and jejunum cDNA as templates. The resulting PCR product was isolated, cloned and sequenced. The 5' RACE system (Life technologies) was then used to obtain the putative full length chicken cDNA. The obtained fragment was subsequently sequenced and its homology with mammalian pIgR genes determined.

Cloning chicken pIgR gene

To obtain the full length genomic sequence of the coding region of the putative chicken pIgR a Bacterial Artificial Chromosome (BAC) library of the chicken genome (kindly provided by Dr. R. Crooijmans; *http://www.zod.wau.nl/vf/research/chicken/body_bac_library.html*) was screened with chicken pIgR specific primers (sense oligo 5'-GGATCCGACGTGCAGATCCAGCTCCTTCGT-3' and antisense oligo 5'-TCACCATCATCGACTTCCCAGAGCAGG-3') as described (Crooijmans et al., 2000). A BAC clone was found (AU bw 061 B21) which showed a band of the expected size. The genomic sequence containing the open reading frame of the putative chicken pIgR was revealed by using oligonucleotides, based on the cDNA sequence, generating overlapping PCR fragments. Products obtained by PCR were cloned and sequenced and used to construct a contig corresponding to the putative pIgR gene.

Sequence and structural analyses

DNA sequences were analysed with Vector NTI Suite, version 8 (InforMax). Homology searches were performed using BLAST at the National Center for Biotechnological Information (NCBI). Prediction of coding sequences in the genomic sequence was done using the GENESCAN algorithm, available at *http://genes.mit.edu/GENSCAN.html*. The structures of the sequences were characterized using SMART (Simple Modular Architecture Research Tool, *http://smart.embl-heidelberg.de*). This algorithm allowed identification of putative signal peptides, location of immunoglobulin-like domains and transmembrane regions.

To compare protein sequences of pIgR immunoglobulin-like domains of different species (*Homo sapiens* pIgR (human), NM_002644 ; *Mus musculus* pIgR (mouse), NM_011082; *Rattus norvegicus* pIgR (rat), P15083; *Oryctolagus cuniculus* pIgR (rabbit), X00412; *Bos taurus* pIgR (cow), LO4797; *Sus scrofa* pIgR (pig), Q9N2H7; *Didelphis* pIgR (opossum), AX282930; *Macropus eugenii* pIgR (wallaby), AF317205; *Trichosurus vulpecula* pIgR (possum), AF091137) multiple alignments were carried out using the BLASTp algorithm at the NCBI server (Tatusova et al., 1999). Standard parameters were applied. The percentage of identity between two sequences was calculated according to Piskurich *et al.* (Piskurich et al., 1995). The phylogenetic trees were established with Neighbor Joining analyses of amino acid sequences (Saitou et al., 1987) performed in PAUP* 4.0B10. To obtain the optimal tree, bootstrap analyses were conducted with 1000 replicates. Additional to the Ig-like domains of the pIgR sequences, two *Xenopus* ESTs with significant homologies to pIgR immunoglobulin-like domains (BU904426 and BG233093, E-values of $8*10^{-13}$ and $1*10^{-17}$ resp.) were included.

Northern blot analysis

RNA was isolated from heart, jejunum, duodenum, caeca, caecal tonsils, bursa of Fabricius, spleen and thymus of a ten-week-old hen. RNA concentrations were equalized by OD and verified by ethidium bromide staining of the gels. RNA was blotted onto nylon membranes and hybridised with radio-labelled probes according to standard procedures.

Production GG-pIgR antiserum (α-SC)

A fragment of 354 bp encoding the fourth immunoglobulin-like domain of chicken pIgR was cloned in the pBAD/TOPO-vector of the thiofusion expression system (Invitrogen). The resulting plasmid, termed pBADthioSCN4 was introduced in TOP10 *E.coli* cells and expression of the fusion protein was induced by arabinose. The protein was purified using a Ni-NTA column under denaturing conditions

with 8M urea according to the manufacturer (Qiagen). After purification the thioredoxin moiety was cleaved using EnterokinaseMAX (Invitrogen) and the remaining pIgR fragment was purified using Ni-NTA mini spin columns (Qiagen). With the purified pIgR fragment a rabbit was immunized according to standard procedures using Freund's Incomplete Adjuvant. Two weeks after the last immunization serum was collected.

Western blot analysis

For detection of the secretory component, serum, bile and fecal extract samples (prepared as described in Dann et al. (2000)) were mixed 1:1 with sample buffer (50 mM Tris·HCl pH 6.8; 2% (w/v) SDS; 10% (w/v) glycerol; 0.01% bromophenolblue) with or without addition of 40 mM DTT. SDS-PAGE was carried out using 7.5 or 10% polyacrylamide gels. After electrophoresis the proteins were transferred to PVDF membranes (Millipore) by semi-dry blotting. The membranes were blocked with PBS containing 0.1% Tween-20 and 5% low fat milk powder.
Secretory IgA or SC was visualized by incubating the blots with either a 1:1000 dilution of the polyclonal rabbit anti-chicken pIgR serum (α-SC) followed by an incubation with a 1:5000 dilution of anti-rabbit Ig alkaline phosphatase conjugate or with a 1:2000 dilution of goat anti-chicken IgA-alkaline phosphatase conjugate (α-IgA). The reaction was visualized using 5-bromo-4-chloro-3-indolyl phosphate as substrate.

Co-immunoprecipitation

Secretory IgA was co-precipitated using CNBr-activated sepharose 4B beads (Amersham Biosciences). Two hundred microgram of Goat anti-chicken IgA was coupled to 200 µl swollen sepharose beads according to instructions of the manufacturer. The affinity beads were incubated o/n at room temperature with 500 µl chicken bile in 5 ml PBS (pH 7.2). Then the beads were washed 5 times with PBS and subsequently the bound proteins were eluted in 50 µl elution buffer (0.2M Na_2CO_3 ; pH 11.0). Eluted fractions were characterized by Western analysis as described above. Uncoated sepharose 4B beads were used as a control.

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Chapter 5

Age related expression and localization of the polymeric immunoglobulin receptor in the chicken intestine

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SUMMARY

More knowledge about the development of the chicken mucosal immune system during the first weeks of life is important for the development of novel vaccines. Recently, the polymeric immunoglobulin receptor (pIgR) of chicken was identified and characterized, enabling expression and localization studies of this important receptor. In this study we show that pIgR is expressed, as in mammals, in epithelial cells of crypts and villi of jejunum and duodenum. Expression levels were low shortly before and after hatch, increased sligthly until day 7 post hatch, and increased strongly thereafter. The greatest expansion in the number of IgA positive plasma cells takes place between week 4 and 7 post hatch. These results suggest that pIgR expression preceeds the production of IgA. Next to expression in jejunum and duodenum, pIgR was also strongly expressed in liver of 7 weeks old chicken. This suggests that in chicken, like in rodents, the hepatobiliary route of IgA transport may play an important role. In young chicken sIgA seems to play a minor role in protection of the intestinal mucosa against invasion of pathogens.

INTRODUCTION

Extension of passive immune therapy to poultry was recently enabled by the elucidation of the molecular structure of the chicken polymeric immunoglobulin receptor (*pIgR*) orthologue. As in mammals, the extracellular domain of the pIgR, the secretory component (SC) associates with IgA (Wieland et al., 2004). In mammals, the pIgR is selectively expressed by mucosal and glandular epithelial cells and mediates transport of polymeric immunoglobulins (pIg) across the epithelial cell layers into the external secretions (Mostov et al., 1999). Upon transport of polymeric IgA the SC is digested and released into the lumen complexed with IgA (sIgA). In this format, IgA is protected against degradation by proteases and pH fluctuations. Secretory IgA plays a key role in the protection of the mucosa by binding to pathogens, thus hampering colonization or invasion of epithelia (Mostov et al., 1999). In mammals the structure, expression, localisation and function of this essential receptor have been studied in detail (Krajci et al., 1992; Piskurich et al., 1995; Norderhaug et al., 1999; Hempen et al., 2002; Uren et al., 2003). In chicken, Northern blots showed *pIgR* expression in jejunum, liver, the Bursa of Fabricius and thymus (chapter 4 of this thesis), suggesting a similar role for chicken sIgA in the mucosal immune system as its mammalian counterpart.

This study aims at further characterizing the role of IgA and pIgR in the avian mucosal immune system. This is achieved by identifying the sites and levels of expression of pIgR and IgA in the intestine of chickens during the maturation of the immune system. A pIgR specific antiserum was developed and characterized. Relative expression levels in intestinal tissues were determined by quantitative real time PCR (RQ PCR). The results presented here show a steady increase of pIgR expression of chicken jejunum from day 7 post hatch, localized in crypts and villi, in accordance to the situation in mammalian species. Combining these observations with data obtained on IgA and IgM expression will result in a better understanding of the mucosal immune system of young chicks and may add to development of optimal immune intervention strategies, such as oral passive immune therapy.

RESULTS

Chicken pIgR is present in crypts and epithelial cells of duodenum and jejunum

An anti pIgR serum was developed to study the localization of the pIgR protein in the chicken intestine by immunohistochemistry. Two rabbits were immunized with two synthetic peptides coupled to KLH. Both peptides were located in domain I of pIgR, enabling detection of pIgR as well as SC. Peptide specific antibodies were purified by affinity chromatography. ELISA experiments showed that a pool of the two affinity purified sera bound strongly to both conjugated peptides, whereas no binding to KLH alone was observed (Fig 1A). This indicates that the affinity purified antiserum is specifically for the synthetic peptides. In addition, Western blot experiments demonstrated that the affinity purified rabbit anti pIgR sera were able to detect recombinant SC and free SC in chicken bile and fecal extracts (Fig 1B).

In a next step, we used the rabbit antiserum for expression profiling of pIgR using sections of duodenum and jejunum. Initially no or very weak staining could be observed. We hypothesized that this was due to the fact that the antisera recognized continuous epitopes. To disrupt the tertiary structure of pIgR, the cryosections were heated in a ureum solution using a microwave. This procedure resulted in staining of the crypts and epithelial cells of the intestinal villi. However, background staining of muscle cells was also observed. To reduce the background staining, the cryosections were treated with periodate to disrupt polysaccharides. This treatment resulted in specific staining of crypts and villi. Staining of cryosections treated in similar way with the preimmune serum resulted in negative staining.

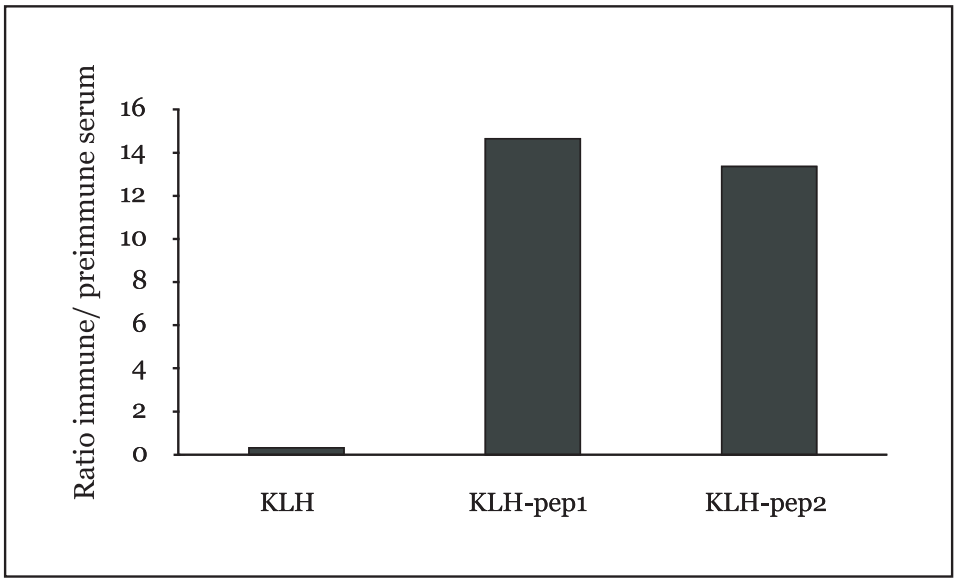
To obtain insight in the expression of pIgR in the intestinal tract during maturation of the immune system, tissue sections of jejunum and duodenum of day 18 embryos and chicks of 3, 7, 14, 28 and 49 days post hatching were stained with anti-pIgR serum. The presence of pIgR was only shown in crypts and epithelial cells along the villi of the jejunum (Fig. 2A and 2C) and the duodenum (results not shown). Incubation of tissue sections with the preimmune serum resulted in negative staining (Fig 2B and 2D).

Determination of pIgR expression by RQ PCR

RQ PCR reactions were performed in order to quantify pIgR expression during different phases of development of the chicken mucosal immune system. As shown in figure 3A, average pIgR expression in the jejunum (n=5 for each time point) increased slightly from embryonic day 18 until day 7 post hatch.

Compared to day 7, the expression levels on day 49 were 16 fold increased. A comparable increase in pIgR expression between day 3 and day 49 was found in duodenum, jejunum and liver (Fig. 3B).

A



B

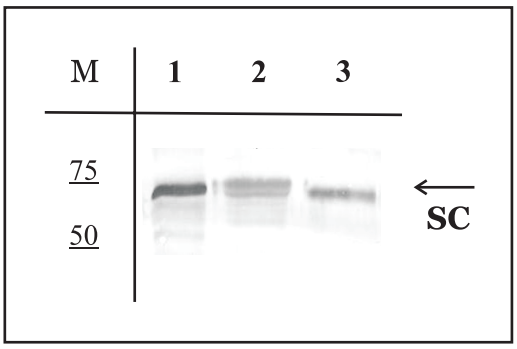


Figure 1 Characterization of the affinity purified rabbit anti-pIgR serum. **(A)** ELISA results show that the anti pIgR serum binds specifically to the two peptides that were used for immunization. Diluted (1:100) affinity purified rabbit serum was incubated with coated ELISA plates. Results were represented as the ratio of the extinctions of the immune- and the preimmune serum. **(B)** Western blots showed specific staining by the anti pIgR serum of recombinant SC (lane 1) as well as SC present in bile (lane 2) and faecal extract (lane 3).

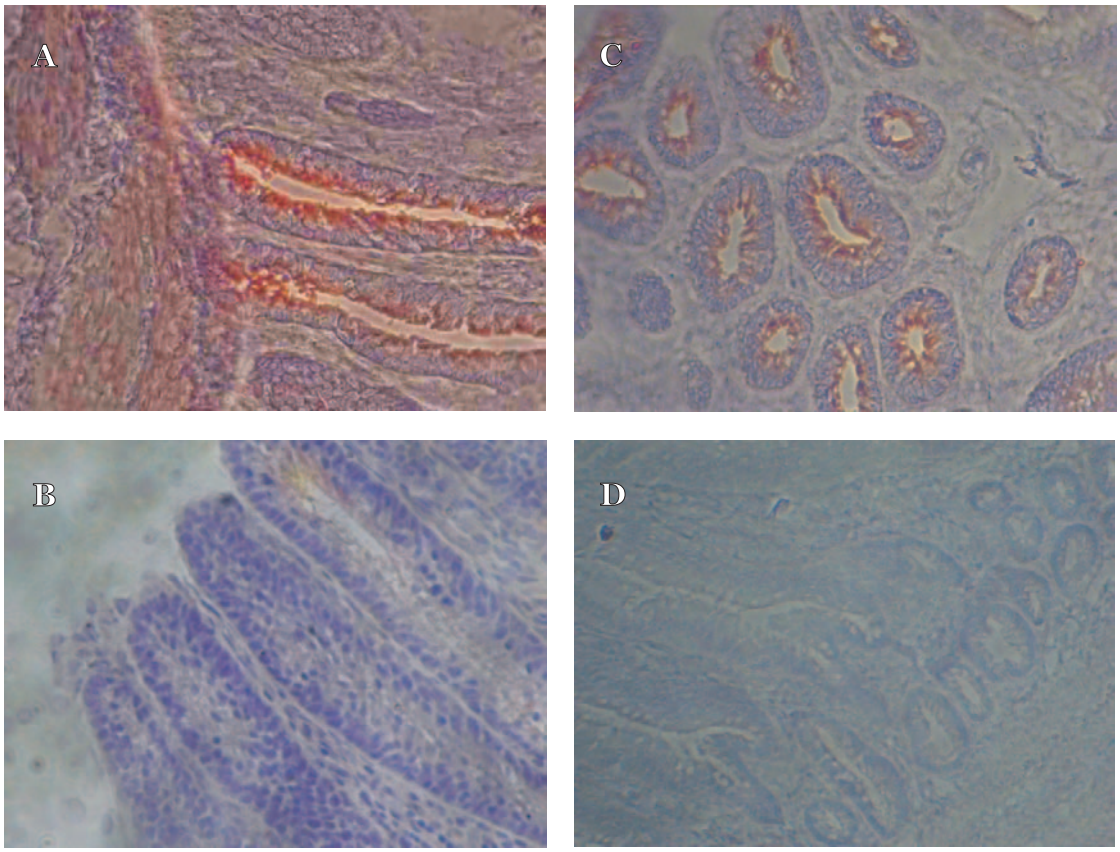
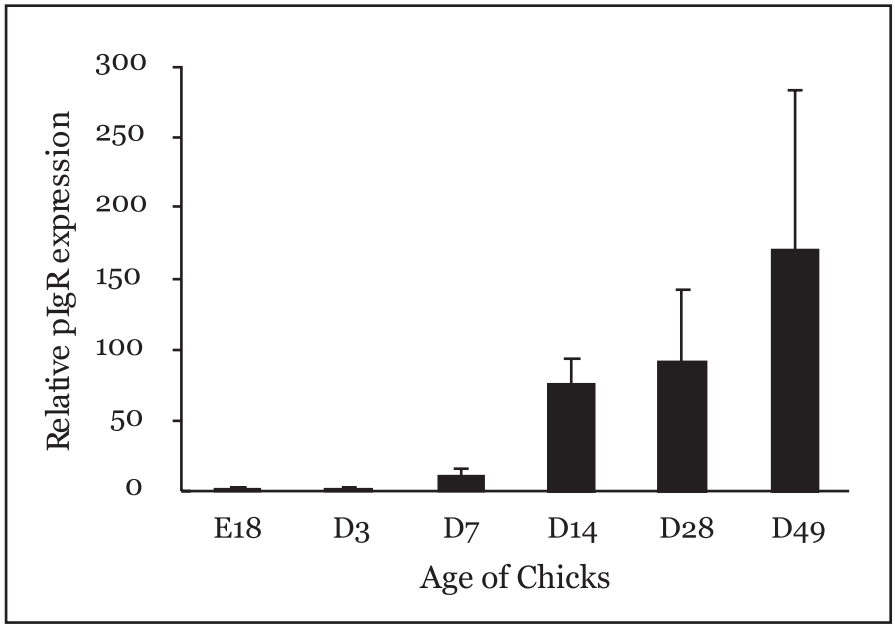


Figure 2 Immunohistochemical detection of pIgR in jejunum of 49 days old chicken. Sections shown in A and C were incubated with affinity purified rabbit anti-pIgR serum. Sections shown in B and D were incubated with rabbit preimmune serum. Clear staining of epithelial cells in villi (A) and crypts (C) is visible. The cryosections were counterstained with haematoxylin.

IgA expression during maturation of the chicken immune system

Cryosections of jejunum and duodenum were stained with anti IgA antibody to obtain insight in the number of IgA positive cells in the intestinal tract during maturation of the chicken immune system. No IgA positive B cells were observed in tissue sections of chickens younger than 7 days post hatch (Fig. 4A, B and C). The number of IgA positive cells in intestinal sections of 14 and 28 days old chicks was low (Fig. D and E). The number of IgA positive cells increased and in tissue sections of 14 days old chicken large numbers of IgA positive cells were found (Fig. 4F). The cytoplasm of the cells was clearly stained, indicating their nature as IgA producing plasma cells. In addition, the intestinal crypts of 49 day old chicks were also clearly stained by the anti-chicken IgA antibody (Fig. 4F). From these results we concluded that before the age of 4 weeks almost no intestinal IgA is produced or transported to the gut lumen.

A



B

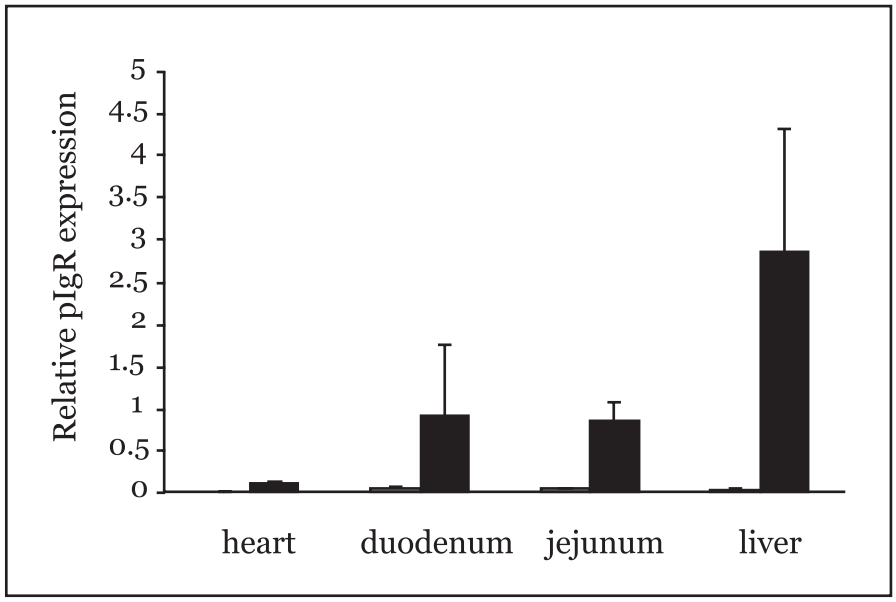


Figure 3 **(A)** Increase in expression levels of pIgR in jejunum during maturation of the immune system. Results at each time point are the averages of 5 individual animals. Expression values are standardized using ß-actin as an internal standard and relative to expression in day 18 embryos. (values are expressed as $2^{-\Delta\Delta Ct}$) **(B)** In another experiment expression levels in heart, jejunum, duodenum and liver were determined of 3 and 49 days old chicken (grey and black bars, respectively). For comparative reasons the values are expressed as $2^{-\Delta Ct}$, using ß-actin as internal standard.

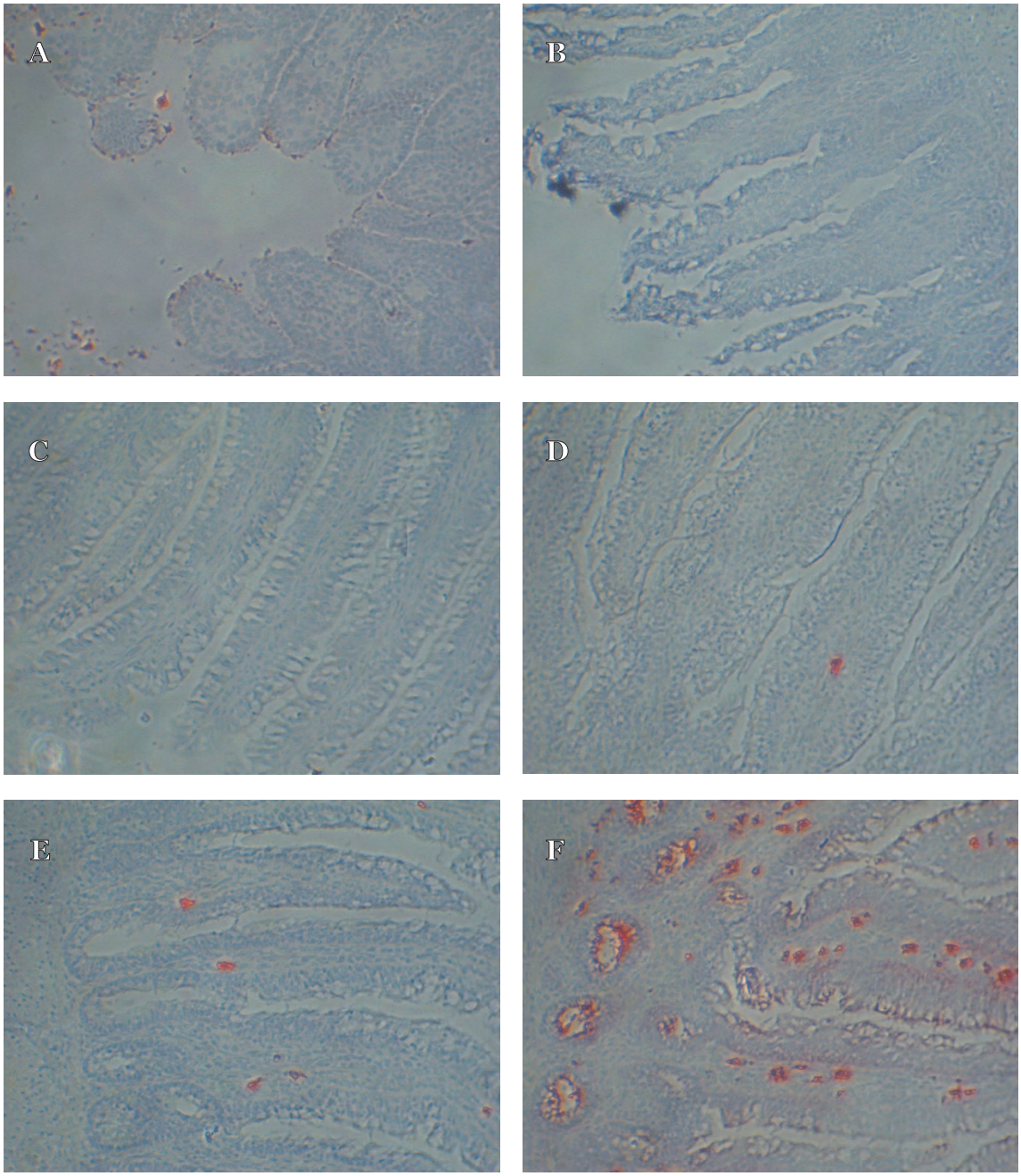


Figure 4 Immunohistochemical staining of jejunum for IgA positive cells. Cryosections of jejunum of day 18 embryos (A), day 3 (B), day 7 (C), day 14 (D), day 28 (E) and day 49 (F) post hatch were stained with anti-chicken IgA antiserum (Bethyl).

DISCUSSION

This study aimed at increasing the knowledge on the maturation of the chicken mucosal immune system, especially with respect to the pIgR expression and localization, and appearance of IgA+ cells. The post hatch period is of major immunological importance, since the chick is, upon hatching, immediately exposed to antigens and, more important, pathogens and very susceptible to infections as the immune system is not yet fully matured. In this study expression levels of pIgR in the intestinal tract of chicken during the first 7 weeks of life were determined by RQ PCR. In addition, intestinal sections obtained from chickens in the same period were immunohistochemically stained for IgA positive cells. PIgR expression was shown to increase strongly after day 7 post hatch, and high numbers of IgA positive cells appeared from week 4 onwards. These observations suggest that pIgR expression precedes IgA production in the chicken intestine. Moreover, regulation of pIgR expression may be related to distribution of IgA cells along the intestinal tract. The observations need to be confirmed by RQ PCR with IgA specific oligonucleotides.

The lack of IgA positive plasma cells at an early age suggests that IgA may not contribute to the protection of the intestinal mucosa in young chicken. Comparable observations were done before (Yamamoto et al., 1977; Jeurissen et al., 1989; Vervelde et al., 1993). Although several studies report that antigen specific sIgA responses can be induced in mature chickens (Muir et al., 2000, and references therein), it was shown that (mucosal) antibody responses could not be induced in chicks that were immunised before 7 to 10 days post hatch (Mast et al., 1999; Klipper et al., 2000; Bar-Shira et al., 2003). Therefore, it is likely that other factors than naturally occurring IgA or specifically induced IgA are involved in the protection of the intestinal mucosa. Possibly the innate immune system plays a role. It is therefore of great interest to study the contribution of the innate immune system to mucosal protection in the intestine of the immunologically immature birds. If the innate immune system plays a significant role in protection of the mucosa of the immature bird, modulation of this system by probiotics and/or bacterial homotopes can probably improve intestinal health.

Chicken pIgR was located in similar structures as in mammals, indicating a comparable function. In mammals *pIgR* expression in intestinal crypts reaches mature levels at 1 or 2 weeks after birth and initial signs of IgA expression are also found at this age (Brandtzaeg et al., 1991). The postnatal increase in pIgR expression is thought to be due to dietary changes and microbial colonization (Mostov et al., 1999). In mammalian cell lines pIgR expression was upregulated by the inflammatory cytokines interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and interleukin-ß (IL-ß) (Mostov et al., 1999, and references therein). Expression of these cytokines is probably enhanced in the chicken intestine after colonization by bacteria and subsequent influx of T cells in the lamina propria.

Influx of T cells may form the trigger for maturation of the chicken mucosal immune system. Bar-Shira et al. (2003) have investigated the maturation of the chicken mucosal immune system during the first two weeks post hatch. They found, indeed, a strong influx of CD3+ cells on day 4 post hatching. After day 4 the CD3+ cell number increased further, but increases were of smaller magnitude. IFN-γ expression, as a marker for effector cell functionality, increased on day 4 and after a short decrease on day 6 a further increase was measured in the second week post hatch. The dynamics of IL-2 expression corresponded also to the number of T cells. Thus, cytokine production during the first weeks post hatch may explain the increase in pIgR expression.

Apart from transport of pIg to luminal sites, pIgR and SC may have other functions in protection against disease. A function for free SC as molecular scavenger that prevents adherence of pathogens to epithelial cells has been proposed (Phalipon et al., 2003). In addition, transport of antigen by pIgR from luminal to apical sites has been reported (Zhang et al., 2000). As pIgR expression in chicken indeed precedes IgA production, free SC may also have a protective role in the young bird.

Like in rabbits and other rodents high pIgR expression was measured in the liver of chickens. This result is in accordance with our previous observations (Wieland et al., 2004). In this study Northern blot experiments showed that expression levels of pIgR was higher in liver than in jejunum. In rabbits pIgR is localized in hepatocytes (Johansen et al., 2004). However, in this study we were unable to localize pIgR in the chicken liver by immunohistochemistry. This might be due to a more dispersed localization of pIgR on the surface of hepatocytes in the liver compared to the concentrated localization in the crypts of the gut epithelium. Rabbits and rodents were found to obtain most of their proximal intestinal sIgA from hepatobiliary transfer of circulating pIgA. Humans in contrast, have no hepatobiliary transport and obtain 95% of intestinal sIgA from mucosal production. The fact that in chicken bile high concentrations of SC and sIgA are present (Rose et al., 1981; Wieland et al., 2004), suggests that the hepatobiliary route of sIgA delivery is also important for avian species.

Immaturity of the immune system is probably the reason for a lack of success in design and application of active mucosal vaccines. Since particularly young chicken become infected by mucosal pathogens, novel mucosal vaccination strategies are required. Application of oral passive immunisation with pathogen specific recombinant secretory IgA in young birds may be a promising alternative approach. For the effectivity of sIgA no active involvement of the immune system is required, because the main action of this immunoglobulin is the formation of immunocomplexes, which results in prevention of adherence of pathogens to and colonization of mucosal epithelial cells.

BIOLOGICAL SAMPLES, MATERIALS AND METHODS

Chicken

Eggs were collected from artificially inseminated hens of a random-bred line of an Isa Brown Warren cross (medium heavy layers). The eggs were incubated at 41 ºC and 55 % humidity. Organs were collected from embryos on day 15 and 18 of incubation and 3, 7, 14, 28 and 49 days post hatch. Small sections of the organs were immediately snap frozen in liquid nitrogen and stored until use at – 70 ºC.

Production of rabbit anti-pIgR serum

Rabbit anti-pIgR sera were prepared by immunisation of two rabbits with two synthetic peptides that were fused to Keyhole Limpet Hemocyanin (KLH). The sequences of the two KLH fused peptides were TSGYRARGYQGR (pep1) and GLSNRVSLDISEGP (pep2), both located in the first immunoglobulin like domain of pIgR. The rabbit sera were affinity purified using peptide coupled Sepharose beads.

ELISA

ELISA plates (middle binding; Greiner, The Netherlands) were coated with 10 µg/ ml KLH-pep1, KLH-pep2, or KLH, respectively in carbonate buffer (pH 9.6) at 37 °C for 1 h. The plates were washed with PBS + 0.05% Tween-20 and blocked with PBS containing 0.05% TWEEN20 and 1% newborn calf serum. Then the plates were incubated with two-fold dilutions of the rabbit sera. Unbound antibodies were removed by washing, followed by incubation with 1:1000 diluted Swine anti-Rabbit-HRP (Dakopatts, Glostrup, Denmark) in PBS/TWEEN20. Tetramethylbenzidine and 0.05% H_2O_2 was added as substrate solution. Colour development was stopped after 15 min at room temperature with 2.5 M H_2SO_4.

Western Blotting

A fragment of 1056 bp encoding the first three immunoglobulin-like domain of chicken pIgR was cloned in the pBAD/TOPO-vector of the thiofusion expression system (Invitrogen). Oligonucleotides used in the cloning process were Sept02SCF1 (5'-GAATTCATGACTTTACTGGCATTCATCCTC-3) and Sept04SCR2 (3'-GGATCCTTCCTTTGATGTTCTCCTCTCCCT-5'). The resulting plasmid, termed pBADthioSC3 was introduced in TOP10 *E.coli* cells and expression of the fusion protein was induced by arabinose.

The protein was purified using a Ni-NTA column under denaturing conditions with 8M urea according to the manufacturer (Qiagen). The recombinant protein comprised next to the first three Ig-like domains fused to thioredoxin and a poly HIS tail. For detection of pIgR, samples of recombinant SC, fecal extract (prepared as described by Dann et al., 2000), and chicken bile were mixed 1:1 with sample buffer (50 mM Tris/ HCL pH 6.8; 2% (w/v) glycerol; 0.01% bromophenolblue; 40 mM DTT). SDS-PAGE was performed using 10% polyacrylamide gels. After electrophoresis the gels were transferred to nitrocellulose membranes by semi-dry blotting. The membranes were blocked with PBS containing 0.1% Tween-20 and 5% low-fat milk powder. SC was visualized by incubating the blots with 1:250 affinity purified rabbit anti-pIgR serum followed by incubation with 1:5000 anti-rabbit alkaline phosphatase conjugate (Jackson ImmunoResearch, USA). The reaction was visualized using Nitroblue Tetrazolium/ 5-bromo-4-chloro-3indolyl phosphate as substrate.

Immunohistochemistry

For detection of pIgR cryosections (0.7 µm) of chicken organs were fixed on polysine microscope slides with acetone for 10 min. The slides were washed twice with PBS-T (0.1 M PBS + 0.05% Tween-20), and subsequently incubated in PBS-T + 1% H_2O_2 to inactivate endogenous peroxidase. After two washes in PBS-T, the slides were boiled in 0.8% ureum in PBS to expose continuous peptide epitopes. Subsequently the slides were incubated in 40 mM $NaIO_4$ in 0.1M NaAc (pH 4.8) for 30 min at RT to reduce background staining. After two washes in PBS-T the slides were incubated with 1:20 diluted affinity purified Rabbit anti-pIgR serum in PBS-T for 2 h. Then the slides were washed 3 times with PBS-T and incubated with 1:50 Swine anti-Rabbit-HRPO in PBS-T. The tissue sections were stained with 3-amino-4-ethyl-carbazole (AEC) in NaAc (pH 4.8).

For detection of IgA positive plasma cells, cryosections were incubated with goat anti-chicken-HRP (Bethyl laboratories, Montogomery, USA). In this case the incubation steps in urea and $NaIO_4$ were omitted.

RNA isolation

Total RNA extraction was performed using TRIzol® LS reagent (Lifetechnologies, Breda, The Netherlands) according to the manufacturer's recommendations. RNA concentrations were determined by spectrophotometric analysis and equalized by dilution in DEPC water. RNA was stored at –70 ºC until use.

DNase treatment and first strand cDNA synthesis

One microgram of total RNA was treated with 1 µl DNase I (Invitrogen, 18068-015) and incubated for 15 min at room temperature in a total volume of 10 µl. DNase was inactivated by adding 1 µl 25 mM EDTA and incubation at 65 °C for 10 min. Then 300 ng random hexamers, 1 µl 10 mM dNTP mix, 4 µl 5 x first strand buffer, 2 µl 0.1M DTT and 10 U RNase inhibitor (Invitrogen, 15518-012) were added and the mixture was incubated for 10 min at 37 °C. To each positive sample (but not the NT controls) 200 U Superscript RNase H^- reverse transcriptase (RT; Invitrogen, 18053-017) was added and reaction were incubated 50 min at 37 °C. All reactions were filled up with demineralized water to a total volume of 1ml and stored at –20 °C for future use.

Real Time Quantitative PCR

Specific primers for chicken pIgR (Genbank acc. nr: AF303371; forward: 5'ATTATGCCAGCACGAGGAAG 3' and reverse: 5' TCGTAGACGTGCAGATCCAG 3') and ß-Actin (Genbank acc. nr: L08165; forward 5' TGCGTGACATCAAGGAGAAG 3' and reverse 5'CCAAGAAAGATGGCTGGAAG 3') were used. For RQ PCR 5 µl cDNA and was added to 20 µl Quantitect Sybr Green PCR Master Mix (Qiagen) containing forward and reverse primers (300nM each). RQ PCR (2 min 48 °C, 10 min 95 °C, 40 cycles of 15 s 95 °C, 30 s 60 °C and 30 s 72 °C) was carried out on a Rotorgene 2000 (Corbett Research, Australia). Amplification efficiencies for the gene of interest (pIgR) and the internal standard (ß-actin) were determined using 10 fold dilutions of cDNA samples. The relative quantitation value was expressed as $2^{-\Delta\Delta Ct}$ (AppliedBiosystems user Bulletin #2; ABI prism 7700 detection system, 2001).

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Chapter 6

Expression of chicken dimeric and secretory IgA in planta

Authors

Willemien Wieland, Diego Orzáez and Arjen Schots

SUMMARY

The chicken secretory immunoglobulin complex, comprising light and α-heavy chains, the J-chain and the secretory component, was produced in tobacco plants using *Agrobacterium tumefaciens* transient expression. The versatility of this system was shown by co-infiltrating with up to four gene constructs. Realization of production of the secretory IgA complex *in planta* opens the way to extend oral passive immune therapy to avian species, thus providing an alternative for the profuse use of antibiotics and other drugs in the poultry sector.

INTRODUCTION

Protection of mucosal surfaces can efficiently be achieved by orally administered (secretory) IgA as demonstrated for many pathogens (Offit et al., 1985; Winner et al., 1991; Czinn et al., 1993; Enriquez et al., 1998). However, large-scale application of sIgA in immunotherapy has been hampered by lack of production systems capable of producing secretory immunoglobulins on a large scale in a cheap and safe manner. In the last 15 years, technological advances in the field of plant biotechnology have not only enabled the production of functional IgG, comprising two heavy and two light chains in plants (Hiatt et al., 1989; Düring et al., 1990), but also immunoglobulin complexes comprising more than 10 indivual polypeptide chains (Ma et al., 1995; Frigerio et al., 2000; Hood et al., 2002).

Secretory immunoglobulins play a key role in the first line of defence at all mucosal surfaces. The main isotype found in mucosal secretions is secretory IgA (sIgA). This immunoglobulin is composed of dimeric IgA, comprising two IgA molecules linked by a J-chain, and a secretory component (SC) enveloping this complex. Assembly of sIgA takes place during a process referred to as transcytosis. Dimeric IgA is bound by the polymeric immunoglobulin receptor (pIgR) at the basolateral surface of mucosal epithelial cells. The complex is endocytosed and transported to the apical surface. Here, the SC portion is cleaved of and remains attached to dimeric IgA, thus forming sIgA (Mostov et al., 1999). In the mucosal lumen, the SC protects IgA from degradation by proteases and low pH (Crottet et al., 1998; Renegar et al., 1998). Furthermore, the highly glycosylated SC “anchors” the IgA in the mucus to the epithelial surface (Phalipon et al., 2003). Here, sIgA serves a protective role through immune exclusion.

Recently, we described the presence of functional secretory IgA in chickens (Wieland et al., 2004). It was shown, that strong similarities exist between avian and mammalian mucosal immune system. In figure 1, a schematic representation of the chicken secretory IgA is presented. So far, only antibodies from human or murine sources, or hybrids of both, have been produced in plants. To extend passive immunotherapy to veterinary medicine, technologies need to be adapted for a variety of animals. The antibody phage display technology enables selection of antibodies of virtually any species. Thus, antibody genes become rapidly available for production in heterologous systems. Here we show the feasibility of the expression and assembly of chicken secretory IgA *in planta* in a transient system using all four genes necessary to produce this antibody complex.

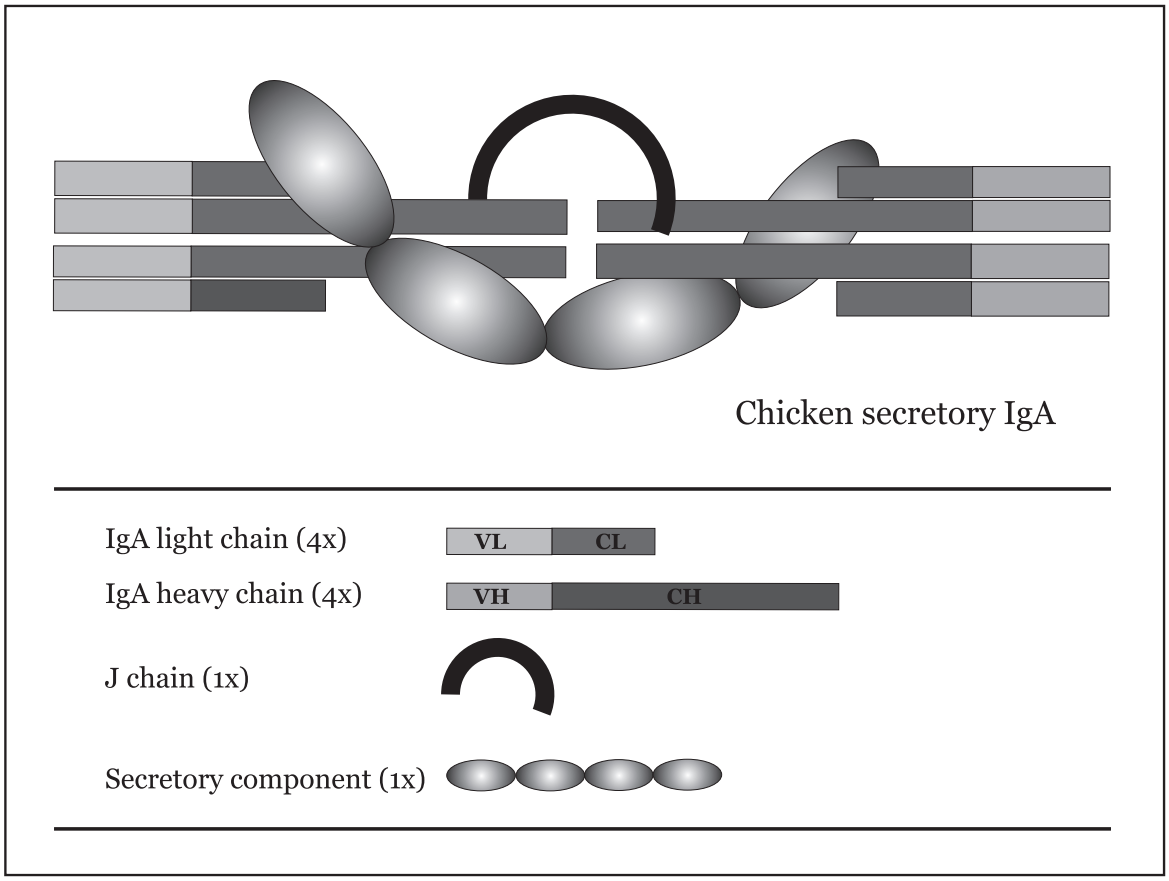


Figure 1 Schematic overview of the structure of chicken secretory IgA. Below, the single proteins forming the complex are shown. The number of proteins of each component necessary for correct assembly of sIgA is given in brackets. The constant domains of the IgA heavy and light chains are indicated by CL and CH, VL and VH show the variable regions.

RESULTS

Plant expression of chicken immunoglobulin genes was accomplished by infiltrating *Nicotiana benthamiana* leaves with combinations of *Agrobacterium tumefaciens* cultures. Each culture harboured vectors with expression cassettes containing either the genes coding for chicken light chain (IgL), alpha heavy chain (IgH), J-chain or secretory component (SC). Three different vectors containing the chicken J-chain were constructed: A pBIN+ -derived vector (J-chain$_{BIN}$), and two pK2GW7-derived vectors (Gateway) containing the J-chain either in a free form (J-chain$_{STOP}$) or fused to a variant of the green fluorescent protein (J-chain$_{GFP}$). A schematic overview of the different combinations used in the infiltrations and the expected protein complexes to be produced is given in figure 2.

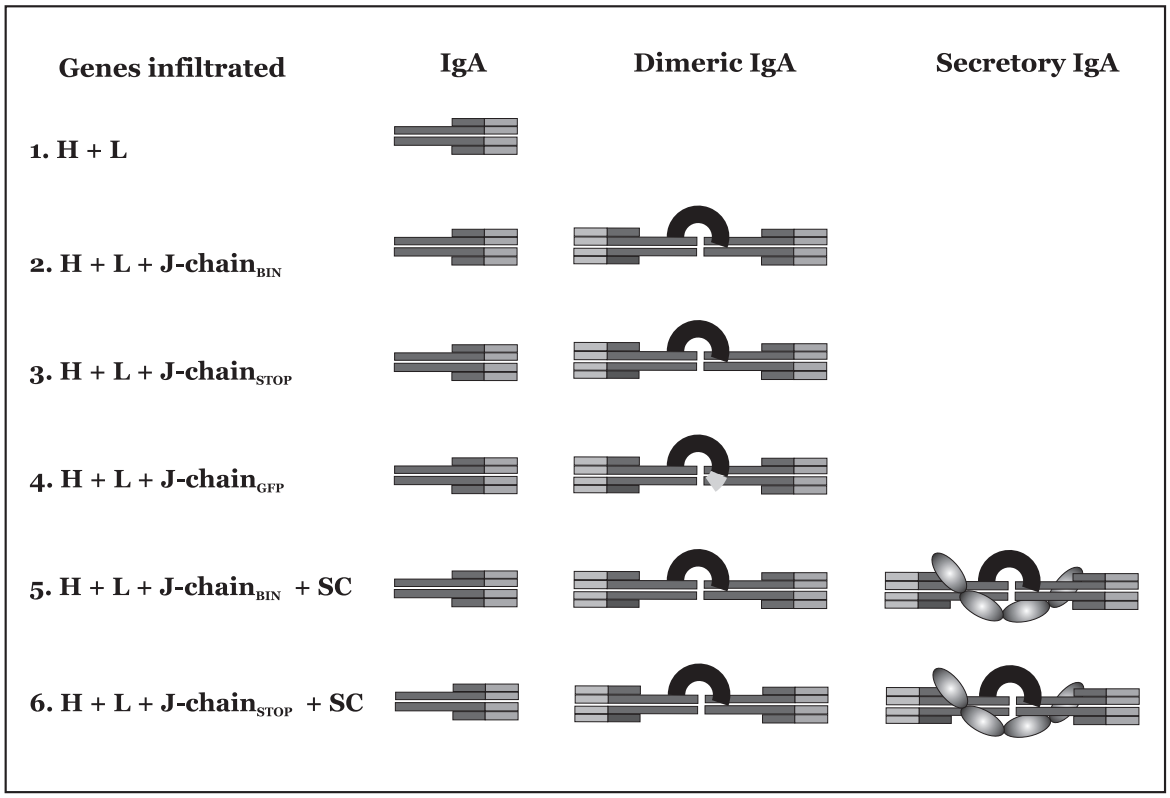
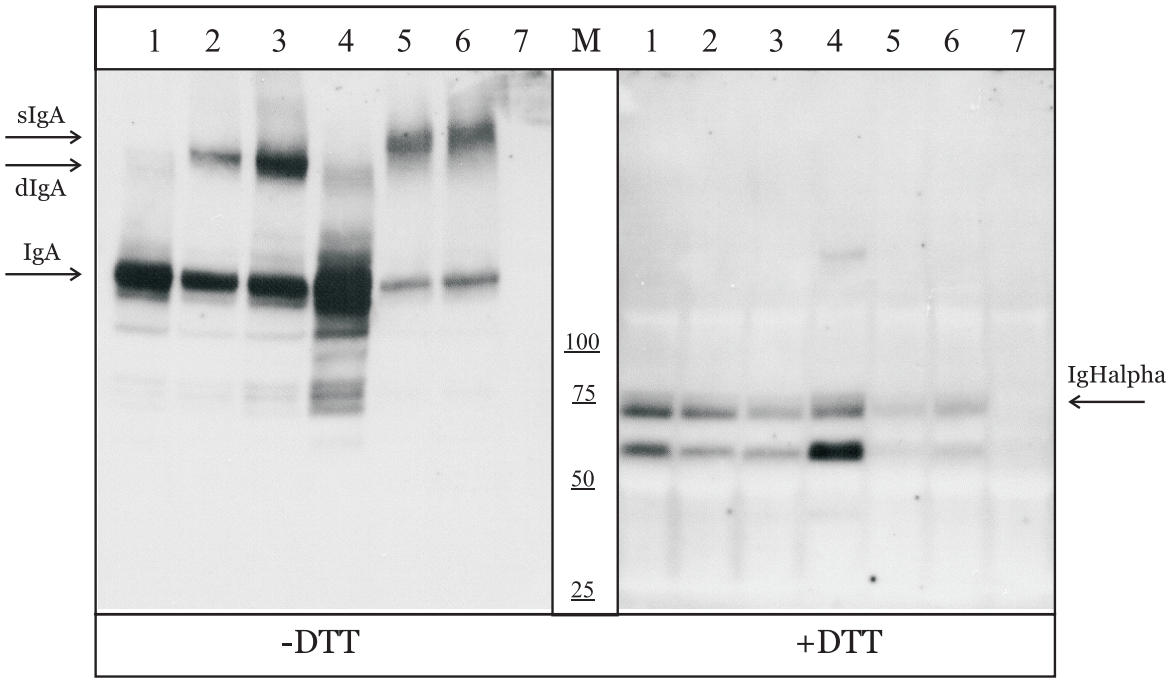


Figure 2 Schematic overview of the genes used in the *Agrobacterium* infiltrations and the expected immunoglobulin complexes to be formed. H, heavy chain; L, light chain; SC, secretory component.

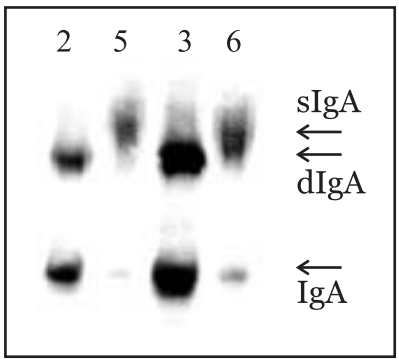
The results of Western blot analyses of infiltrated leaf samples are shown in figure 3. Different high molecular weight bands were detected under non-reducing conditions (figure 3A). In all infiltrations other than the control infiltration with a non-related protein construct (yellow fluorescent protein, YFP), monomeric IgA with a size of approximately 170 kDa was detected. This is comparable to the size of serum-derived chicken IgA. Co-infiltrations of IgA with J-chain$_{STOP}$ and J-chain$_{BIN}$ rendered high molecular weight products, indicating formation of a dimeric IgA complex. This band was not present in the co-infiltration of IgA with Jchain$_{GFP}$. This indicates that oligomerization is probably hampered by steric hindrance of the C-terminal GFP-fusion.

The two bands detected in the lanes representing co-infiltrations of IgA with J-chain and secretory component appeared to be larger. The size difference was confirmed in a second blot using a protein gel that had run longer, allowing better size separation of the formed complexes (figure 3B). This size difference is likely due to assembly of the secretory component with dimeric IgA.

A


1 2 3 4 5 6 7 M 1 2 3 4 5 6 7
sIgA
dIgA
IgA
100
75
50
25
IgHalpha
-DTT
+DTT

B


2 5 3 6
sIgA
dIgA
IgA

C

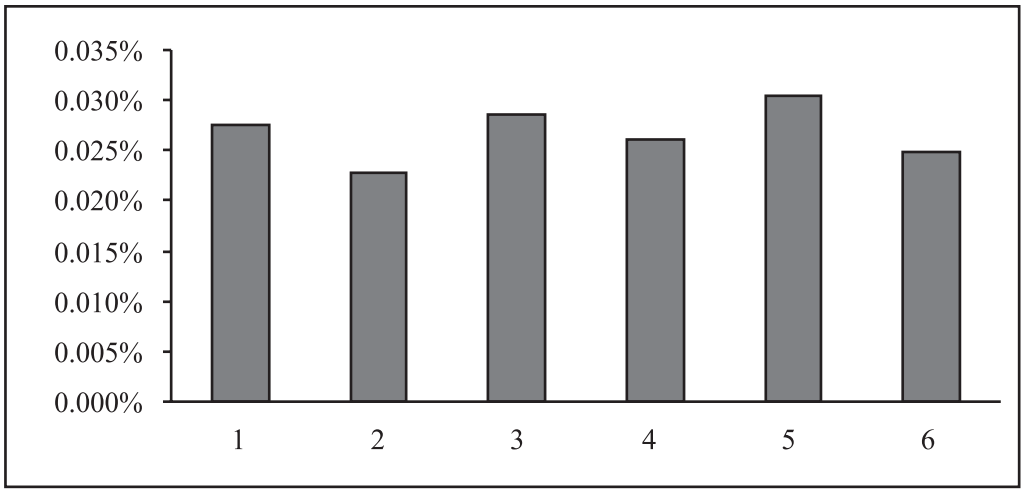


Figure 3 Analysis of infiltrated plants by Western blot **(A and B)** and ELISA **(C)**, IgA yields are shown as a percentage of total soluble protein (TSP)). Numbering of the samples correspond with figure 2. Lane "7" in the Western blot was loaded with a control sample (leaves infiltrated with a construct for YFP expression). IgA, monomeric immunoglobulin A; dIgA, dimeric IgA; sIgA, secretory IgA; IgHalpha, heavy chain of the alpha isotype.

Plant-produced IgA levels were quantified in a double antibody sandwich ELISA. IgA in crude leaf extracts was captured with anti chicken IgH_{alpha} and subsequently detected by anti chicken IgL antibodies conjugated to peroxidase. Chicken serum with known IgA concentration was used as a reference. Controls with either heavy or light chain alone gave no signal. As can be concluded from the data shown in figure 3C, no strong differences were observed among different infiltrations. This was observed, while the number of *A. tumefaciens* bacteria carrying constructs for IgA (H+L) was lower when J-chain or SC was co-infiltrated as the total density was identical for each infiltration (table 2). Apparently, the co-expression of J-chain and SC has a stabilizing effect resulting in increased levels of assembled IgA.

CONCLUDING REMARKS

Here we show that the possibility to express avian secretory immunoglobulins, comprising chicken IgA, J-chain and secretory component, *in planta*. The findings demonstrate the feasibility of extending plants as a production platform for veterinary immune therapeutic applications. The efficiency and versatility of the transient expression system using *Agrobacterium* was demonstrated. From the successful formation of the secretory immunoglobulin complex we can conclude that it is possible through *A. tumefaciens* mediated transient expression to co-express at least 4 different polypeptides in a single cell assembling into one protein complex, namely secretory IgA.

MATERIAL AND METHODS

Vector construction

In this study, expression cassettes pRAPIgL1 and pRAPIgH1 containing the clone "A2" from phage display antibody library Eckα1 were used. Eckα1 was constructed with the phagemid vector pChick3. The detailed description on construction of pChick3, the anti-*Eimeria* library and selection for *Eimeria* binding clones can be found in chapter 2 of this thesis; the construction of plant expression cassettes is described in detail in chapter 3.

The expression cassette for chicken secretory component was based on the GG-pIgR cDNA described by Wieland et al. (2004). A clone coding for SC, based in GG-pIgR but excluding the signal peptide and the transmembrane and cytoplasmic domains, was amplified by PCR with oligos 1 and 2 (table 1) and introduced in pBAD/TOPO-vector of the thiofusion expression system (Invitrogen). This vector was used as a template for oligos 1 and 3 to obtain an insert for transfer to pRAPIgL vector using *Sal*I and *Xba*I restriction sites. The entire expression cassette was then transferred to the pBIN+ vector using *Pac*I and *Asc*I restriction enzymes

Table 1
Overview of oligos used for construction of plant expression vectors for the single proteins forming the secretory IgA. Restriction sites in oligos 1 – 5 are depicted by normal letters. Capital letters in oligos 6-10 represent overlapping regions necessary for Gateway cloning procedure.

Oligo	Sequence
1 (*Sal*I)	5'CCCAGAgtcgacTTAAACCCAGTGTGTGGACCGCAG 3'
2 (*Sma*I)	3' CCCCAGcccgggTGGCTCTGCCTGCGGAACGGCCTC 5'
3 (*Xba*I)	3' GGAGACCGTtctagaTCAATGGTGATGGTGATGATG 5'
4 (*Nco*I)	5' GAAGGCAccatggCGAGCTCTTTGCCGTGGGTGGCTTTG 3'
5 (*Sma*I)	3' CAGTGGcccgggctaTTCGGCATAGCAGGATGTTGG 5'
6	5' ACAAAAAAGCAGGCTatgaagagctctttgccgtg 3'
7	3' ACAAGAAAGCTGGGTgctattcggcatagcaggatg 5'
8	3' ACAAGAAAGCTGGGTgttcggcatagcaggatgttg 5'
9	5' ggggacaagtttgtACAAAAAAGCAGGCT 3'
10	3' GGGGACCACTTTGTacaagaaagctgggt 5'

J-Chain$_{BIN}$ was constructed in a similar way as SC but conserving its endogenous signal peptide, by using oligos 4 and 5 (table 1, based on J-chain sequence as elucidated by Takahashi et al. (2000)). In parallel, the J-chain was cloned into Gateway vector pK7FWG2. Entry clones were obtained performing a two-steps PCR standard Gateway protocol. Oligos 6 and 7 were used for J-Chain$_{GFP}$ construct, whereas entry clones in J-Chain$_{STOP}$ were constructed using oligos 6 and 8. Note that oligo 8 incorporates a stop codon precluding fusion with GFP. A second, nested PCR step made use of oligos 9 and 10 (table 1). The plant

expression vectors carrying constructs for SC or J-chain expression were transferred to *A. tumefaciens* (MOG101 strain) all vectors constructed using the Gateway system were transferred to *A. tumefaciens* (LB404 strain).

Transient expression of chicken immunoglobulin genes in *N. benthamiana* leaves

Preparation for infiltration was performed as described (Kapila et al., 1997; Vaquero et al., 1999). Induced Agrobacterium solutions (both pMOG101 and LB404) were equalized to an OD_{600nm} of 1.0 for all infiltrations, mixed as shown in table 2, and used to infiltrate four week old Nicotiana benthamiana plants. Infiltrated areas were marked and harvested 4 days post-infiltration Upon harvesting, leaf material was frozen immediately in liquid nitrogen.

Table 2
Overview of co-infiltrations leading to expression of monomeric, dimeric or secretory IgA. The last column shows the percentage of (H+L) carrying *Agrobacterium tum*. in the infiltration solutions prior to plant infiltration .

Complex	Constructs	Ratio	% (IgA)
IgA	(H+L)	1:1	100%
dIgA	(H+L) + $Jchain_{BIN}$	(1:1): 1	50%
dIgA	(H+L) + $Jchain_{STOP}$	(1:1): 1	,,
dIgA-(GFP)	(H+L) + $Jchain_{GFP}$	(1:1): 1	,,
sIgA	(H+L) + $Jchain_{BIN}$ + SC	((1:1): 1): 1	25%
sIgA	(H+L) + $Jchain_{STOP}$ + SC	((1:1): 1): 1	25%

Detection of plant-expressed antibodies

Soluble protein was extracted by grinding frozen leaf material using a mortar and pestle in PBS containing 1 mM pefablock (Boehringer Mannheim, Germany) and 0.05% Tween 20 (PBS-T). Cell debris was removed by centrifugation in an Eppendorf centrifuge (13,000g/5 min/4°C). Supernatant was stored at -80°C for further analyses. For the detection of expressed proteins or protein complexes, plant samples were mixed 1:1 with sample buffer (50 mM Tris·HCl pH 6.8; 2% (w/v) SDS; 10% (w/v) glycerol; 0.01% bromophenolblue) with and without the addition of 40 mM DTT. SDS-PAGE was carried out using Novex 3-8% Tris-acetate gels (Invitrogen, Roosendaal, the Netherlands). After electrophoresis the proteins were transferred to nitrocellulose membranes (Millipore, the

Netherlands) by semi-dry blotting according to standard procedures. A-specific binding sites on the membranes were blocked with PBS containing 0.1% Tween-20 and 5% low fat milk powder. The immunoglobulin heavy chains were visualized by incubating the blots with a 1:7,500 dilution of goat anti-chicken IgA-peroxidase (Bethyl, Montgomery, Texas).

To estimate total concentration of plant-produced IgA microtitre plates were coated o/n with anti chicken IgA (1:2,000; Bethyl, Montgomery, Texas,) in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). Non-specific binding sites were blocked with 2% low fat milk powder in PBS-T.
As standard, chicken reference serum with known IgA concentration was used (Bethyl, Montgomery, Texas). Dilution of plant crude extracts and reference serum were made in PBS-T. Plates were incubated for 2 hours at room temperature with the samples. Bound antibodies were detected with peroxidase-labelled goat anti chicken Ig light chain (1:5,000; Bethyl, Montgomery, Texas) and developed with ABTS (Amersham). Reactions were carried out for 25 minutes at room temperature. Concentrations of IgA in the plant extracts were calculated by non-linearized regression using the GraphPad PRISM 4.0 algorithm.

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Chapter 7

SUMMARY AND CONCLUDING REMARKS

The frequency and spectrum of infections with pathogens harbouring resistance to antibiotics and other drugs has dramatically increased over the last years. One of the main causes is the extensive use of antibiotics and other drugs in human and veterinary medicine because of their efficiency as therapeutics against a broad variety of pathogens. Parasites, such as *Eimeria* causing coccidiosis in chicken and pathogenic bacteria like *Salmonellae* and *Campylobacter* are examples of pathogens that acquired resistance. The latter can colonize both animals and humans (zoonosis) and infection with resistant strains is difficult or even impossible to treat with conventional antibiotics. Furthermore, continuous use of drugs in diets of animals kept for human consumption increases the risk of residues in food, that possibly affect human health. A detailed overview of the negative effects of the use of antibiotics is given in chapter 1. These drawbacks of antimicrobial drugs have led to a demand for alternative treatments. In this thesis an alternative approach for prevention of coccidiosis in chicken is described, based on immune intervention by passively administered, plant produced, secretory IgA.

Selection of anti-Eimeria Fab fragments

As a first step to fulfill the prerequisites for passive immune therapy in chicken, *Eimeria* binding IgA fragments were selected. To identify suitable antibodies, we applied the phage display technique. The variable regions of light and heavy chains are displayed on the phage surface as a functional antigen- binding antibody fragment fused to a coat protein. The chimeric gene encoding the variable regions is incorporated in the phage, thus physically linking phenotype and genotype. Thus, selection is enabled from a pool of millions of different antibody fragments each having their own specificity, e.g. against the *Eimeria* parasite. After selection of a binder, the sequence encoding the variable regions can be transferred to other expression systems. The phage display system was adapted to be used for the display of chicken Fab fragments. A newly constructed vector, named pChick3, allows straightforward cloning of chicken variable antibody domains in frame with the constant domains of the chicken light chain

and the first constant domain of the IgA heavy chain. The functionality of pChick3 was demonstrated by construction of an *Eimeria*-specific antibody library using lymphoid tissues (bursa of Fabricius, cecal tonsils and spleen) of *Eimeria acervulina* infected chickens. This library was named Eckα1 and comprised $1.5*10^7$ primary transformants. From Eckα1, ten antibodies were selected with specificity for *Eimeria* antigens.

Production of chicken IgA in planta

In order to be used in oral passive immunization approaches, chicken IgAs need to be produced as full length antibodies. We have chosen plants as production system. The main advantages of plants are their eukaryotic glycosylation pattern, high yield, cost-effectivity and safety, since plants, as opposed to mammalian cell systems, do not harbour human pathogens. Furthermore, when therapeutic proteins are expressed in animal feed plants, they can easily be administered to animals in a needle-free way. In chapter 3, the design and construction of new plant expression vectors, the transfer of ten phage display derived antibodies to this vector system and their subsequent expression *in planta* as full size IgA is described.

Upon expression of the ten selected anti-*Eimeria* antibodies, differences up to 500-fold in yield were observed. Effects downstream the transcriptional level caused these differences, since immunoglobulin chain specific mRNA levels did not correlate with protein yields. Absolute IgA concentrations were dependent on physiological and environmental conditions However, relative expression levels remained constant. Although some of the differences observed could be explained as a consequence of disadvantageous codon usage, the poor performance of some clones required additional explanation. Other factors, like compatibility of heavy and light chain also play an important role. Since heavy and light chains were encoded on separate vectors, this could elegantly be shown by swapping heavy and light chains from four antibodies to obtain 16 combinations. This comparison showed that poor expression characteristics of either heavy or light chain have a dramatic effect on the final antibody accumulation levels. When one of the two polypeptides is intrinsically unstable, the other polypeptide cannot elevate its low expression e.g. by stabilization. Furthermore, the influences of 5 different silencing suppressors, small proteins that minimize breakdown of mRNA of the recombinant protein by the plant itself, were tested for their abilities to boost IgA expression. Effects were found to be clone and suppressor dependent.

These observations lead to the conclusion that an extra *in planta* selection step is inevitable for successful integration of phage display and plant expression systems.

Does mammalian pIgR have an avian counterpart?

In the mammalian gut, IgA is present as a multi-protein complex. This complex is formed by two IgAs held together by a small peptide called the J-chain, and enveloped by the secretory component (SC). The latter comprises the extracellular part of the polymeric immunoglobulin receptor (pIgR), a protein responsible for transcytosis of dimeric IgA from the body inside to the lumen of the intestine or other mucosal sites like the respiratory and urogenital tracts. Upon transport, the SC-part of the pIgR is digested and remains bound to IgA (sIgA). The secretory IgA complex is highly stable, and protects IgA against degradation by proteases or pH-fluctuation. For bird species, the molecular structure of the pIgR or SC had not been elucidated. In chapter 4, a detailed description is given of the characterization of the chicken pIgR. Interestingly, the chicken SC comprises only four immunoglobulin-like domains compared to five found in mammals. Molecular comparison revealed that the second Ig-like domain in mammalian SC does not have a homologous domain in chicken. Rabbits and bovine species possess a shorter form next to the full size SC, which lacks the second and third Ig like domains due to alternative splicing. In chicken, only four domains were found in the genome, thus excluding alternative splicing as a cause for this discrepancy in length. The chicken SC is functional, since association with IgA was detected and found in bile and faeces, but not in serum, in accordance with the situation in mammals.

In chapter 5 the expression of the pIgR in various chicken tissue in relation to age is described. Immunohistochemical analyses revealed that pIgR is produced, as in mammals, in epithelial cells of crypts and villi of jejunum and duodenum. pIgR was detectable by immunohistochemical staining in chickens of 7 weeks old, IgA positive B-cells were clearly visible in chickens of 4 weeks. These results suggest that IgA precedes pIgR expression. The pIgR ontogeny was clarified by real time PCR (RQ-PCR), a sensitive technique to quantify gene expression. These measurements revealed a steady increase of pIgR expression starting approximately 1 week post hatching until at least 7 weeks post hatching. RQ-PCR showed also expression of pIgR in the liver of 7 week old chickens, suggesting that in chicken, IgA is transported through the hepatobiliary route by pIgR, comparable to rodents but unlike the situation in humans.

Expression and assembly of chicken secretory IgA in tobacco

In a final step, the elucidated secretory IgA complex was expressed in tobacco plants (chapter 6). One of the antibodies selected from Eckα1 by phage display and cloned into plant expression vectors, was co-expressed with the other components of secretory IgA, the chicken J-chain and the secretory component. Next to the J-chain construct, a construct was made in which the N-terminus of the J-chain was fused to the green fluorescent protein (J-chain$_{GFP}$). Analysis of

these samples showed formation of differently sized complexes with IgA. Co-infiltrations with IgA, J-chain and SC rendered larger complexes than IgA alone. The complex formed by combination of IgA and J-chain was slightly smaller in size than the expression of IgA, J-chain and SC. No complex was formed between IgA and J-chain$_{GFP}$, probably due to steric hindrance.

These observations demonstrate the possibility to transiently express, through *Agrobacterium tumefaciens*, at least 4 different polypeptides in a single cell. The four peptides necessary to form secretory IgA, assemble into one protein complex when produced in tobacco plants.

CONCLUDING REMARKS

With the final achievement of the production of *Eimeria*-specific secretory IgA in plants, the prerequisites for chicken passive immune therapy were fulfilled. An integrated system for both selection and expression of immunoglobulins was developed. This system has been applied for the selection and expression of *Eimeria*-specific antibodies, which are now ready for application in passive oral immune therapy. The studies described in this thesis show that plants are not only able to produce functional antibodies originating from mammalian or avian species, but also complex proteins like secretory IgA. As opposed to yeast- or bacteria-based production systems, plants are able to perform most of the posttranslational modifications that are required for therapeutic applications of recombinant proteins. Thus, plants represent a safe and profitable alternative to animal cells and broad application in human and veterinary medicine should be considered.

In future research, *in vitro* and *in vivo* testing has to substantiate the effectivity of plant produced (s)IgA. Our objective, to provide a prophylaxis for coccidiosis in chickens by orally administered sIgA, can easily be tested in an *in vitro* invasion assay. In this assay, epithelial cells are invaded by the *Eimeria* parasite and the possibility to intervene in this process by plantibodies can be assessed. Once *in vitro* effectivity has been demonstrated, a challenge study with Eimeria infected chicken will be undertaken.

The system described here can easily be adapted for other pathogens that threaten poultry. As mentioned, *Salmonella* and *Campylobacter* spp. represent a major threat, notably for human health. The pChick3 vector can be applied in the construction of microbe-specific libraries, from which antibody fragments can be selected. Subsequent transfer, as described for *Eimeria*-specific binding antibodies, will lead to full size, specific antibodies. The system described here can be applied for virtually any pathogen, and will yield functional antibodies within limited time. This is very promising in light of newly emerging zoonoses.

Next to practical applications, the research described in this thesis has added substantial new insights in the (maturing) avian mucosal immune system. More detailed studies on the role of secretory immunoglobulins in birds are now possible, a start has been described in chapter 5. Improved knowledge on the avian mucosal immune systems will lead to a sound basis for the design of vaccines and other therapeutics for immune intervention in (young) birds. Furthermore, elucidation of the molecular structure of the chicken polymeric immunoglobulin receptor provides –due to its distinct structure- more insight on the evolution of proteins with immunoglobulin-like domains.

Since avian and mammalian mucosal immune systems are more alike than was generally believed, comparative immunological studies can now be undertaken, shedding new light on avian and mammalian immune systems and mucosal immunology in general.

Samenvatting

Ten behoeve van de gezondheid van mens en dier worden op grote schaal antibiotica toegepast. Dat dit niet zonder nadelige gevolgen blijft, blijkt uit het feit dat veel ziekteverwekkers resistentie ontwikkeld hebben tegen deze medicijnen. Dit bemoeilijkt, of maakt het zelfs onmogelijk, om infecties veroorzaakt door deze pathogenen te bestrijden omdat de ziekteverwekker niet meer reageert op antibiotica. Het bekendste voorbeeld is wellicht de multiresistente ziekenhuisbacterie *Staphylococcus aureus* (Lemmen et al., 2004; Witte, 2004). Door overvloedig gebruik van antimicrobiële middelen in ziekenhuizen zijn stammen ontstaan met resistentie tegen meerdere antibiotica. Infectie met de "ziekenhuisbacterie" is daardoor vaak niet te behandelen en besmette patiënten worden uit ziekenhuizen geweerd uit angst voor verspreiding van de besmetting. Op vergelijkbare wijze hebben *Eimeria* parasieten, welke hevige diarree veroorzaken bij vogelsoorten, resistentie ontwikkeld tegen bijna alle beschikbare middelen (Chapman, 1997). *Eimeria* veroorzaakt, naast enorme economische schade wereldwijd, veel dierenleed in de pluimveehouderij.

De ontwikkeling van bacteriële resistenties heeft verstrekkende gevolgen die niet beperkt blijven tot problemen binnen ziekenhuizen en agrarische bedrijven, maar over de hele breedte van de maatschappij gevoeld worden. Het ontstaan van resistentie is direct gekoppeld aan het toegenomen gebruik van antibiotica in de landbouwsector (Endtz et al., 1991; White et al., 2001). Bij kippen komen micro-organismen voor die op de mens overdraagbaar zijn (dit proces noemen we zoönose), zoals *Salmonella-*, *Campylobacter-* en *Listeria*-soorten. Verschillende studies hebben aangetoond dat het aantal humane infecties met resistente stammen van deze ziekteverwekkers onrustbarend stijgt. Dergelijke ontwikkelingen vormen met name een gevaar voor mensen met een zwakker immuunsysteem, zoals kinderen, ouderen en zieken (Cohen, 1992; Shea, 2003). Bovendien kunnen residuen in dierlijke producten zoals eieren, melk en vlees via consumptie in de mens terechtkomen en op deze manier een bedreiging voor de menselijke gezondheid vormen, b.v. door het opwekken van allergische reacties.

Vaccinatie vormt veelal een goed alternatief voor het gebruik van antibiotica en andere geneesmiddelen. In de intensieve veehouderij, en met name in de kippensector zijn er echter enkele praktische bezwaren. Naast het feit dat vaccinatie een zeer tijdrovend karwei is resulteert het in veel stress bij de dieren met alle nadelige gevolgen van dien, zoals een verminderde afweer en agressief gedrag. Ook is vaccinatie niet direct effectief omdat de ontwikkeling van een afweerreactie enige tijd vergt. Tijdens deze periode is het dier nog steeds gevoelig voor infecties. Kippen in de intensieve veehouderij worden bovendien veelal niet

oud genoeg om een goede immuunrespons te ontwikkelen. Bovendien is het moeilijk een vaccin te ontwikkelen dat bescherming biedt tegen alle stammen van een ziekteverwekker. Dit blijkt ook het geval te zijn bij *Eimeria*, het pathogeen dat model heeft gestaan voor de studies beschreven in dit proefschrift.

Een alternatieve aanpak vormt het passief ondersteunen van het immuunsysteem. Dit is te vergelijken met het geven van moedermelk aan pasgeboren baby's. De melk bevat naast voedingsstoffen ook antilichamen, die een eerste bescherming bieden tegen ziekteverwekkers. Via het voer toedienen van antistoffen die specifiek zijn voor bepaalde ziekteverwekkers, kan op eenzelfde manier bescherming geven aan kippen en andere dieren.

In dit proefschrift zijn de praktische stappen beschreven die nodig zijn om te komen tot de productie van beschermende antilichamen voor toepassing in passieve immuuntherapie bij kippen. Bij zoogdieren komen antilichamen (IgA en IgM) in de darm voor als complex met twee andere eiwitten, de J-keten en de secretoire component (SC). In deze vorm zijn de antilichamen beschermd tegen afbraak door proteases en grote pH-verschillen en kunnen zo actief zijn tijdens de gehele darmpassage. Bij kippen waren er slechts indirecte aanwijzingen voor aanwezigheid van een vergelijkbaar complex in de darm. Om meer inzicht te krijgen in het mucosale afweersysteem van de kip, dat wil zeggen het afweersysteem dat een rol speelt in de bescherming van de darm, maar ook b.v. in de neus, werd de moleculaire structuur van de SC ontrafeld. Deze kippen-SC vormt een functioneel complex met IgA en hiermee werd aangetoond dat het mucosale immuunsysteem van de kip, en waarschijnlijk van alle vogels, op eenzelfde manier werkt als dat van zoogdieren. Voor een passieve immuuntherapie in de kip is het secretoire antilichaamcomplex dus de optimale vorm.

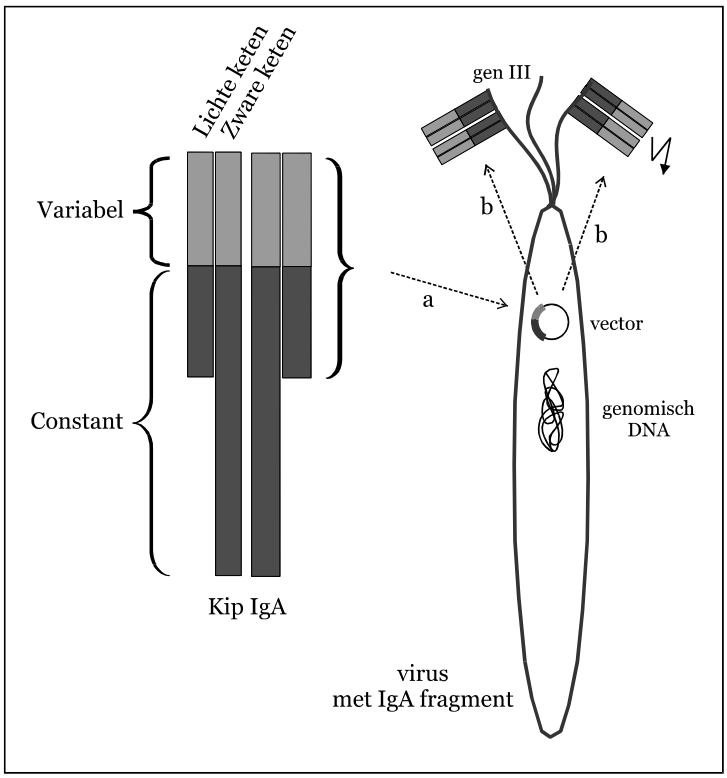
Hiernaast is de techniek om specifieke antilichamen te selecteren toegepast op kippenantilichamen van de subklasses (het isotype) die veel in de darm voorkomt, namelijk IgA. Deze antilichamen zijn bijzonder geschikt voor passieve immuuntherapie vanwege hun ontstekingsremmende eigenschappen, en worden daarom in deze studie toegepast. Daarnaast is een systeem ontwikkeld waarmee geselecteerde antilichamen eenvoudig en snel konden worden overgezet naar een systeem, waarmee ze in tabaksplanten geproduceerd konden worden. In een laatste stap werd het gehele secretoire antilichaam complex, welke in een gezonde kip optimale bescherming biedt tegen ziekteverwekkers, in planten tot expressie tot expressie gebracht.

Selectie van *Eimeria* specifieke antilichamen

In **hoofdstuk 2** staat de ontwikkeling van een systeem beschreven waarmee specifieke kippenantilichamen geselecteerd kunnen worden voor later gebruik in passieve immuuntherapie. In een eerste stap werden volwassen kippen geïnfecteerd met de *Eimeria* parasiet. Als afweer worden o.a. antilichamen aangemaakt, die aan de parasiet binden en deze zo neutraliseren. Antilichamen bestaan uit twee zware en twee lichte ketens, zoals schematisch weergegeven in figuur 1. De ketens hebben een constant gedeelte en een variabel gedeelte. De variabele gedeeltes van een zware en een lichte keten vormen samen een unieke herkenningsplaats voor b.v. binding aan een parasiet.

Elk dier of mens heeft miljoenen verschillende antilichamen ter beschikking en bij een infectie worden de cellen die meest effectieve antilichamen produceren geactiveerd. Uit deze antilichaam producerende cellen van geïnfecteerde kippen werd de genetische informatie verzameld van alle antilichamen die deze kippen als afweer aangemaakt hadden. Zo werd een zogenaamde antilichaambibliotheek verkregen. Om hieruit antilichamen te selecteren die specifiek binden aan de *Eimeria* parasiet, en die dus effectief kunnen zijn bij passieve immunisatie, werd gebruik gemaakt van de phage display techniek in een voor kippen aangepaste vorm.

Bij de phage display techniek wordt de genetische informatie voor antilichaamfragmenten uit de antilichaambibliotheek tot expressie gebracht op het oppervlak van virussen (of "phages"), gekoppeld aan het genIII-eiwit. Het gen coderend voor dit eiwit bevindt zich op een plasmide, ook wel een vector genoemd. Plasmiden zijn relatief kleine, circulaire DNA-moleculen die in bacteriën voorkomen naast het genomisch DNA. Plasmiden worden in de biotechnologie gebruikt om specifieke genen in bacteriën tot expressie te brengen. Door fuseren van de genetische informatie van een antilichaamfragment met het genIII-eiwit brengt elk virus, een uniek antilichaamfragment op zijn oppervlak tot expressie. Ter verduidelijking is een overzicht van de techniek gegeven in figuur 1.



Figuur 1
Schematisch overzicht van phage display techniek

Links een antilichaam van de kip (IgA), de donkergrijze delen zijn constant, de lichtgrijze zijn variabel en bepalen waaraan het antilichaam bindt (b.v. aan *Eimeria*). DNA coderend voor deze delen wordt overgezet in faag (a). Het virus leest de informatie en maakt op zijn oppervlak een deel van het antilichaan aan (b).
De pijl helemaal rechts geeft de bindings-plaats weer.
De relatieve verhoudingen van de tekeningen zijn voor de duidelijkheid aangepast.

De hier gebruikte virussen kunnen zichzelf vermenigvuldigen door infectie van *Escherichia coli* (*E.coli*) bacteriën. Een geïnfecteerde bacterie zal aangezet worden tot het kopiëren van het virus. De virussen met antilichaamfragmenten die specificiek zijn voor de *Eimeria*-parasiet kunnen eenvoudig uit de populatie gevist worden door ze te laten binden aan de parasiet. De bindende virussen, die genen met de code voor het antilichaam bevatten, werden gebruikt om opnieuw bacteriën te infecteren. Na een aantal van deze selectierondes werden de tien best bindende virussen geïsoleerd en gekarakteriseerd.

Productie van kippenantilichamen door planten

In een volgende stap werden tien aldus gevonden *Eimeria*-bindende antilichaamfragmenten als volledige antilichamen geproduceerd (**hoofdstuk 3**). Voor grootschalige productie van antilichamen werden planten gekozen vanwege de lage kosten van dit systeem en de veiligheid (geen contaminatie door menselijke pathogenen, wat b.v. wel het geval is bij productiesystemen met humane cellijnen). Bovendien kunnen planten gevoerd worden aan dieren waardoor injectie overbodig is en de antilichamen direct op de plek terecht komen waar ze bescherming moeten bieden. Om te testen of kippen-IgA geproduceerd

kan worden door planten, werd hier gewerkt met “tijdelijk transgene” tabaksplanten, omdat deze techniek snel en eenvoudig werkt. Bij succes kunnen in een latere fase b.v. maisplanten permanent transgeen gemaakt worden.

Bij de door ons gebruikte techniek worden bladeren van tabaksplanten geïnfiltreerd met *Agrobacterium tumefaciens* bacteriën. Deze bacteriën komen in de natuur voor en kunnen planten binnendringen op plaatsen waar het weefsel beschadigd is. Een deel van hun genen kunnen ze in het DNA van een plant laten integreren. Aldus zetten ze plantencellen aan tot ongeremde groei, hetgeen zichtbaar is als een kankergezwel bij bomen en struiken. Door modificatie van deze bacteriën zijn we in staat om elk willekeurig gen in de plant in te bouwen en de plant allerlei andere eiwitten te laten produceren, zoals b.v. IgA antilichamen. Dit gebeurt ook met behulp van plasmiden, die wij extra genen voor aanmaak van de antilichamen hebben meegegeven. De variabele gedeeltes van de met phage display gevonden antilichamen kunnen in deze plasmiden ingebouwd worden, zodat *Eimeria*-specifiek IgA aangemaakt wordt in de plant. Binnen een paar dagen zijn geproduceerde antilichamen te vinden in de planten.

Deze werkwijze maakt snelle en grootschalige analyse van verschillende eigenschappen van de geselecteerde antilichamen mogelijk. Zo werd ontdekt dat niet alle antilichamen met dezelfde efficiëntie door de plant gemaakt worden. De helft van de geselecteerde antilichamen werd niet of nauwelijks geproduceerd. Twee andere antilichamen daarentegen juist heel goed, de overige drie iets minder. Voor toepassing in passieve immuuntherapie is een grote opbrengst noodzakelijk. Om duidelijkheid te krijgen welke oorzaken aan deze verschillen in expressie ten grondslag liggen, is een aantal experimenten gedaan.
Hieruit bleek dat de totale IgA opbrengst sterk afhangt van de compatibiliteit van de lichte en de zware keten van het antilichaam. Deze kunnen elkaar namelijk stabiliseren, wat leidt tot een hogere opbrengst. Bovendien kunnen voorspellingen ten aanzien van de expressie worden gedaan op basis van karakteristieken van het DNA dat codeert voor het variabele gebied, omdat planten bepaald DNA minder goed kunnen vertalen. Dit was al bekend, maar in dit systeem werd duidelijk dat de voorspellende waarde niet voor alle antilichamen gold. Dit is de eerste beschrijving van een dergelijk geïntegreerd systeem waarbij ‘phage display’ en plant expressie gecombineerd worden.

Lijkt het secretoire IgA van de kip op dat van zoogdieren?

Voor zoogdieren is vrij goed beschreven hoe IgA in de darm terecht komt. In zoogdieren produceren antilichaam producerende witte bloedcellen (B-cellen) IgA in het weefsel rondom de darm, de luchtwegen, het urogenitaal stelsel (nieren en geslachtsorganen). In de B-cellen wordt ook de zogenaamde joining (J-)keten geproduceerd, een klein eiwit dat twee IgA moleculen verbindt tot dimeer IgA. Dit

dimeer IgA bindt aan een vierde soort eiwit, de polymere immuunglobuline receptor (pIgR) die zich aan de lichaamszijde van de epitheelcellen, die onder andere de darm afschermen van de buitenwereld, bevindt. Dit complex wordt door deze cellen opgenomen en aan de buitenzijde, b.v. in het lumen van de darm, uitgescheiden. Het overgrote deel van de receptor blijft daarbij aan het dimere IgA gebonden en heet dan secretoire component (SC). Alle onderdelen samen vormen zo het secretoire IgA. Een schematisch overzicht van dit transport is te vinden in de introductie (blz 6) en de verschillende vormen zijn schematisch weergegeven in hoofdstuk 6 (blz 109). Een groot voordeel van deze secretoire vorm is de stabiliteit van het complex, IgA wordt op deze manier beschermd tegen schommelingen in de zuurgraad en tegen eiwit afbrekende enzymen, die veel voorkomen in de darm voor de spijsvertering.

De moleculaire structuur van de polymere immunoglobuline receptor. pIgR, van de kip was tot nog toe niet opgehelderd. In hoofdstuk 4 is de karakterisering van het gen en structuur van het eiwit beschreven. Hoewel de functie van de kippenreceptor gelijk is aan die van het overeenkomstige eiwit uit zoogdieren, blijkt de structuur iets af te wijken van de zoogdiervorm. Het deel van de kippenreceptor dat bindt aan het IgA blijkt iets korter dan bij zoogdieren. Door de structeren van alle bekende pIgRs te vergelijken vonden we dat dit veroorzaakt werd doordat de kip pIgR opgebouwd is uit 4 ipv 5 domeinen, het tweede zoogdierdomain “mist” in de kip.

De ontdekking dat deze receptor niet alleen bij zoogdieren maar ook bij vogels voorkomt en functioneel is werpt een nieuw licht op de rol van deze receptor binnen het afweersysteem: blijkbaar zijn secretoire antilichamen evolutionair gezien ouder dan tot nu toe aangenomen. In hoofdstuk 5 is de expressie van de pIgR in de kip nader onderzocht. Met behulp van immunohistochemische kleuringen van darmcoupes werden de celtypes gevonden die de pIgR aanmaken in de darm. Daarnaast werd onderzocht in welke fase pIgR en IgA aangemaakt worden in jonge kipjes.

Aanmaak van het secretoire IgA complex in planten

Passieve immuuntherapie is er op gericht om het immuunsysteem te ondersteunen door het aanbieden van IgA dat specifiek bindt aan parasieten en deze zo uitschakelt. Omdat IgA in de kippendarm als secretoir complex aanwezig is, is het zinvol dit complexe eiwit ook bij passieve immuuntherapie te gebruiken. Voor vorming van het secretoire complex zijn vier componenten nodig: de lichte keten en de zware keten die samen een antilichaam vormen, de J-keten en de secretoire component. In **hoofdstuk 6** staat de co-expressie van deze onderdelen in de plant beschreven. De data wijzen erop, dat het gehele complex aangemaakt

wordt. Secretoire antilichamen zijn al eerder geproduceerd in planten (Ma et al., 1995; Frigerio et al., 2000; Hood et al., 2002). Echter, dit is de eerste keer dat het secretoire antilichaamcomplex van een vogelsoort geproduceerd is en dat daarvoor een systeem gebruikt is waarbij geen permanent genetisch gemodificeerde planten worden gebruikt.

EINDCONCLUSIE

Eigenlijk kan hier beter gesproken worden van een beginconclusie. De onderdelen die noodzakelijk zijn voor het realiseren van grootschalige passieve immuuntherapie bij kippen zijn nu beschikbaar. Er is een systeem ontwikkeld voor de snelle selectie van antilichamen die specifiek binden aan een ziekteverwekker en de aansluitende productie van het secretoire antilichaamcomplex in de plant. In een volgende fase zal de effectiviteit van de "plantibodies" getest gaan worden, eerst in een *in vitro* systeem en vervolgens *in vivo* met kippen die besmet worden met de *Eimeria* parasiet. Kortom, het echte werk kan beginnen!

Zaligmakend is de hier beschreven aanpak echter niet: passieve immuuntherapie zal nooit het gebruik van antibiotica en andere middelen volkomen vervangen. De combinatie van de hier beschreven aanpak met andere vaccinatie- en immuuninterventie-strategiën zal er echter wel toe bijdragen dat het gebruik van antibiotica en andere middelen in de intensieve dierhouderij in de toekomst sterk kan worden verminderd.

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Curriculum Vitae

Willemien werd op 31 augustus 1974 als Wilhelmina Hermina Wieland geboren in Arnhem. In 1993 behaalde zij het Abitur aan het Gymnasium in Kannenbäckerland in Höhr-Grenzhausen en begon ze met de studie Biologie aan de toenmalige Landbouwuniversiteit te Wageningen. Haar eerste afstudeervak heeft zij gedaan bij de vakgroep Toxicologie, waar zij een *in vivo* test met nematoden ontwikkelde om effecten van bodemverontreigingen op opeenvolgende generaties te testen. Hierop aansluitend deed zij een stage aan de Michigan State University (USA) waar gewerkt werd aan een risico evaluatie voor siliconen in het aquatisch milieu. Een tweede afstudeervak volgde bij de vakgroep Celbiologie en Immunologie, waar zij werkte aan de ontwikkeling van een permanente cellijn van karperlymphocyten. In september 1998 studeerde zij af met als specialisatie Cel. Aansluitend ging zij bij Elti Support in Nijmegen aan de ontwikkeling van biochemische testen voor vervuilingen (bv pesticiden) in oppervlaktewater werken. In juni 2000 keerde zij weer terug naar Wageningen, om aan het Laboratorium voor Moleculaire herkenning en Antilichaamtechnologie te werken aan het promotieonderzoek beschreven in dit proefschrift.
Momenteel is zij werkzaam als postdoc op het LMA om het effect van plantibodies op een *Eimeria* infectie in de kip te bepalen.

The training and supervision plan was completed at the graduate school Experimental Plant Sciences (EPS)

1. Participation in postgraduate courses:

Bioinformatics, EPS winterschool 2001
Ethics for Life Scientists, 2003

2. Oral and poster presentations at international conferences:

VIIIth International Coccidiosis Conference, Palm Cove, Australia (2001).
Poster: Recombinant plant monoclonal antibodies for preventive immunotherapy of poultry against coccidiosis.

15th European Immunology congress, Rhodes, Greece (2003).
Poster and oral presentation: Identification and characterization of the first non-mammalian polymeric immunoglobulin receptor: the chicken pIgR lacks the 2nd Ig-like domain.

International symposium on Plant-derived vaccines and antibodies: Potential and Limitations, Annecy, France (2004).
Poster: From phage display to plant expression: Producing chicken IgA plantibodies for oral immune therapy.

3. Participation in a diversity of EPS organized happenings:

Participation at annual EPS students day (2000-2003, with a poster presentation in 2003), annual EPS theme symposia (2000-2004) and EPS flying and other seminars (2000-2004).

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