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Goose-Derived Igy: A Potential Therapeutic Antibody For The Treatment Of Infectious Disease

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GOOSE-DERIVED IGY: A POTENTIAL THERAPEUTIC ANTIBODY FOR THE TREATMENT OF INFECTIOUS DISEASE

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This dissertation, submitted by Ashley Fink in partial fulfillment of the requirements for the Degree of Doctor of Philosophy from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

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Ashley L. Fink
November 25, 2014

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ABSTRACT

IgY, the predominate avian antibody isotype, has biochemical properties that make it attractive for a human immunotherapy: IgY does not bind to mammalian Fc receptor (FcR) or rheumatoid factor; IgY does not activate the mammalian complement; and IgY has no heteroagglutinins. Anseriformes (waterfowl, e.g. ducks and geese) produce 2 isoforms of IgY, full length IgY and alternatively spliced IgY, (IgYΔFc) lacking the Fc region and a stable equivalent to mammalian $F(ab')_2$ fragment, and the predominate isoform following hyperimmunization. We, and others, have demonstrated that egg-derived avian polyclonal antibodies are prophylactic or therapeutic for a variety of different infectious agents including bacteria, viruses, and parasites.

Several routes of administration have been utilized for IgY, although the most intriguing has been those that are administered orally. The targets for the successful oral administrations have been associated with the gastrointestinal tract, other organ systems, and systemic infections. However, bioavailability of orally administered IgY has not been determined. As part of the research presented here, we administered purified goose-derived IgY via oral gavage to mice and determined seroconversion. Oral IgY is bioavailable and can be detected in the serum by 24 hrs. Multiple dosing and buffering to pH 8.0 resulted in higher serum titers, with the buffered IgY preparations not detected until 48 hrs. IgY was detectable up to 7 days post oral administration. Goose-derived IgY

was relatively resistant to intestinal trypsin and chymotrypsin digestion but sensitive to gastric pepsin digestion, as demonstrated by others with IgY from other avian sources. This demonstration that orally administered IgY is bioavailable significantly increases the potential applications of IgY therapy. Furthermore the ability to administer IgY orally versus injection provides a novel and efficient means to treat disease worldwide.

In addition to determining the bioavailability of goose-derived IgY, we tested its therapeutic potential in two unrelated disease models. One organism of interest for the development of therapeutic IgY is dengue virus (DENV). At present, there are no anti-viral agents or vaccines approved to treat dengue-induced disease. Dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), both disease manifestations originating from dengue virus infection are severe and life threatening. These disease states are mediated by serotype cross-reactive antibodies that facilitate antibody dependent enhancement (ADE) by binding to viral antigens and then Fcγ receptors (FcγR) on additional myeloid cells. In this study we hypothesized that avian IgY, which does not interact with mammalian FcγR, would provide a novel therapy for DENV. Polyvalent anti-DENV2 IgY was purified from eggs of DENV2-immunized geese and tested for its ability to neutralize and enhance a DENV2 infection both *in vitro* and *in vivo*. Our data suggests that DENV2 IgY is able to effectively neutralize DENV2 in the absence of inducing ADE. DENV2-specific epitopes were determined in both the full length and alternatively spliced (IgYΔFc) goose IgY populations and were used to develop affinity purified DENV2 epitope specific antibodies.

The second organism used to test the therapeutic potential of IgY was *Plasmodium berghei*, the causative agent of murine cerebral malaria. Human cerebral

malaria is a severe manifestation resulting from the infection of *Plasmodium falciparum*. Although there are successful antimalarial drugs on the market, there are increased reports of isolated strains that are drug resistant, therefore highlighting the need for new drug molecules or therapies to be used in combination therapy. Here we generated goose-derived IgY that was specific for *Plasmodium berghei*, or the merozoite surface protein 1 (MSP-1) antigens, and determined their ability to treat cerebral malaria in a murine model. Mice survived significantly longer and had decreased parasitemia when both malaria specific IgY were administered together on days 2 and 4. These data suggest that malaria specific IgY is a potential therapeutic candidate to be used in combination therapy in order to prolong death and provide time for the combination drug to be effective.

CHAPTER I

INTRODUCTION

Passive immunization

Passive immunization refers to the transfer of antibodies from an immune donor to a non-immune individual. Immunity is often artificially transferred by the injection of antibodies or serum isolated from an immune human or animal. Passive immunity can also occur naturally from the mother to the fetus during the placental or colostral transfer of IgG or IgA. In contrast to active immunity, passive immunization has the ability to provide rapid and immediate protection (1, 2). Passive immunization was first reveled by Emil Adolf von Behring and his partner Kitasato Shibasaburo when they demonstrated that blood serum from an infected animal could be injected into another animal to provide immunity. Eventually the blood serum was named antitoxin and was used during the early 1900s as a curative agent (3). In the following years, serum transfer was used to treat many infectious diseases. Today, passive immunization of immunoglobulin isolated from hyperimmune donors is used to treat numerous infectious diseases both prophylactically and therapeutically (4, 5).

Most of the antibodies currently used in passive therapeutics are of human or murine origin (4). In order to obtain sufficient human antibody titers for the use in therapeutics, pre-screened volunteer donors are generally immunized with the

antigen, and their plasma is then collected. Alternatively, convalescent sera is collected from patients who have successfully recovered from an infectious disease (6). Although human-derived antibodies are very effective treatment molecules, there are several restrictions and limitations to obtaining them. For example, there are limitations on the types of vaccines used, the number of immunizations permitted per individual, the adjuvant used, and the amount of total plasma that can be collected (7). In addition, it is unknown whether or not individual serum samples are further contaminated with virus or other potential microbial species. Some of these limitations can be overcome by using other animals as antibody sources.

Following the development of mouse hybridoma technology there has been an increase in the number of therapeutic antibodies of murine origin. Although many of these antibodies are currently being used in the clinic, they may not be ideal candidates. Murine antibodies have a high potential to be immunogenic when administered to humans. Often times the murine antibody is seen as a foreign antigen by the human immune system and this triggers an immune response that can lead to the formation of human anti-murine antibodies (HAMAs). HAMAs remain in the blood for a period of time and can cause adverse reactions in patients, especially if the antibodies are re-activated during a secondary exposure. Unfortunately, the HAMA response can also lead to antibody inactivation and clearance by the host immune system before the treatment antibodies are able to provide protection. In order to overcome such challenges, researchers designed a technique to genetically modify murine antibodies to make them humanized. During this process the Fc portion of the murine antibody is replaced with the Fc portion of a human antibody. Often this technique is used with monoclonal antibodies

that are specific for a disease antigen. Humanized antibodies are currently being used to treat several diseases, and there are even greater amounts that are in clinical trials (8). Although humanized antibodies may offer sufficient protection, like the naturally occurring human antibodies, they are highly susceptible to unwanted interactions with conserved human proteins, and are laborious and costly to generate (9). In recent years there has been an increased concentration on the development of avian-derived therapeutic antibodies as an alternative to murine-derived passive immunotherapeutics, whereas several studies have demonstrated their effectiveness in the absence of unwanted host interactions.

Avian IgY and the Avian Immune System

Passive immunization with avian derived antibodies has emerged as an attractive alternative approach to treat human and animal diseases. The avian immune system differs from those of mammalian species specifically in the genes, molecules, cells and organs it possesses, and also in functional mechanisms that it has evolved (10). Most of the research that has been performed in order to better understand the avian immune system has been done using a chicken model. Although chickens (galliformes) are closely related to geese (anseriformes), it is important to understand that the chicken is just a model and not all published research directly applies to all avian immune systems.

The avian immune system is comprised of both primary and secondary lymphoid organs. The primary lymphoid organs include the thymus, bone marrow, and the Bursa of Fabricus (BF). The secondary lymphoid organs are the spleen, harderian gland, germinal centers, and other diffuse lymphoid tissues. The thymus is located in the neck along the jugular vein and is where T lymphocytes both develop and mature. The thymus also

includes a subpopulation of B lymphocytes. The bone marrow is the source of precursor stem cells for both T and B cells that eventually migrate to either the thymus or the BF where they are further differentiated. The BF is located on the dorsal surface of the cloaca and consists of thousands of bursal follicles. The BF is the sole site of B cell maturation and differentiation (10). In mature birds, the spleen is the major site of antigen processing and antibody production. The harderian gland is located behind the eyeball within the orbit and is the major secondary lymphoid organ within the head. This gland is comprised of a large number of plasma cells with the majority of these cells being B cells.

Like the mammalian immune response, the avian immune response is divided into an innate and adaptive response. The innate immune response has traditionally been considered a non-specific response, however there is increasing evidence that there are responses that are specific to different classes of pathogens (10). During the innate response the first lines of defense are the physical and chemical barriers; the skin, mucosal epithelium, and gastric secretions. The cellular components of the innate immune response that confer specificity include the natural killer cells, macrophages, and heterophils. Unlike mammals, the avian species do not have eosinophils, basophils, or neutrophils (10). Heterophils are the predominate granulated leukocyte that are present during the acute inflammatory response in galliformes (11). Heterophils are phagocytic cells that both uptake foreign antigens via phagocytosis and release granules. They are similar in function to neutrophils but have different granule components (11). Natural killer cells are found in the spleen, peripheral blood, thymus, BF and intestine and are cytotoxic cells that are important for completing antibody-dependent cellular cytotoxicity (ADCC). Macrophages are phagocytic cells that serve as antigen presenting cells and

bridge the innate and adaptive immune responses (10). Dendritic cells (DCs) are also a population of very efficient antigen presenting cells that are found both in tissues that are in contact with the external environment and in the bone marrow (12).

There are several cells in the avian innate immune response that have receptors that will bind to foreign antigens and initiate the production of cytokines and chemokines. These receptors are called pattern recognition receptors (PRRs) and they recognize certain conserved molecules called pathogen associated molecular patterns (PAMPs). Some of the most studied PRRs in the avian immune system are the toll like receptors (TLRs) that recognize both cell surface and endocytic molecules. When TLRs recognize PAMPs they initiate signaling pathways that can lead to the activation of NF-κB, type I interferons (IFN) and the production of pro-inflammatory cytokines and chemokines (10).

The adaptive immune response in avian species involves the targeted recognition of specific molecular features on the surface of a pathogen, resulting in a series of events intended to eliminate the pathogen and prevent future infection (13). Although protection conferred by the innate response may be sufficient, the adaptive immune response is generally required to clear pathogens and generate immunological memory (10). Protection conferred during the adaptive immune response is mediated preferentially by either a cell-mediated response or a humoral response, although non-exclusive (13). The of presence of certain cytokines and chemokines, and sub sequential $CD4^+$ T cell subset (Th1/Th2) at the sight of inflammation is what determines the activation of the cell-mediated or the humoral response, respectively. During an adaptive immune response antigen presenting cells (APCs) present antigen within major histocompatibility (MHC)

molecules to both T and B cells which recognize the peptide antigen. As a result of cell mediated immunity, either $CD4^+$ or $CD8^+$ T cells are stimulated to proliferate and become activated (10). $CD8^+$ T cells, also known as cytotoxic T cells are involved in the direct lysis of pathogen infected cells and tumor cells (13). $CD4^+$ T cells have both effector and regulatory functions; specifically they activate macrophages by secretion of cytokines and stimulate B cell growth and differentiation.

The production of antibodies specific for foreign antigens is a result of the activation of the humoral immune response. Antibodies are secreted by plasma B cells and are found in both body fluids and tissue spaces. Present in the avian immune system are three antibody isotypes, IgM, IgY, and IgA (14). Avian IgM, IgY, and IgA have been identified as genetic homologs of mammalian IgM, IgG, and IgA (15, 16). IgM is primarily found in the serum and egg white and is present on the surface of B cells (17). IgM is generally the first antibody isotype secreted following an infection and it functions to bind and activate the avian complement system. IgA is secreted continuously and is found primarily in serum, bile, saliva, and at mucosal surfaces, but also found in the egg white (17). IgY is the primary serum antibody but is also transferred from the serum to the egg yolk during embryonic development. IgY is a homolog of mammalian IgG and to some extent functions like both IgG and IgE, and has some characteristics of IgA. In anseriformes there are two isoforms of IgY present, the full length IgY, and a smaller IgY isoform formed by alternative splicing (IgYΔFc) that lacks the Fc antibody portion. During egg formation, both IgY isoforms are passed from the blood to the egg yolk via receptors that are specific for IgY translocation (18, 19). The amount of IgY that is

transferred from the blood to the yolk is directly proportional to the serum concentration (18).

The generation of antigenic diversity is remarkably different in the avian immune system as compared to the mammalian immune system. In the avian system genetic rearrangement contributes little diversity because both the heavy (H) and light (L) chain loci consist of only one functional variable (V) and one functional junctional (J) gene. Avians use three mechanisms to generate diversity in the antibody repertoire: somatic gene hyper conversion, V-J flexible joining, and somatic point mutations (20-23). B cell formation takes place in the BF during embryonic development and for a few weeks after hatching. The BF is colonized in the embryo between days 8-14 of incubation by immature progenitor cells that give rise to differentiated progenitors. The gene rearrangement leading up to this takes place at both the H and L chain loci during the early colonization process (24). All B cells use the same V_L and J_L segments (20) so little diversity is gained during the VJ_L rearrangement process. Heavy chain rearrangement also generates little diversity because there is only one V_H and one J_H and approximately 15 diversity (D) segments used (21). Subsequent V region diversity is generated by somatic gene conversion events occurring on days 15-17 of incubation in which sequences within the rearranged V region genes are replaced with sequences derived from upstream pseudo-V region gene families (20, 21). These pseudogenes are located upstream of both the VL and the VJ regions and are defined as pseudogenes because of their lack in promoter sequences necessary for transcription, a truncated 5' or 3' coding region, or lack in recombination signal sequences (20). Once the DNA is transferred from pseudo V genes to the recombined VJ_L or VDJ_H regions of the immunoglobulin genes,

the result is the production of mature B cells that migrate out of the BF and are competent to form a functional humoral immune response (9). Approximately 5% of the B cells in a newly hatched bird appear to be derived from rapidly dividing extrabursal precursors and are potentially the source of peripheral B cells in older birds after bursal involution. In mature birds B cells are often located in germinal centers within the spleen or other mucosa associated lymphoid tissues. The activation of avian B cells to antibody secreting B cells occurs via a similar mechanism to that in mammals. The avian B cell receptor of germinal center B cells is modified by somatic hypermutation and gene conversion to generate antibody diversity. Immunoglobulin class switching is also occurring simultaneously in the avian germinal centers. In the terminal differentiation step following antigen encounter, B cells first become plasmablasts and subsequently plasma B cells. These cells are able to secrete antibody (IgM, IgA, and IgY) that is specific for the antigen encountered (25).

Structure and Function of IgY

IgY is the primary immunoglobulin isotype in oviparous animals and is the functional equivalent to mammalian IgG but also has the ability to sensitize tissues to anaphylactic reactions (26). IgY is a low molecular weight serum antibody that contains two heavy (H) and two light (L) chains and has a molecular mass of 180kDa, which is larger than mammalian IgG (159kDa) (27, 28). The H chain of IgY possesses a variable domain (V_H), four constant domains (Cυ1-Cυ4) and lacks a hinge region. In contrast, the H chain of IgG consists of a V_H and three constant domains (Cγ1-Cγ3), where Cγ1 is separated from Cγ2 by a hinge region (27). Also present in anseriform birds (waterfowl e.g. ducks and geese) is a truncated (120kDa) form of IgY lacking Cυ3 and Cυ4. The

truncated form of IgY coexists with the full length IgY and is the structural equivalent of a $F(ab')_2$ fragment (28). Both full length IgY and IgYΔFc are generated by the same gene but different pathways of mRNA processing (29, 30). It is important to distinguish the difference in function between full length IgY and IgYΔFc. During the activation of the immune response in avian species IgYΔFc is found later than full length IgY and is unable to fix complement and sensitize tissues to anaphylaxis (31).

Physiochemical properties of IgY and IgYΔFc

IgY and IgYΔFc differ from mammalian IgG in their β-sheet content, hydrophobicity, and isoelectric points. IgY has decreased β-sheet content specifically in its constant domain (32) and thus a potentially more disordered conformation than IgG. Full length IgY also has a larger Fc fragment than IgG and is therefore a more hydrophobic molecule (33). When comparing the isoelectric point of IgG to IgY, IgY is between 5.7-7.6, which is much lower than that of IgG (34).

IgY is a relatively stable molecule that is functional at a wide pH range. IgY is functional between pH 4.0 -11.0 but changes if above or below (35, 36). IgY activity is decreased in a pH 3.5 solution and is completely lost at pH 3.0 (32). IgY is also fairly heat stable with minimal loss in activity at temperatures of 60-65°C and significant loss in activity when heated to 70°C for at least 15 minutes (32, 35, 37). One way researchers have overcome the temperature and pH limitations of IgY is to add additional sugars to the antibody solution (38).

The stability of chicken IgY has also been tested in the presence of several digestive enzymes, specifically pepsin, trypsin and chymotrypsin. The stability of IgY in the presence of pepsin is highly pH dependent whereas activity remains high at a pH of 4

but decreases significantly at pH 3.5 (39) IgY has moderate resistance to chymotrypsin and trypsin and has very little loss in activity after an 8 hour incubation period in the respective enzymes (39).

Advantages of IgY for Passive Immunotherapy

The use of IgY in therapeutics has many advantages over the traditional use of IgG. One important advantage is the genetic background and phylogenetic distance that distinguishes birds from mammals. IgY that is isolated from birds has a higher avidity for mammalian antigens and has the ability to recognize different epitopes that may be non-immunogenic in mammals (9, 27). There are data that suggest that when mammals and chickens are immunized with the same antigen, the antibody repertoire that is present post vaccination is different between the two animals (40, 41). In one study, chickens and rabbits were both immunized with human papilloma virus type 16E7, and antibodies were collected and used for epitope mapping with eight peptides. Antibodies isolated from the chicken recognized all eight peptides, whereas rabbits only recognized two (40). Furthermore it has been demonstrated that avian IgY that is specific for insulin receptors in rats was able to inhibit insulin binding, but mammalian (rabbit) antibodies with the same specificity could not (41). Another advantage that arises from the phylogenetic distance of these two antibody reservoirs is the ability of IgY to be used as an antibody against conserved mammalian proteins; proteins so highly conserved that it would otherwise be impossible to use a mammalian antibody for treatment (42).

The ability of IgY to be easily isolated from the egg yolk poses another key advantage to using IgY as an alternative to mammalian IgG. Eggs can be collected from laying hens and the egg yolks used as a source of IgY. This is a rather rapid process that

enables researchers to avoid serum collection while still maintaining high antibody yields. The concentration of IgY present in the egg yolk of immunized birds depends on the species, breed, age of the bird, and the antigen injected. Concentrations range from 60-150mg per chicken egg and 100-500mg per goose egg (43). An average laying hen will produce upwards of 1000 to 2800mg of antibody per month, while geese will produce even greater amounts. These concentrations are superior to what can be isolated from the serum of rabbits or other animal sources (44). It has also been demonstrated that older laying hens (2 years) will lay fewer eggs but will produce more IgY per egg yolk (43, 45). It is important to note that the IgY isolated from these chicken eggs remained stable and was functional over this extended period of time (43). The isolation technique used for isolating IgY from egg yolks is also a fairly straightforward process that has been exploited by many research groups. Briefly, the egg white is separated from the egg yolk, and IgY is extracted using a series of centrifugation and precipitation steps and finally purified using chromatography techniques. At present there are a number a industrial processes set up for the collection and separation of eggs, making large scale production of IgY a feasible option (27).

IgY has a number of different Fc mediated effector functions that suggest the use of these antibodies would be better tolerated than mammalian antibodies. The Fc portion of IgY functions different from IgG and therefore could prevent unwanted interactions including the activation of complement, binding to rheumatoid factor (RF), HAMA, and the binding to human and bacterial Fc receptors.

The purpose of the complement system is to initiate the recruitment of inflammatory cells, which eventually leads to the clearance of the pathogen from the host. When

mammalian antibodies are used for treatment, the Fc portion of the treatment antibody has the ability to bind to self-antigens and activate complement. When complement is activated under these conditions it can lead to unwanted inflammatory reactions due to the production of anaphylatoxins C4a and C5a and antibody dependent cell-mediated lysis. When anaphylatoxins are produced they have the ability to stimulate the release of cytokines that can lead to the induction of shock in the patient (46, 47). The activation of complement can also promote illness similar to serum sickness; immune complexes bind to complement leading to the activation of leukocytes and even tissue damage. Although IgY can activate complement in the avian system, its distinct structure prevents it from being able to activate human complement (48). The Fc region of an antibody is composed of different carbohydrates and the composition of this region is what determines its ability to activate complement. It has been suggested that IgY is lacking the necessary Fc carbohydrates that would allow it to promote activation (49). Therefore, the advantage of using IgY would be its potential to prevent the aforementioned possible complications.

RF is an autoantibody that is generally associated with patients diagnosed with rheumatoid arthritis, but not exclusively (50). RF will interact with the Fc portion of IgG but will not interact with IgY. This is important because when RF antibodies bind to the treatment antibodies, the treatment will likely be less effective or require a higher dosage to be protective.

There is also evidence that suggests that IgY does not bind to the bacterial proteins *Staphylococcal* protein A or *Streptococcal* protein G. Both of these proteins are used during IgG affinity purification processes and other immunological bioassays. Briefly, the bacterial protein is immobilized within a column matrix, the crude sample is

passed through the column, and during this time the bacterial proteins will bind to the Fc portion of the antibody in the sample. This becomes problematic if the assay is used to purify IgG from a source containing treatment antibodies. There have been several studies done to determine the binding capacity of the IgY Fc portion to these bacterial antigens. Initially it was reported that chicken-derived IgY does not bind to *Staphylococcal* protein A or *Streptococcal* protein G (51-54). However, recent reports suggest that the reactivity of IgY may depend on the source. Specifically, Justi-Valliant *et al*. demonstrated that when tested by direct ELISA, duck-derived IgY, but not chicken-derived IgY, reacted with *Staphylococcal* protein A. It was also determined in this study that neither duck-derived nor chicken-derived IgY interacted with *Streptococcal* protein G. When the reactivity of IgY was compared to IgG, IgY proved to have much lower reactivity to these bacterial proteins regardless of the source (55). Although these data suggest that IgY may have the potential to bind to bacterial proteins, this binding is not likely to be Fc mediated due to the structural differences between IgG, IgY, and IgYΔFc.

Oral Passive IgY Immunotherapy

Polyclonal avian IgY is currently being studied for its passive immunization applications both in human and veterinary medicine. IgY has been effective against several human pathogens and diseases both *in vitro* and in animal and clinical settings. The therapeutic efficacy of IgY has been specifically demonstrated for a variety of different infectious agents including bacteria, viruses, and parasites. Some of the most prominent research studies suggesting the use of IgY for the treatment of disease will be discussed below.

Pseudomonas aeruginosa

One of the most successful clinical applications of IgY has been in the prevention of *Pseudomonas aeruginosa* (*P. aeruginosa*) infections in cystic fibrosis patients. Cystic fibrosis patients often present with repeated lung infections caused by *P. aeruginosa*, which causes rapid deterioration in lung function and is the major cause of mortality in these patients. In an attempt to clear the infection, these patients undergo antibiotic treatment. If initial antibiotics fail to eradicate infection, the patient often becomes chronically infected and will be subject to continual treatment with antibiotics. Antibiotic treatment for prolong periods of time has detrimental effects to the host; specifically it can lead to bacterial resistance, secondary infections, antibiotic toxicity, and the reduction of normal flora (56-59).

There are ongoing clinical trials in cystic fibrosis patients using a mouth rinse containing purified anti-*P. aeruginosa* IgY. Patients were asked to gargle the IgY mouth rinse for two minutes and then swallow the rinse, after their last meal and brushed teeth. Patients who were treated with the mouth rinse had an increased amount of time between subsequent *P. aeruginosa* infections compared to the control group. Further study indicated that those patients treated long term with the mouth rinse had fewer positive *P. aeruginosa* cultures than the control group, and none of the patients in the experimental group became chronically infected (60). Results of these experiments were similar in another almost identical study researching the long term effects of an anti-*Pseudomonas* IgY mouth rinse (61).

Nilsson *et al*. elucidated the mechanism by which IgY causes decreased colonization by *P. aeruginosa.* These researchers determined that anti-*P. aeruginosa*

binds to flagellin present on the surface of the bacteria. This binding may prevent the bacteria from adhering to host proteins and reduce bacterial motility and thus decrease or prevent *P. aeruginosa* infection (62).

Rotavirus

Rotavirus is viral pathogen that causes severe acute gastroenteritis specifically in infants and children and the young of other mammalian and avian species, including calves and piglets. At present, there are two orally administered vaccines that are approved for use against human rotavirus and they are widely distributed and effective throughout the United States. However, due to the lack of established health care in developing countries, it has been difficult to institute an effective vaccination protocol and therefore rotavirus induced diarrhea remains the cause of death for millions of children (63). The need for a treatment alternative has fueled several researchers to assess the potential of rotavirus specific IgY to be used as an oral passive immunization therapy.

Human trials have been done to test anti-rotavirus IgY in children suffering from virally induced diarrhea. Specifically, children that had confirmed cases of virally induced diarrhea were treated orally with anti-rotavirus IgY or naïve IgY and stool output was determined. By day 4 post treatment, 74% of children treated with anti-rotavirus IgY no longer had diarrhea whereas less than 50% of the control group no longer had diarrhea (64). Another study was done using gnotobiotic piglets that were experimentally infected with human rotavirus at 24 hours of age and treated twice a day for 9 days (days 3-12) with cow milk supplemented with rotavirus specific IgY. Treatment with rotavirus specific IgY dose dependently protected the piglets from diarrhea and significantly reduced virus shedding (65).

Bovine rotavirus is a common cause of diarrhea in neonatal calves typically through 8 weeks of age (66). Bovine rotavirus in calves becomes a major issue for the cattle industry because of financial loss due to a reduction in body weight and the expense of treating the disease. The current treatment strategy is to vaccinate the mothers and rely on passive antibody transfer from the mother to the newborn calf. Vaccination has been somewhat successful, however it does not prevent virus infection. Passive immunization with bovine rotavirus specific IgY has proven to be an attractive alternative treatment (65, 66)

Vega *et al.* experimentally inoculated 2-day-old calves with bovine rotavirus and then treated them with milk containing 6% bovine rotavirus immune egg yolk, or non-immune control egg yolk twice a day for 14 days. In this study, 80% of the infected calves that were fed the bovine rotavirus immune egg yolk were protected and the antibodies could be detected in the feces at 21 days post infection (66).

Helicobacter pylori

Helicobacter pylori (*H. pylori*) is the causative agent of gastritis and gastric ulcers. *H. pylori* produces several virulence factors but one of the most important for bacterial colonization is the urease enzyme. Urease is able to hydrolyze urea into carbon dioxide and ammonia to permit the bacteria to survive in the gastrointestinal tract (67). Gastritis and ulcers are caused by the *H. pylori* induced disruption of the gut epithelium. This infection is generally treated with antibiotics although treatment is not always successful. Purified anti-*H. pylori* IgY has been shown to decrease bacterial adhesion, growth, and urease activity *in vitro* and decrease *H. pylori* induced gastric mucosal injury and inflammation *in vivo* (68). Patients suffering from *H. pylori* infections were also

given a yogurt that contained 1% anti-urease IgY as well as *Lactobacillus acidophilus* and *Bifidobacterium* species. The patients who drank the yogurt had suppressed *H. pylori* infection compared to the control group (69). *In vivo* studies using mice have also been exploited to test the efficacy of *H. pylori* antigen specific IgY treatment. In this study mice were infected with *H. pylori* and the anti-Hp58 IgY was administered on day 1, week 1, week 4, or week 12-post infection. When administered one week post infection there was a significant difference in the degree of gastritis and a higher recovery rate compared to the control group (70).

Streptococcus mutans

Streptococcus mutans (*S. mutans*) is one of many bacteria present in the mouth that can cause infections, specifically dental carries. Most treatments are focused on eliminating the bacterium or suppressing virulence factors. There have been several studies using various forms of *S. mutans* IgY that provide evidence that IgY can be used to treat these infections. Hatta *et al*. evaluated the efficacy of oral anti- *S. mutans* IgY mouth rinse to prevent bacterial colonization. Those patients who gargled the mouth rinse had a decreased level of *S. mutans* as well as a higher protection from dental carries (71). In a randomized, double blind, placebo-controlled study, Nguyen *et al*. also reported that anti-cell associated glucosyltransferase IgY could significantly suppress oral colonization by salivary *S. mutans* (72). Cell-associated glucosyltransferase is essential for the production of glucans by *S. mutans* and is an important virulence factor.

Salmonella

Salmonella enterica serovar Enteritidis (*Salmonella* ser. Enteritidis) and *Salmonella enterica* serovar Typhimurium (*Salmonella* ser. Typhimurium) cause major

outbreaks in humans but also in other animal species such as chickens. Salmonella species have several virulence factors that are targets for the creation of passive immunotherapies. One of the virulence factors targeted is the fimbria surface antigen of *Salmonella* ser. Enteriditis. Fimbria is implicated in the bacterial adherence to the mucosal epithelium of the host. Peralata *et al*. demonstrated protection with IgY specific for the *Salmonella* ser. Enteriditis fimbria (SEF) 14. Briefly, SEF-14 specific IgY was isolated from the egg yolk of immunized laying hens and given to mice that were infected with *Samonella* ser. Enteriditis. Mice that were treated with the IgY had increased survival rate compared to the control mice treated with naïve IgY. When given the highest antibody titer the survival rate was 77%, the lowest titer survival rate was 59.3% and the naïve IgY survival rate was only 32% (73). The SEF-14 IgY was also tested *in vitro* using isolated murine small intestinal cells. In this experiment the SEF-14 IgY decreased the adherence of *Salmonella* ser. Enteritidis to the small intestinal cells when compared to the naïve IgY. These researchers suggest that although the mechanism of protection is unknown, it is likely that the SEF-14 IgY is preventing initial attachment of the bacteria to the cell surfaces (73).

Others have also done research with *Salmonella* specific IgY targeting the outer membrane proteins (OMP) of the bacterium (74, 75). Yokoyama isolated IgY from chickens that were immunized with OMP, lipopolysaccharide (LPS), and flagella from either *Salmonella* ser. Enteritidis or *Salmonella* ser. Typhimurium. These three proteins are associated with bacterial virulence. To test the efficacy of the antigen specific IgY, mice were infected with either bacterial strain and then orally administered OMP, flagella, or LPS specific IgY antibodies. Antibodies were administered three times a day

for three days total. The mice that were infected with *Salmonella* ser. Enteritidis had a 80% survival rate with OMP IgY, 60% survival rate with the flagella IgY, 47% survival rate with the LPS IgY, and 20% survival rate with the control naïve IgY (75). The mice that were infected with *Salmonella* ser. Typhimurium had 40% survival rate with OMP IgY, 30% survival rate with LPS IgY, 20% survival with the flagella IgY, and all control naïve mice died (75). These research studies demonstrate that IgY specific for *Salmonella* or *Salmonella* virulence factors has the potential to be used for the passive treatment of human salmonellosis.

Escherichia coli

Enterotoxigenic *Escherichia coli* (ETEC) is an enteric bacterium that causes severe diarrhea in both animals and humans. ETEC is one of the most frequent causes of childhood diarrhea prevalent in developing countries. ETEC is also the cause of diarrhea in newborn calves and in piglets. ETEC uses adhesion molecules to adhere to the small intestine and secrete enterotoxins. These enterotoxins cause the increased secretion and reduced absorption in the small intestine (76). ETEC is often treated through the use of antimicrobial agents and thus finding alternative treatment options is necessary.

ETEC specific IgY has been used in research studies to determine its efficacy in treating animals infected with the bacterium. Porcine ETEC fimbrial antigens K88, K99, and 987P are associated with bacterial adhesion and therefore are good targets for the production of IgY. IgY that was produced against these antigens showed to decrease ETEC binding to porcine epithelial cells and intestinal mucus *in vitro*. When piglets were treated with the antigen specific IgY, there were no adherent ETEC along the intestinal epithelial surface (77).

Other researchers have used freeze-dried K88 fimbiral antigen specific IgY to treat piglets or neonatal pigs that were experimentally challenged with ETEC. These antibodies were made into a powder and administered orally post infection at different time points. K88 IgY was able to protect both the neonatal and 21 day weaned piglets from ETEC induced diarrhea in just 24 hours post treatment. Piglets that were given naïve IgY powder continued to have diarrhea and only 37.5% of them survived (78).

Ikemori *et al.* has exploited the K99 pilus virulence factor to create K99 specific IgY powder that can be administered in colostrum to newborn calves in order to protect them from ETEC induced diarrhea. In this study the calves were challenged with ETEC and then treated with either the K99 specific IgY or naïve IgY powder in milk. The calves that received a high titer IgY treatment had only temporary diarrhea and did not experience weight loss or dehydrateion, whereas the control group developed severe diarrhea and died 3 days post challenge (79).

Oral therapeutic IgY summary

The aforementioned research studies represent a significant amount of evidence for the efficacy of orally administered IgY to treat various diseases both contained within the gastrointestinal (GI) tract and potentially within other organ systems. Despite this, there are no published findings demonstrating the oral bioavailability of these antigen specific IgY antibodies.

Other therapeutic IgY

Venom IgY

In many parts of the world snakebites and bites from other venomous animals are medical emergencies. According to the World Health Organization (WHO) report in 2007 on snakebites, there are about 5 million snakebite incidences resulting in 2.5 million envenoming, and 125,000 deaths that occur annually (80). Envenomation is usually performed using anti-venoms that are derived from horse sera. A deleterious outcome of using horse sera anti-venoms is that they often contain large amounts of non-specific horse proteins that can cause side effects such as serum sickness and anaphylactic shock when used for treatment in patients (81-83). Several studies have examined the potential of anti-venom IgY for envenomation in order to avoid the unwanted reactions between the currently available anti-venoms and the human immune system.

One of the first demonstrations of IgY anti-venom was the purification of IgY from the egg yolks of hens that were vaccinated with either Cortalid snake venom or *Leiurus quinquestiratus hebraeus* scorpion venom. In this study the neutralizing potential of the IgY was tested by mixing the anti-venom IgY with a lethal dose of each venom and then injected into mice. All ten mice administered the anti-Cortalid IgY and 7 of the 8 mice administered the *Leiurus quinquestiratus hebraeus* IgY were alive at 24 hours post infection. All but one of the control mice died by 24 hours post infection (84). Several other studies have been done that also suggest the neutralizing potential of anti-venom IgY is very high (85-87).

West Nile Virus IgY

Past research in our lab has exploited geese as a source of IgY. Initial studies aimed to determine if the serum from West Nile Virus (WNV) immune geese could protect against WNV infection in geese. Geese were given immune serum prior to and post WNV infection and mortality was assessed. Of the ~10,000 geese per group there was a 65% reduction in mortality when serum was administered as a prophylactic and 62% reduction in mortality when administered as a treatment. These experiments were repeated with purified anti-WNV IgY and similar results were observed. Purified anti-WNV was also tested in WNV infected golden hamsters. All of the hamsters treated with anti-WNV IgY survived and had viral titers of zero, compared to the sham treated hamsters that had 100% mortality and 65% viral titer (Bradley et al., unpublished data).

Summary

As evidenced by the significant body of related research, passive immunization with avian antibodies is an unconventional and appealing strategy for the treatment of infectious diseases. The following chapters will describe the current research in our lab focused on the bioavailability of goose-derived IgY, the ability of *Plasmodium* specific IgY to treat murine cerebral malaria, and the epitope mapping and viral neutralization capacity of anti-dengue virus IgY.

CHAPTER II

BIOAVAILABILITY OF ORAL IGY

Abstract

Avian IgY exhibits biochemical properties that make it an attractive human immunotherapeutic. Unlike mammalian IgG, IgY does not bind to mammalian Fc receptor (FcR) or rheumatoid factor, does not activate mammalian complement, and has no heteroagglutinins. The prophylactic and therapeutic efficacy of IgY has been demonstrated for a variety of different infectious agents including bacteria, viruses, and parasites. The targets for the successful oral administrations have been associated with the gastrointestinal tract, other organ systems, and systemic infections. However, bioavailability of orally administered goose-derived IgY has not been determined. In this study we administered purified goose-derived IgY via oral gavage to mice and determined seroconversion. Oral IgY is bioavailable and can be detected in the serum by 24 h. Multiple dosing or increasing the pH resulted in higher serum titers. IgY was detectable up to 7 days post oral administration. Goose-derived IgY was relatively resistant to intestinal trypsin and chymotrypsin digestion but sensitive to gastric pepsin digestion, as previously demonstrated with IgY from other avian sources. This demonstration that orally administered IgY is bioavailable significantly increases the potential applications of IgY therapy. Furthermore the ability to administer IgY orally provides a novel and efficient means to treat disease worldwide.

Introduction

Immunoglobulins are well-established affinity molecules that have applications ranging from biochemical analysis, diagnostics, and therapeutics. Mammalian immunoglobulins are the most commonly used and have proven to be adequate for most approaches. They are however highly susceptible to unwanted interactions with conserved proteins and can promote unnecessary immune mediated pathologies. Mammalian antibodies have the ability to activate the human complement system and thus have the potential to reduce the binding capacity of the antibody to the target antigen (88). Furthermore, when complement is activated the anaphylatoxins that are released, C4a and C5a, have the ability to initiate unnecessary inflammatory reactions such as stimulating the release of TNF-α. Another example is the binding of mammalian IgG to the auto-antibody rheumatoid factor (RF). RF is found primarily in patients with rheumatoid arthritis but is also found in a small percentage of healthy blood donors (50). When IgG binds non-specifically to RF, the total IgG available to interact with the disease antigen is decreased. These characteristics among others hamper the use of mammalian or humanized immunoglobulins for use as therapeutics.

The development of unconventional methods to generate antibodies that are suitable for use as therapeutics is an area of active research. Immunoglobulin Y (IgY) is the major immunoglobulin in oviparous animals. Many distinct properties of this antibody type allow it to overcome several of the limitations of the current mammalian derived therapeutic antibodies (9). During egg formation, IgY is passed from the blood to the egg yolk through receptors that are specific for IgY translocation (18, 19). IgY is the equivalent of mammalian immunoglobulin G (IgG), although there are differences in

structure and function (6). IgY is a low molecular weight serum antibody that contains two heavy (H) and two light (L) chains and has a molecular mass of 180 kDa, which is larger than IgG (159 kDa) (27, 28). The H chain of IgY possesses a variable domain (V_H), four constant domains (Cυ1-Cυ4) and lacks a hinge region. In contrast, the H chain of IgG consists of a V_H and three constant domains (Cγ1-Cγ3), where Cγ1 is separated from Cγ2 by a hinge region (27). Also present in anseriform birds (ducks and geese) is an alternatively spliced (120 kDa) form of IgY lacking Cυ3 and Cυ4, IgYΔFc. IgYΔFc coexists with the full length IgY, is the structural equivalent of a $F(ab')_2$ fragment, and is the predominant isoform produced during hyperimmunization (28). The use of IgY in therapeutics has many advantages over the traditional use of IgG. One important advantage is the genetic background and phylogenetic distance that distinguishes birds from mammals. This allows IgY to target those antigens or epitopes that may be non-immunogenic in mammals (9, 27). Avian antibodies will also recognize different epitopes than mammalian antibodies, providing an antibody repertoire that is distinct from mammalian antibodies (27, 89).

Most of the biological effector functions of immunoglobulins are associated with the Fc region. The Fc mediated secondary functions of IgY, compared to those of IgG, make IgY antibodies highly suitable for use as therapeutics (28). IgY lacks many of the interactions with mammalian immune components that promote unwanted cross-reactivities (9). IgY does not activate mammalian complement, and similarly does not bind to human and bacterial Fc receptors on cell surfaces (27, 90). IgY does not interact with mammalian IgG, nor does it bind to rheumatoid factors (27, 91, 92) or bacterial antigens staphylococcus protein A or Streptococcus protein G (53, 89).

Polyclonal avian IgY is currently being studied for its passive immunization applications in both human and veterinary medicine. Unlike most of the mammalian derived immunoglobulin therapeutics that require routine injections, there is increasing evidence that the oral administration of IgY is an effective approach for passive protection of both humans and animals. One of the most successful clinical applications of IgY has been in the prevention of *Pseudomonas aeruginosa* (PA) infections in cystic fibrosis (CF) patients. There are ongoing clinical trials in CF patients using a mouth rinse that contains purified anti-PA IgY and when administered on a continuous basis has shown to reduce and even prevent PA colonization, lessening the need for antibiotic treatment (60-62, 93). Researchers determined that anti-PA IgY remained active in the saliva for 8 hours and could be used for immunotherapy over a long period of time in the absence of negative side effects (62, 94). The clinical application of IgY in humans is also being studied in the prevention of *Helicobacter pylori* infections, the causative agent of gastritis and gastric ulcers. Anti-*H. pylori* IgY has been shown to decrease bacterial adhesion, growth, and urease activity *in vitro* and decrease *H. pylori* induced gastric mucosal injury and inflammation *in vivo* (68). Patients suffering from *H. pylori* infections were given a yogurt that contained 1% anti-urease IgY as well as *Lactobacillus acidophilus* and *Bifidobacterium* species resulting in suppression of *H. pylori* infection (69). Furthermore, studies using rotavirus immune bovine colostrum passively administered during an outbreak in children both significantly reduced the risk for rotavirus induced gastroenteritis associated diarrhea and the number of days children experienced diarrhea (95, 96). Similarly, Vega *et al*. showed that anti-human rotavirus

IgY passively administered to neonatal piglets as a cow milk supplement protected piglets against human rotavirus (65).

In veterinary medicine, IgY produced against porcine enterotoxigenic *Escherichia coli* (ETEC) fimbrial antigens decreased *E. coli* binding to porcine epithelial cells and intestinal mucus *in vitro* (77, 97). These antibodies were given to piglets orally and protected against *E. coli* infection in a dose-dependent manner (77). Similarly, IgY specific for *Salmonella enterica* Serovar Enteritidis and *Salmonella enterica* Serovar Typhimurium reduced *Salmonella* adhesion to epithelial cells *in vitro* and when chickens were fed egg powder that contained the specific IgY there was a decrease in fecal shedding, cecal colonization and the rate of *Salmonella*-contaminated eggs (74, 98, 99). Another successful application of IgY has been in treating newborn calves with egg yolks containing anti-bovine rotavirus (BRV) IgY to reduce BRV induced diarrhea (66).

Although there have been several studies demonstrating the efficacy of orally administered IgY in treating gastrointestinal pathogens, the ability of IgY to cross the intestinal barrier and enter the blood remains to be determined. Vega *et al.* reported that no human rotavirus IgY was detected in the serum samples from IgY treated piglets, however, it was not clear at what time post treatment the serum samples were obtained (65). It is important to establish the bioavailability of IgY and the circumstances where IgY may be bioavailable in order to increase the potential therapeutic applications of IgY. There are several reasons to consider the possibility of seroconversion. IgY is a highly stable molecule and has moderate resistance to some of the digestive enzymes, specifically trypsin and chymotrypsin (35). The stability of IgY in the presence of pepsin is highly pH dependent whereas activity remains high at a pH of 4 but decreases

significantly at a pH of 3.5 and below (35, 100). Furthermore, IgY is temperature stable up to 60°-70° C (100). In this study we used a murine model to evaluate the ability of goose derived IgY to undergo seroconversion in a dose dependent manner both at neutral pH and pH 8.0. We also determined the remaining IgY activity following incubation with several proteolytic enzymes.

Materials and Methods

Ethics Statement

All research was conducted in compliance with the Animal Welfare Act and adheres to principles stated in the Guide for Care and Use of Laboratory Animals (8th ed.), National Research Council, 2011. All animal experiments were performed under the approval of the University of North Dakota IACUC.

Purification of IgY and IgYΔFc from Goose Egg Yolk

Yolks were isolated and rinsed with water and then punctured to drain the contents and diluted 1:10 with cold deionized water, stirred, and acidified to pH 5.0. The diluted yolk was centrifuged at 10,000 x *g* for 30 minutes, and the supernatant was filtered. IgY was further purified via column gradient chromatography (Avianax LLC, 13 March 2014, US20140073766 A1 patent application).

Mice

The common lab strain, B10.T(6R) mice (originally obtained as a gift from Chella David, Mayo Clinic and College of Medicine, Rochester, MN) were bred in laminar flow containment and were maintained in a clean conventional area within the Center for Biological Research (CBR) at the University of North Dakota (UND).

Administration of Antibody

Six to eight week old B10.T(6R) male mice were administered 0.1 mg or 1 mg purified IgY or 0.1 mg IgG diluted in 1x PBS by oral gavage with a 20-gauge stainless steel oral feeding needle (George Tiemann & Company, Hauppauge, New York). Each experimental group consisted of 7-8 mice, and experiments were performed in duplicate.

Serum collection

Sera were collected into BD Microtainer serum separator tubes by retro-orbital bleeding using Natelson blood collecting tubes (Plain, Fisherbrand) prior to the administration of antibody and on various days post administration (as noted). Blood was centrifuged for 10 min at 10,000 x *g* and stored at -80°C until assayed.

ELISA

The presence of IgY or IgG in the sera was determined using an ELISA. Briefly, microtiter plates were coated with 100 µL of the capture antigen diluted in 1x PBS (Rb α-Whole goose IgY, 2.5µg/mL for IgY detection; Donkey α-Goat Fc – unlabeled for IgG detection) and stored at 4°C overnight. Plates were then washed 3 times with wash buffer (1X PBS, 0.05% Tween-20 (Fisher Scientific) pH 7.4) and blocked with 400 µL per well of blocking buffer (0.25% BSA (Fisher Scientific), 0.05% Tween-20 (Fisher Scientific)1X PBS) and incubated at room temperature for 30 min. Plates were washed 3 times and 50 µL of each serum sample was added in triplicate and serially diluted down the plates. IgY or IgG standards and naïve serum were prepared in blocking buffer and added to the plates in duplicate. The plates were allowed to incubate for 30 min at 37°C. The plates were then washed 3 times and blocked with blocking buffer for 10 min at room temperature. Plates were washed 3 times and 50 µL of capture antibody (biotinylated rabbit anti-goose IgY for IgY detection; biotinylated donkey α-goat IgG for

IgG detection) was added to each well and incubated for 30 min at 37°C. Plates were then washed 3 times and blocked with blocking buffer for 10 min at room temperature. 50 µL of diluted streptavidin-HRP (Invitrogen) was added to each well and allowed to incubate for 30 min at 37°C. The plates were washed 3 times and 50 µL of prepared OPD was added to each well. After 15 min the reaction was terminated with the addition of 50µL of 1N H_2SO_4 and the absorbance was measured at A_{490} (Biotek).

Western Blot

Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). 27 µL of each serum sample containing IgY (0.1mg IgY from 1 day post administration, 0.1mg IgY from 2 days post administration, and 1mg IgY pH 8.0 from 3 days post administration) was diluted in 9 µL of 4x Laemmli sample buffer and loaded into each of the three respective wells. The positive control naïve IgY was diluted in 1x PBS and 9 µL of 4x Laemmli sample buffer and loaded in the control well. Proteins resolved by SDS-PAGE were transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) using Tris-Glycine buffer. IgY was visualized using 1:10,000 dilution of Rb anti-chicken IgY-AP (Sigma-Aldrich) and protein bands were visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate.

The effects of proteolytic enzymes on IgY

For the examination of the effect of pepsin on IgY, pepsin was dissolved in 0.07 M sodium acetate buffer (pH 2.0 or pH 4.0) at a concentration of 5 µg/mL as previously described [30]. The enzyme solution was immediately mixed with either 1 mg or 0.1 mg 99% purified goose IgY at a weight ratio of 1/200 pepsin to IgY. The mixture was then incubated at 37°C at pH 2.0 or 4.0 for the appropriate incubation period (0-4 h). After

incubation a 0.45 mL sample of the mixture was combined with 0.05 mL of 5.0% sodium carbonate (for the pH 2.0 sample) or 0.05mL of 1.0% sodium carbonate (for the pH 4.0 sample) to inactivate the enzyme. The remaining IgY activity was measured by ELISA. Trypsin or Chymotrypsin was dissolved at a concentration of 2.0 mg/mL in 50 mM Tris buffer containing $CaCl_2$, pH 8.0. The enzymes were mixed with either 1 mg IgY or 0.1 mg IgY at a ratio of one enzyme to 20 of IgY by weight and incubated at 37°C for the appropriate time (0-8 h). Following incubation a 0.45 mL sample of each mixture was mixed with 0.05 mL of phenylmethyl sulfonyl fluoride (PMSF) solution to inactivate the enzymes. The remaining IgY activity was measured by ELISA.

Statistical Analysis

Two way repeated measures ANOVA and the bonferroni post hoc tests were performed for the data sets given in figures 1-3. P values are represented in the figure legends. All statistics were done using GraphPad PRISM Version 5.0d for Macintosh.

Results and Discussion

Bioavailability of IgY

Following the administration of 0.1 mg IgY by oral gavage, intramuscular (IM) injection, or subcutaneous (SC) injection, the level of IgY present in the serum was determined by ELISA at the specified time points. We detected IgY in the serum as early as 24 h post administration for all routes, and the peak IgY titer was at 24 h post administration by oral gavage at a concentration of 21158.16 ng/mL (Table 1). Furthermore, it is important to note that at 24 h all administration routes induced similar levels of IgY in the serum. IgY was present in the serum until day six for both the IM and

SC routes and until day five for the oral gavage (Figure 1 and Table 1). Serum samples from mice administered 0.1mg IgY orally were also analyzed by western blot on day 1 and 2 post administration (Figure 3). Western blot analysis confirms that IgY is present in the serum post oral administration. We believe this is the first demonstration of bioavailability of whole IgY. It is not clear if the titer of IgY demonstrated here is sufficient for protection. Reports demonstrating the efficacy of orally administered IgY support the potential of this antibody to be therapeutic. The presence of whole IgY in the serum suggests that orally administered IgY may indeed by therapeutic for targets beyond those located in the gastrointestinal tract. The orally available titers of IgY necessary for protection would need to be determined based on the specific microorganism or disease being treated. The level of IgY in the serum declines slightly faster in the orally administered IgY group, compared to administration by other routes. The advantages of the potential to administer IgY orally, most importantly eliminates the specially trained medical personnel necessary for IM or SC injections, may outweigh this pitfall. Without such requirements not only would the cost of administration decrease but the potential for these therapeutics to be widely distributed would expand.

TABLE 1 Serum IgY and IgG concentrations (ng/mL)

Day post administration	Antibody concentration and route of administration							
	0.1 mg IgY Oral	1 mg IgY IM	0.1 mg IgY IM	1 mg IgY SQ	0.1 mg IgY SQ	0.01 mg IgG Oral	0.01 mg IgG IM	0.01 mg IgG SQ
1	21158.16	105258.3	17325.23	152502.1	19193.46	73.23	420.93	515.68
2	2957.59	91490.04	7780.93	64601.88	7598.07	0	428.49	575.87
3	967.77	15403.16	1977.65	16978.34	2127.87	0	557.14	726.53
4	435.83	24286.17	854.23	6418.9	984.17	0	579.28	558.29
5	301.55	2616.86	447.09	3596.2	371.04	0	307.3	384.56
6	0	728.06	180.16	207.37	168.77	0	9.8	10.16
7	0	0	0	21.98	0	0	0	0
14	0	0	0	0	0	0	0	0

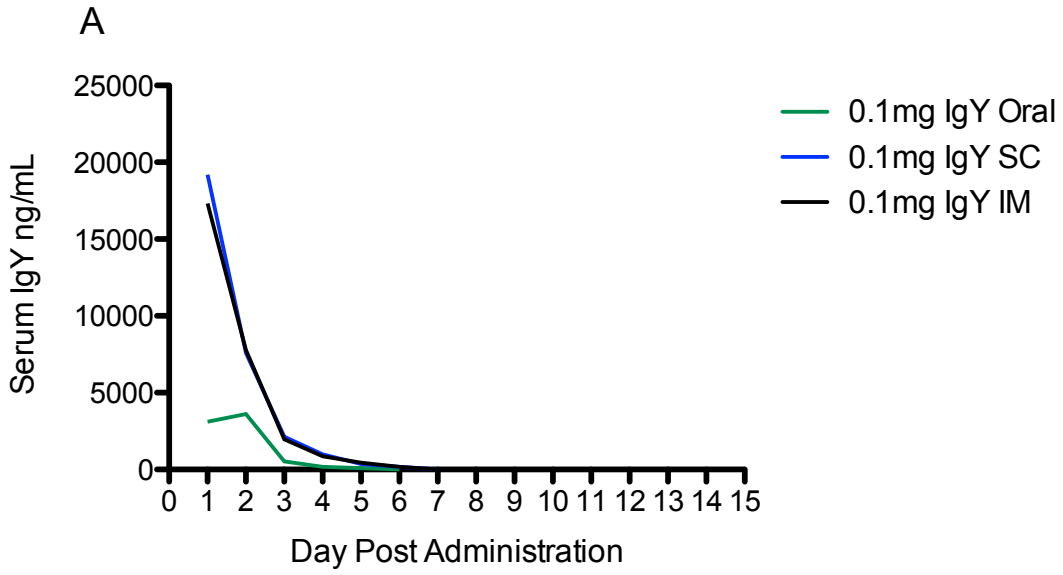


Figure 1. Bioavailability of IgY. IgY (0.1 mg) was administered by oral gavage, intramuscular injection or subcutaneous injection in PBS pH 7.2. The concentration of IgY present in the serum at various time points post administration was determined by ELISA. Data is representative of two experiments, samples analyzed in triplicate, n=7-8. Two-way repeated measures ANOVA and the bonferroni multiple comparisons tests were performed. Column factor p value = 0.0029 (Figure 1A). Oral and SC administration are significantly different on days 2 and 3 ($P < 0.0001$ and $P < 0.01$ respectively). Oral and IM are significantly different on days 1 and 2 ($P < 0.0001$). IM and SQ are significantly different on day 1 ($P < 0.0001$).

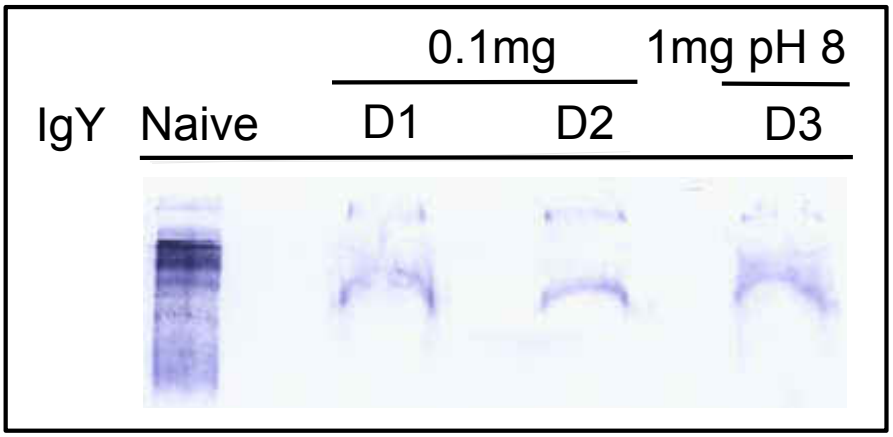


Figure 2. IgY detected in serum post administration. Serum samples from mice administered 0.1mg IgY and 1mg pH8.0 were used to do western blot analysis. Lane one is naïve IgY, lane two is serum collected 1 day post oral administration of 0.1mg IgY, lane three is serum collected 2 days post oral administration of 0.1mg IgY, and lane four is serum collected 3 days post oral administration of 1mg IgY pH 8.0.

Bioavailability of IgG

The bioavailability of IgG was determined for three injection routes, IM, SC, and by oral gavage. IgG was present in the serum 24 hours post administration for all routes and was able to be detected in the serum as late as five days post SC or IM injection (Table 1, Figure 3). IgG administered orally had a peak serum titer at one day post administration whereas the peak serum titer for SC and IM injections were at days three and four, respectively (Figure 1B). When comparing the oral bioavailabilty of IgG to that of IgY on a molecular level, these data clearly demonstrate that there is a greater amount of IgY than IgG in the serum at all-time points post administration. The importance of this demonstration is that therapeutic IgY may uniquely be given orally, whereas there are currently no approved IgG treatments that are administered orally, likely due to the very low bioavailability.

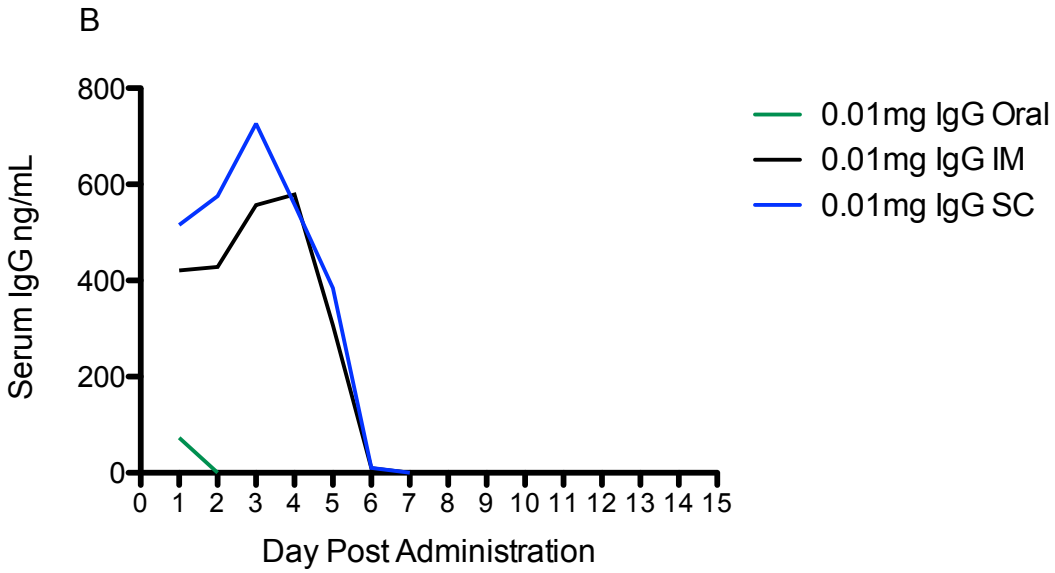


Figure 3. Bioavailability of IgG. IgG was administered by oral gavage, intramuscular injection, or subcutaneous injection in PBS pH 7.2. The concentration of IgG present in the serum at various time points post administration was determined by ELISA. Data is representative of two experiments, samples analyzed in triplicate, n=7-8. Two-way repeated measures ANOVA and the bonferroni multiple comparisons tests were performed. Column factor p value = 0.0006 (Figure 1B). IgG SC and IgG oral are significantly different on days 1-5 ($P < 0.001$). IgG IM and IgG oral are significantly different on days 1-5 ($P < 0.001$). IgG IM and IgG SC are different on day 2 ($P < 0.05$).

Bioavailability of orally administered IgY (1 mg) after multiple doses

To determine if the titer of bioavailable IgY could be increased by increasing the dose or 1 mg of IgY was administered by oral gavage either once, or three times as part of a multiple dose schedule at 0 h, 12 h, and 24 h. With multiple administrations, IgY was detected in the serum as early as 24 h after the final antibody dose (48 h after the first dose). However, when a higher concentration (1 mg) of IgY was administered as a single dose, IgY was undetectable the serum at any time point post administration (Figure 4). This was surprising, as the previous experiments using orally administered IgY at a lower dose demonstrated the presence of IgY in the sera. We expected that IgY administered at a higher dose (1 mg) would also be bioavailable. The mechanism by which IgY seroconversion occurs only at a lower dose during single administration is unknown and is currently being investigated. Overall, these data suggest that depending on the concentration of IgY administered, it would be advantageous to design a therapeutic strategy that would include a multiple dosing schedule. When IgY was administered at a higher concentration (1mg) dose the bioavailability followed a similar trend in the IM and SC groups but not in the orally administered group (Table 1).

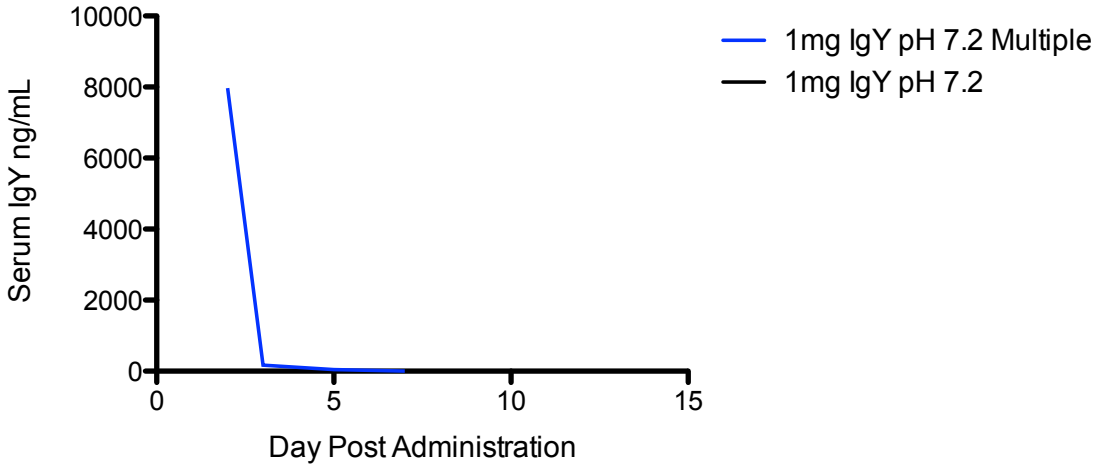


Figure 4. Bioavailability of IgY increases with multiple doses. IgY was administered by oral gavage in PBS pH 7.2 either once or at multiple time points (0 h, 12 h, 24 h). The concentration of IgY present in the serum at various time points post administration was determined by ELISA. Data is representative of two experiments, samples analyzed in triplicate, n=7-8. Two-way repeated measures ANOVA and the bonferroni multiple comparisons tests were performed. Column factor p value = 0.0008. On days 2 and 3 $P < 0.0001$.

Bioavailability of IgY in PBS pH 8.0

To further investigate why IgY was not able to seroconvert at a higher dose we looked into ways to alter the antibody conditions without changing the dose. In this experiment IgY was administered by oral gavage in PBS at either pH 7.2 or pH 8.0 and the level of IgY present in the serum was determined at the specified time points. When IgY was administered in PBS buffered to pH 8.0, the orally administered IgY was detected in the serum at 48 h post administration at a level of 45,760 ng/mL. Seroconversion still did not occur when IgY was administered as a single dose in a pH 7.2 solution, as demonstrated above (Figure 5). We were able to demonstrate that by increasing the pH of the solution the higher concentration of IgY became bioavailable. These data suggest that increasing the pH of the antibody solution is better than both the low dose and the multiple dose strategy. The level at 24 hours is over double that of the 0.1mg IgY administration and approximately sixty times higher than the oral administration of IgG. Furthermore, when the pH is increased the IgY remains at a higher level in the serum over time. At day five, 1mg IgY in pH 8.0 is at an average of 3807 ng/mL whereas at day five 0.1mg IgY is at an average of 301.5 ng/mL and IgG is absent by day five. Further investigation is ongoing to elucidate a mechanism as to why increasing the pH proves to be advantageous, however one possible explanation is that the increased pH solution is able to buffer the acidic environment of the stomach. If the stomach acid is in some way changing the behavior or structure of the IgY in a way that prevents it from being passed into the small intestine and absorbed, then by adding a buffer to counteract the acid may offer some protection to these antibodies allowing them to seroconvert. Furthermore, these data do suggest that other potential formulations and

manipulations of IgY may be beneficial in order to be used orally. One alternative would be to microencapsulate the IgY, which may aid in both protecting IgY from proteolytic enzymes in the gastrointestinal tract and increasing the bioavailability.

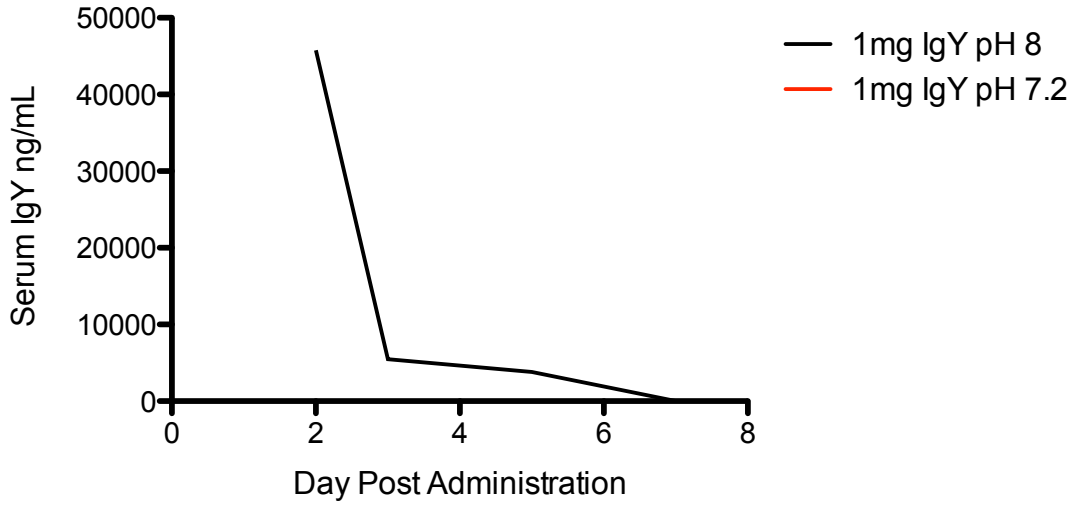


Figure 5. Bioavailability of IgY increases in PBS pH 8.0. IgY was administered by oral gavage in PBS pH 7.2 or PBS pH 8.0. The concentration of IgY present in the serum at various time points post administration was determined by ELISA. Data represented follows the same trend in both experiments, samples analyzed in triplicate, n=7-8. Two-way repeated measures ANOVA and the bonferroni multiple comparisons tests were performed. Column factor p value < 0.0001. On days 2 and 3 $P < 0.0001$, on day 5 $P < 0.01$.

Behavior of IgY against proteolytic enzymes

Previously it has been shown that chick IgY is more resistant to specific proteolytic enzymes, compared to mammalian IgG. Therefore we want to determine if goose-derived IgY also demonstrated this resistance, and to determine if the resistance was concentration-dependent, in light of the ability of buffering the high dose orally administrated IgY to obtain bioavailability. In these experiments we determined the amount of IgY activity remaining following the incubation of either 0.1 mg or 1 mg IgY in pepsin (pH 2.0), pepsin (pH 4.0), trypsin, or chymotrypsin. We detected no dose-dependent difference in the proteolytic resistance, regardless of the IgY dose. IgY incubated with pepsin (pH 4.0) retained more activity than IgY incubated with pepsin (pH 2.0). Incubation of IgY with trypsin or chymotrypsin had much less effect on the degradation of IgY, with 50-70% of the antibody activity still remaining after 8 hours of incubation. The degradation of IgY incubated in PBS alone was measured as a control, and 100% activity remained after 8 hours (Table 2). These results are in agreement with previous studies (39), and suggest that when administered orally IgY is able to withstand partial activity, which is highly advantageous over the use of IgG that tends to be less stable. Unfortunately these data did not explain the difference we see in the bioavailability between the high and low doses of IgY.

TABLE 2 Behavior of IgY against proteolytic enzymes

Incubation period	0.1 mg IgY Pepsin pH 2.0		1 mg IgY Pepsin pH 2.0		0.1 mg IgY Pepsin pH 4.0		1 mg IgY Pepsin pH 4.0	
	Titer	%Recovery	Titer	%Recovery	Titer	%Recovery	Titer	%Recovery
0 hours	97272.23	100	1180302.43	100	97272.23	100	1180302.43	100
1 hour	29137.86	29.9	387101.53	32.7	38518.23	39.5	557631.03	47.2
2 hours	27091.4	27.8	295364.4	25.0	41580.6789	42.7	388228.96	32.8
4 hours	22102.74	22.7	297198.6	25.1	41944.74	43.1	389606.12	33.0

Incubation period	0.1mg IgY Trypsin		1mg IgY Trypsin		0.1mg IgY Chymotrypsin		1mg IgY Chymotrypsin	
	Titer	%Recovery	Titer	%Recovery	Titer	%Recovery	Titer	%Recovery
0 hours	97272.23	100	1180302.43	100	97272.23	100	1180302.4	100
1 hour	82685.38	85.0	873275.47	73.9	79241.61	81.4	928249.76	78.6
2 hours	77365.61	79.5	817156.15	69.2	56926.25	58.5	752834.10	63.7
4 hours	64414.82	66.2	703863.25	59.6	75315.67	77.4	673075.81	57.0

CHAPTER III

IGY TREATMENT FOR MURINE CEREBRAL MALARIA

Abstract

Increasing evidence for drug resistant *Plasmodium* strains suggests there is a substantial need for new or additional therapeutic options. Cerebral malaria (CM), a manifestation of severe *Plasmodium falciparum* infection is a clinical disease that can lead to lasting neurological and cognitive disorders and is yet to be cured. Although the pathogenesis of CM is not well characterized, the use of *Plasmodium berghei* ANKA has been accepted as a commonly used model organism to study CM in mice. Here we used this murine model of CM to evaluate the efficacy of a combination of two anti-malaria IgY antibodies: anti-*Plasmodium* IgY and anti-MSP-1 IgY.

C57BL/6 male mice were infected intraperitoneally with 1.0×10^7 *Plasmodium berghei* ANKA infected red blood cells (iRBC). Mice were treated subcutaneously on days 2 and 4 or days 2 and 5 post infection with a combination of anti-*Plasmodium* IgY and anti-MSP-1 IgY, naïve IgY, or PBS. Survival and clinical signs of CM were monitored daily and thin blood smears were collected and Giemsa stained to determine parasitemia daily as well.

Mice that were treated with a combination of anti-*Plasmodium* and anti-MSP-1 had significantly increased survival rates as compared to both the naïve IgY treated and PBS treated control mice. Parasitemia was also reduced in the malaria antigen IgY treated

mice and this decrease corresponded to the timing of treatment. IgY therapy delayed mortality in the murine model of CM.

Goose-derived anti-*Plasmodium* and anti-MSP-1 IgY is able significantly extend the times to death in mice infected with *Plasmodium berghei* ANKA. These data provide evidence for anti-malaria IgY as a potential candidate to be used in antimalarial combination therapy.

Introduction

The decreasing efficacy of the currently approved anti-malarial drugs for the treatment of severe malaria facilitates the need for new drugs and/or the exploitation of different drug classes. Most of the drugs that have been approved for treatment of malaria patients are artemisinin derivatives (101). Artemisinin derived anti-malarial drugs are highly important because of their efficacy against multidrug-resistant strains of *Plasmodium falciparum* (*P. falciparum*). One disadvantage of artemisinin drugs is the occurrence of recrudescence when given in short course monotherapy (102). Therefore, artemisinin derivatives are often used in combination with other antimalarial drugs to provide both rapid and long-lasting protection. Artemisinin was isolated in 1972 by a group of Chinese researchers from the *Artemisia annua* plant. Artemisinin derivatives work at the early trophozoite and ring stages of parasite development, unlike other drugs that work only during the later stages (trophozoite and schizont). Therefore, artemisinin has been successful in treating patients suffering from uncomplicated malaria, but unfortunately its efficacy is reduced in complicated cases of malaria, such as cerebral malaria (CM). Even after intramuscular treatment with artemether, an artemisinin derivative, the mortality rate of children with CM is 15-25% (102). Furthermore,

researchers have identified human-infecting *Plasmodium* strains that are resistant to artemisinin drugs (103). The mechanism of drug resistance in such parasites is an area of active research and debate (104).

Malaria is a global disease that affects millions of people every year. Approximately 3.4 billion people within 106 countries and territories live in areas that are at risk for malaria transmission, and in 2012 the World Health Organization estimates that malaria caused over 207 million clinical episodes and approximately 627,000 deaths (105). The life cycle of the parasite involves both hepatic and erythrocytic stages and the clinical manifestations of disease occur following erythrocyte rupture (106). There are five species of *Plasmodium* that are infectious to humans, however *P. falciparum* is the parasite responsible for the development of severe malaria (107).

CM is a clinical syndrome, a neurological complication, which is the result of a severe infection of the parasite *P. falciparum*. CM is characterized by the presence of parasites in the blood, and as a diffuse encephalopathy resulting in an altered level of consciousness (e.g. coma) in the absence of other causes of encephalopathy (108). CM is the leading cause of hospitalization and mortality of children under the age of five in sub-Saharan Africa and accounts for approximately 80% of all fatal cases of malaria (109, 110). In the absence of treatment, CM is nearly universally lethal and, even with intervention, the mortality is still 15-20% (111). Following treatment for CM, patients often exhibit permanent residual symptoms, including cognitive, behavioral, and motor changes (112).

There are two leading theories describing the pathogenesis of CM: the first is the obstruction of cerebral microvasculature by sequestered *Plasmodium*-infected red blood

cells (iRBC); and the second is an immunopathology caused by the exacerbation of the host inflammatory response (113). The assessment of CM pathogenesis in humans is especially difficult due to the invasiveness required to accurately assess human brain tissue. Experimental cerebral malaria (ECM) is currently modeled in mice using *Plasmodium berghei* ANKA (PbA) infection. ECM has been valuable providing a wealth of information about the pathogenesis of CM despite the functional differences in *Plasmodium* pathogenesis between humans and mice (114). In the ECM model the neurological syndrome is associated with severe vasculopathy and a systemic inflammatory response. There are several factors that contribute to this disease state including the activation of leukocytes, cytokine production, and increased expression of endothelial adhesion molecules (115).

Merozoite surface protein 1 (MSP-1) is the most abundant protein on the surface of *P. falciparum* merozoites, which are the invasive form of the parasite (116). MSP-1_{42} (42 kDa) is synthesized initially as a large precursor during intracellular merozoite development and then is expressed as a GPI-linked protein in complex with MSP-6 and MSP-7 on the surface of a replicating merozoite inside of the iRBC (116). Between merozoite release and the completion of erythrocyte invasion, MSP-1_{42} undergoes proteolytic processing that results in the formation of two MSP fragments, MSP-1_{33} and MSP-1_{19}. The majority of the MSP-1 complex, the MSP-1_{33} fragment, is then shed from the parasite surface leaving only the C-terminal MSP-1_{19} fragment intact. Antibodies that are specific for MSP-1_{19} were able to block merozoite invasion of RBCs and the development inside the iRBC in an *in vitro* assay (117). Other reports have described the potential of anti-MSP antibodies in malaria vaccines (118-120). There is also evidence

that MSP-1 is essential for parasite survival as efforts to disrupt or knock out the *msp1* gene have been unsuccessful (121).

In this study we tested the ability of goose derived anti-MSP-1 and anti-*Plasmodium* IgY antibodies to treat ECM. IgY is the predominant antibody isotype found in birds and is located in both the serum and egg yolk. Also present in anseriform birds (waterfowl, e.g.ducks and geese) is an alternatively spliced IgY isoform called IgYΔFc that lacks two constant domains present in full length IgY and is the functional equivalent of an IgG $F(ab')_2$ fragment. The IgYΔFc isoform is also the most prevalent antibody produced following hyperimmunization (28). Although IgY and IgG are functionally similar molecules, there are several characteristics of IgY that make it an attractive alternative to using conventional mammalian antibodies for the treatment of infectious agents in mammals. The genetic background and phylogenetic distance between birds and mammals is critical difference between these two antibody sources that allows avian IgY to recognize epitopes that may not be recognized by mammals. IgY does not bind to mammalian complement to induce an inflammatory reaction (48). IgY does not bind to human rheumatoid factor, and has decreased binding to bacterial proteins *Staphylococcus* protein A or *Streptococcus* protein G (51-54, 92). The binding of IgY to Fc receptors is also drastically reduced when compared to the binding of Fc receptors by IgG (90). There have been several reports of experimental and clinical trials indicating the efficacy of IgY for the treatment of infectious diseases (27, 39, 61, 64, 68, 71, 75, 79, 93, 98, 100, 122-124). We demonstrate here that anti-*Plasmodium* IgY in combination with anti-MSP-1 IgY is able to increase the time to death and decrease the parasitemia in PbA infected

mice. These data suggest that IgY is a putative therapeutic that could be used in new combination therapy for malaria.

Materials and Methods

Ethics statement

All research was conducted in compliance with the Animal Welfare Act and adheres to principles stated in the Guide for Care and Use of Laboratory Animals (8^{th} ed.), National Research Council, 2011. All animal experiments were performed under the approval of the University of North Dakota IACUC committee.

Plasmodium berghei ANKA

Plasmodium berghei ANKA (PbA) was obtained from BEI resources and maintained by successive infection of C57BL/6 male mice. On day 6 p.i. blood was collected by retro orbital bleeding with a Natelson blood collecting tube (Fisherbrand) and placed into a BD vacutainer with sodium heparin to prevent clotting. Experimental mice were inoculated intra-peritoneally with 1.0×10^7 iRBC in 100µL of PBS. Blood smears were collected daily starting at day 3 post infection and stained to analyze parasitemia.

Mice

Mice were bred in our breeding colony and maintained within a clean conventional area in the Center for Biological Research at the University of North Dakota. C57BL/6 male mice between the ages of 6-8 weeks were used for the infection studies. All mice were assessed for survival and signs of CM were monitored daily.

Generation of blood Smears for determining parasitemia

Blood was collected from all experimental mice via tail clip and a drop of blood was placed on a clean slide (Fisherbrand). A second slide was used to create a thin smear. Slides were then fixed with methanol and allowed to dry for 30 minutes. Each slide was stained in a dilute solution of Giemsa stain (Sigma-Aldrich) for 1 hour and allowed to air dry. Slides were viewed using a 100x oil immersion lens.

Parasitemia was determined by counting the number of iRBC/500 RBC in Giemsa stained blood smears. Parasitemia was calculated for each experimental mouse and the daily average parasitemia was calculated for each group.

Anti-*Plasmodium* IgY

Geese, three for each antigen, were vaccinated subcutaneously with killed whole *Plasmodium berghei* parasite previously isolated from iRBCs or the MSP-1 antigen (yeast secreted recombinant 19 kDa carboxy-terminius of MSP-1 from *Plasmodium yoelii* lethal strain (XL), ATCC) at day 0, followed by booster injections on weeks 2, 4, and 6. Geese were then bled between 6-8 weeks and serum was obtained using a 10,000 x g, 30 minute spin at 20°C. The serum was filtered through a PES 0.22 μm filter unit and a series of precipitation steps were carried out to obtain the antibody populations. Following this, MEP HyperCel Chromatography was performed using a 30 mL MEP HyperCel (Pall Biosciences) column at 5 mL/minute. The column was washed and IgY was eluted using a 50 mM acetate buffer step gradient. Antibody fractions were combined and placed into a 500 mL chamber of Water's LabScale Tangental Flow Filtration (TFF) apparatus with a Pellicon XL TFF PES membrane (100,000 MW cutoffs). Samples were concentrated and diafiltration was performed with 1x PBS.

Antibody treatment

Experimental mice received a combination of anti-*Plasmodium* or anti-MSP-11 antibody on either on days 2 and 5 post infection (p.i.), or on days 2 and 4 p.i. subcutaneously, as outlined. Each antibody type was injected separately into a different site on the mouse as to avoid potential unwanted interference. Specifically mice were injected into the loose skin above the neck, and into the loose skin further down the back near the dorsal rump. Multiple injections were performed within 15-20 minutes of each other to prevent any leakage from the initial injection site.

Statistical analysis

Survival curves were analyzed using the log-rank (Mantel-Cox) test and p values are reported in the text and figure legends. All statistics and figures were made using GraphPad PRISM version 5.0d for Macintosh.

Results

IgY therapy of CM

Treatment on days 2 and 5 p.i.

C57BL/6 mice infected with PbA were administered anti-MSP-1 IgY, anti-*Plasmodium* IgY, a combination of both antibodies, or PBS via a subcutaneous route of infection on days 2 and 5 p.i.. Parasitemia was calculated from daily blood smears. Mice that received the combination of the two antibodies showed the greatest initial survival, although all treatment groups had similar survival rates at 21 days p.i. with approximately 30% survival (Figure 6). All mice, regardless of treatment, did eventually succumb by 23 days post infection. There was no statistically significant difference between any of the treatment groups. However, these data suggested that the anti-*Plasmodium* and anti-MSP-

1 IgY antibodies offered protection that allowed these mice to survive longer than the PBS control treated mice.

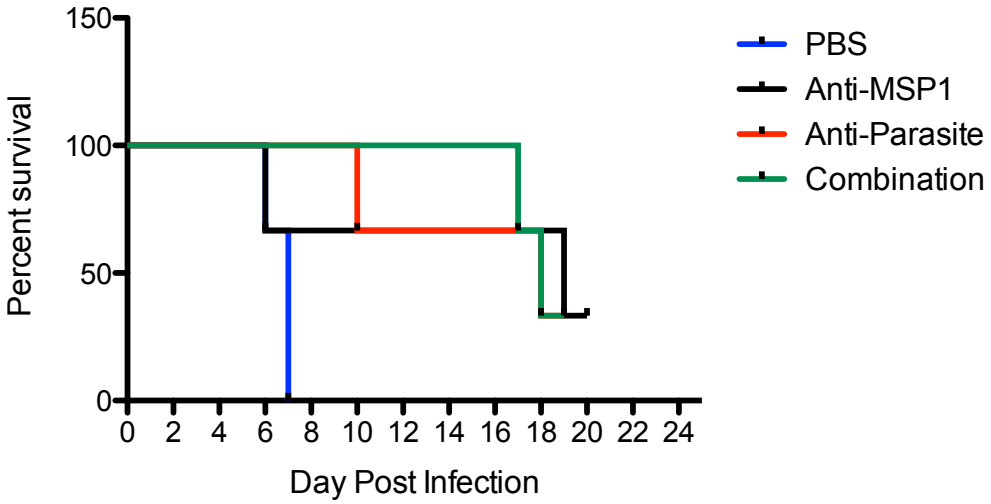


Figure 6. Prolonged day to death following IgY treatment on days 2 and 5 p.i. C57BL/6 male mice ages 6-8 weeks were infected by intraperitoneal route with 1.0 x 10^7 infected red blood cells previously harvested from an infected mouse. Cells were injected in a 1x PBS vehicle solution. On days 2 and 5 post infection a groups of mice (n=7-8/group) were administered anti-*Plasmodium* IgY (anti-Parasite), anti-MSP-1 IgY, a combination of both antibodies (combination) or PBS via subcutaneous route. Survival was monitored daily.

Treatment on days 2 and 4 p.i.

We observed, as others have reported (125) that death associated with PbA infection typically started within 7 days post infection. Therefore, in order to examine the kinetics of treatment and provide potentially better protection with the anti-*Plasmodium* and anti-MSP-1 antibodies, the administration schedule was altered to days 2 and 4 p.i. with PbA. Mice treated with the combination of anti-*Plasmodium* and anti-MSP-1 antibodies survived significantly longer, day 21 p.i. compared to day 7 with PBS treatment and day 10 with the naïve IgY antibody treatment, $p = 0.0007$ and $p= 0.045$ respectively (Figure 7). This demonstrated that malaria-specific IgY was able to significantly prolong the day to death in mice infected with PbA.

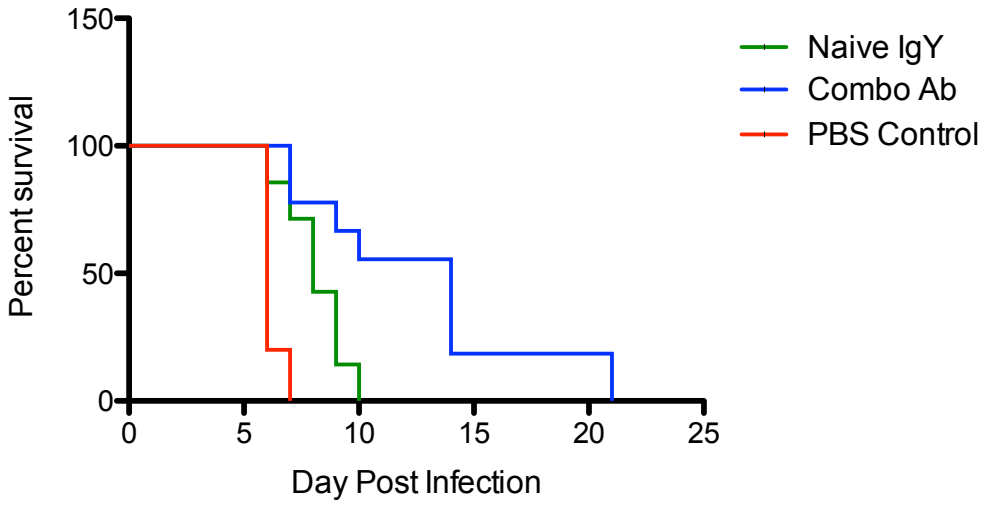


Figure 7. Prolonged day to death following IgY treatment on days 2 and 4 p.i. C57BL/6 male mice ages 6-8 weeks were infected i.p. with 1.0 x 10^7 infected red blood cells previously harvested from an infected mouse. Cells were injected in a 1x PBS vehicle solution. On days 2 and 4 post infection groups of mice (n=7-8/group) were administered anti-Plasmodium IgY and anti-MSP-1 IgY (combo Ab), naïve IgY, or PBS via subcutaneous route. Survival was monitored daily. A log rank (Mantel-Cox) test was performed between the PBS control group and the combination IgY treatment group p value = 0.0007. A log rank (Mantel-Cox) test was performed between the naïve IgY and combination IgY treatment p value = 0.045.

To better understand the protection provided by the anti-*Plasmodium* and anti-MSP-1 IgY antibodies, parasitemia by Giemsa-stained thin film blood smears was determined. Parasitemia was calculated daily starting on day 3 post infection as shown in Figure 8. Parasitemia was lower in the antibody treated group by day 4 p.i., rapidly post treatment on day 2. Both the PBS control and naïve IgY control groups had higher parasitemia on all days following treatment with the greatest notable difference at day 8 post infection. Between days 12 and 13 the parasitemia started to rise again in the malaria-antigen specific IgY treated groups until the day of death (Figure 8).

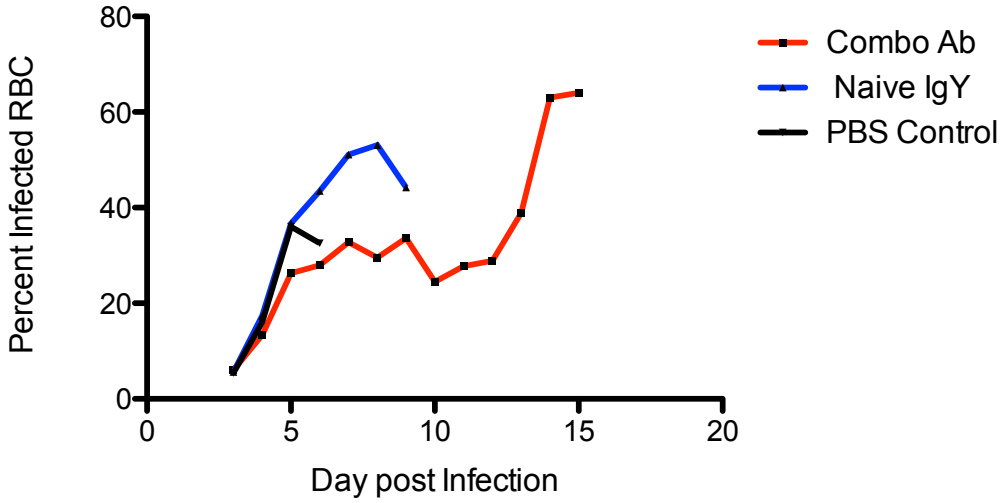


Figure 8. Decreased parasitemia in mice treated with IgY on days 2 and 4 p.i. C57BL/6 male mice ages 6-8 weeks were infected by intra peritoneal route with 1.0 x 10^7 infected red blood cells previously harvested from an infected mouse. Cells were injected in a 1x PBS vehicle solution. On days 2 and 4 post infection groups of mice (n=7-8/group) were administered anti-Plasmodium IgY and anti-MSP-1 IgY (combo Ab), naïve IgY, or PBS via subcutaneous route. Thin blood smears were made from the blood of each mouse starting at day 3 post infection. Each slide was fixed and stained with dilute Giemsa stain. Parasitemia was calculated by counting the number of infected red blood cells per 500 red blood cells for each mouse.

Discussion

The current situation regarding the parasitic resistance to artemisinin derivatives in malaria patients has pushed the World Health Organization to recommend artemisinin based combination treatments instead of a monotherapy approach to treating malaria infections. There have been several studies determining the efficacy of different artemisinin derivatives in combination with other anti-malaria compounds (126). Unfortunately there are indications of resistance to drugs that may be used in artemisinin based combination therapies, e.g. Malarone ® which is a combination of atovaquone and proguanil (127). Artemisinin treatments, despite many interventions, have not been able to cure severe complicated malaria and overall there has been no significant difference in the neurological sequelae with this treatment (102). These data highlight the need for new anti-malaria compounds or immunotherapies that could be used as part of an artemisinin based or other combination therapy. In this study we demonstrate that PbA infected mice that are treated with a therapeutic antibody on either days 2 and 4 or on days 2 and 5 p.i. succumb to the infection at a slower rate than those without treatment. We also show that mice that receive IgY treatment have decreased parasitemia that correlates with when the antibodies were administered and the time of death. The administration of malaria antigen-specific IgY at a later time point did change the statistical significance of the data although the trends remained similar (data not shown). It has been previously demonstrated that polyclonal antibodies can prevent erythrocyte invasion by merozoites *in vitro* (128, 129). It has also been demonstrated in several studies that IgY is safe for human consumption and has less unwanted reactivities with host proteins than does other mammalian antibody treatments. We understand that IgY is not a candidate for

monotherapy treatment of malaria, however we do propose that anti-*Plasmodium* IgY and anti-MSP-1 IgY would be a good alternative cocktail for use in combination therapies. Further studies are necessary to determine what antibody-drug combinations and treatment regimes provide the best protection, and if antibody therapy is able to prevent neurological damage to a greater extent than other drugs used in combination therapy for severe cerebral malaria.

Conclusions

In summary, the data presented in this study offer evidence that a combination treatment with other potential drug candidates and anti-*Plasmodium* and anti-MSP-1 IgY may offer increased protection. Here we demonstrate that IgY treatment delayed the time between infection and death and has decreased the parasitic load in these infected animals. Elaboration on this preliminary study will include the investigation of the efficacy of IgY in combination with other antimalarial drugs.

CHAPTER IV

PASSIVE IMMUNIZATION OF IGY FOR THE TREATMENT OF DENGUE VIRUS INFECTIONS

Abstract

Dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) are severe disease manifestations following secondary heterotypic dengue virus (DENV) infections. At present, there are no licensed therapies or vaccines to treat DENV induced disease. DHF and DSS are mediated by serotype cross-reactive antibodies that facilitate antibody dependent enhancement (ADE) by binding to viral antigens and then Fcγ receptors (FcγR) on additional myeloid cells. Balsitis et al. have verified using genetically engineered DENV specific antibodies that the interaction between the Fc portion of the serotype cross-reactive antibodies and the FcγR is required to induce ADE (130). Additionally, they demonstrated that these antibodies were as neutralizing as their non-modified variants, were incapable of inducing ADE, and were therapeutic following a lethal, antibody-enhanced infection. We therefore hypothesized that avian IgY that do not interact with mammalian FcγR, would provide a novel therapy for DENV induced disease. In this study, polyvalent anti-DENV2 IgY were purified from the eggs of DENV2-immunized geese. The neutralization and enhancement capacity of anti-DENV2 IgY was tested *in vitro*, and the therapeutic efficacy against lethal challenge was tested *in vivo*. It was determined that anti-DENV2 IgY neutralized DENV2 and did not induce

ADE *in vitro*. Anti-DENV2 IgY was also protective *in vivo* when administered 24 hours following a lethal DENV2 infection. The anti-DENV2 IgY were separated into the "full length" and "alternatively spliced (Δ)" antibody populations and DENV2-specific epitopes were mapped and compared to the well-characterized DENV and flavivirus epitopes. Anti-DENV2 IgY recognized some but not all well characterized DENV epitopes, the two antibody populations recognized different epitopes, and both populations recognized uncharacterized epitopes. Peptide selection of the anti-DENV2 antibodies based on the unique DENV2 epitopes was completed to test the neutralization capacity *in vitro*. These findings support the potential of avian antibodies as a new treatment for DENV infection.

Introduction

Dengue virus epidemiology

Almost half of the world is at risk for dengue virus infections with up to 390 million possible infections occurring in nearly 100 endemic countries annually (131). Dengue is a fast emerging disease with a 30-fold increase in disease incidence reported in the past 50 years (132). Dengue has established itself globally in both endemic and epidemic transmission cycles and is currently regarded as the most important arboviral disease internationally (91, 131, 133).

DENV is a member of the Flavivirus family of RNA viruses. There are four distinct serotypes (DENV1, DENV2, DENV3, DENV4) that differ at the amino acid level by 25-40% (134). It has been estimated based on genetic studies of the four different serotypes that these serotypes emerged from a common ancestor virus that circulated between non-human primates and mosquitoes some 500 years ago. Following

this it is likely that each virus serotype emerged separately into a human urban transmission cycle (135). DENV is primarily transmitted by the *Aedes aegyptii* mosquito and *Aedes albopictus* is a secondary vector. Mosquitos transmit DENV to humans by feeding on previously infected human hosts. Following the incubation period in the mosquito, the mosquito is infectious and can release the virus upon feeding on another human host (136, 137).

It has been suggested that the principle mosquito vector *Aedes aegyptii* originated in Africa and that the exportation of DENV to the Western Hemisphere occurred approximately 400 years ago. The exportation likely occurred in multiple introductions in association with the slave trade (138). The first recognized dengue outbreaks occurred early in the 17th century with the firsts reported on the Caribbean islands of Martinique and Guadeloupe. During these outbreaks patients reported symptoms including fever, severe headache, lassitude, and pains in the legs. During a 1780 outbreak in Philadelphia Benjamin Rush coined the term "break-bone fever" (91). In the early years DENV was considered a nuisance disease with very low mortality rates. The earliest known cases of possible DHF/DSS were in India during the 1870s as the 4 different serotypes began to spread globally and reinfection became common (139). During the early 20th century the mosquito vector began to spread and DENV became prevalent in more tropical areas of the globe. There have been several suggested factors that explain the expansion of the mosquito vector including the international travel and urbanization and globalization following World War II and an increase in standing water as a result of poor housing situations, water systems, and improper sewer and waste management systems (133). During the 1950s the first documented epidemics of DHF occurred, first in the

Philippines (1953-1954) and Thailand (1958) (133). In the Americas, epidemic dengue was controlled in most of the region by the eradication program that eliminated the Aedes aegypti mosquito vector from 23 countries until the program was terminated in the early 1970s (140). Following this the mosquito vector was rapidly reestablished and new DENV serotypes and strains emerged, causing the co-circulation of multiple DENV serotypes (133).

In 1980 DENV1 was isolated from a 5-year-old girl in Brownsville, Texas, representing the first indigenous case of dengue since the initial case in 1945. (141). Since then there have been further reported cases of dengue in Brownsville, TX and in Florida and the presence of DENV1 has been detected in *Aedes aegypti* mosquitos in Florida (141, 142).

It is important to realize that the spread of the virus is ultimately dependent on the mosquito vector and thus vector control is crucial. Unfortunately, to effectively control the mosquitoes that live in close contact with the human host, every house and office in the city must be visited on a daily basis and this has proven to be nearly impossible without effective community outreach programs (133). It has become clear that in order to control the disease in the absence of a strong vector control program there needs to be the development of new antiviral therapies and vaccines.

DENV characteristics and replication

DENV is an enveloped positive-strand RNA virus. The mature DENV contains three structural proteins, the capsid protein (C), membrane protein (M), and the envelope protein (E); and seven nonstructural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5 (143). The structure of the virus includes a viral nucleocapsid surrounding the RNA

genome composed of multiple copies of the C protein. Surrounding the nucleocapsid is a host-cell-derived lipid bilayer, in which 180 copies of the M and E proteins are anchored. The M protein is proteolytic fragment of the precursor protein Pre-membrane (PrM).

DENV naturally infects cells of the mononuclear phagocyte lineage (e.g. monocytes, macrophages, and dendritic cells) as well as the skin-resident Langerhans cells (144, 145). It is unknown what receptor(s) DENV attaches to the on the cell surface to facilitate entry but it has been suggested that possible receptors are DC-SIGN, and the closely related L-SIGN (146, 147). In addition to these two receptors it was recently demonstrated that the carbohydrate moieties on the DENV E protein can bind to the mannose receptor expressed on human macrophages (148). DENV use clathrin-mediated endocytosis to enter the host cell and upon internalization they are delivered to Rab5-positive early endosomes. Once the endosome as matured into a Rab7-positive late endosome the virus and host cell membrane fusion occurs (149). Following this the viral nucleocapsid is released into the cytoplasm. As the replication machinery of positive strand RNA viruses is not packaged in the viral particle, the DENV must undergo an initial round of translation, to generate viral replicase, upon entry into the cell. The DENV genome is translated as a single polyprotein and is later co-and post-translationally cleaved into respective proteins. During this initial translation the structural proteins are anchored in the endoplasmic reticulum (ER). Following the proteolytic cleavage of proteins, dengue virions are assembled on the membrane of the ER. The C protein remains associated with the ER and the PrM and E proteins form heterodimers on the luminal side (150).

After the initial translation episode the virus begins to replicate inside the host cell cytosol. Several nonstructural proteins are required for successful replication of the dengue RNA. Negative strand synthesis begins upon assembly of NS5 with the other components of the DENV replicase, NS3, NS1, NS2A, NS4A and NS4B. Negative strand synthesis results in the formation of a double stranded replicative form. The replicative form undergoes transition into a replicative intermediate, and this is a process that likely involves genome cyclicization. Viral replication proceeds on this replicative intermediate asymmetrically and semi-conservatively resulting in the predominant production of positive strand RNA. Once the newly synthesized RNA is present the C protein forms a nucleocapsid around the RNA genome. Immature virions are formed after heterodimers of PrM and E are oriented into the lumen, as previously stated. The immature virion travels through the trans-golgi network where the acidic environment causes dissociation of the PrM and E proteins. Dissociation allows the PrM proteins to be cleaved by furin protease to form a mature virion. As the new virus exits the golgi it fuses with the host cell membrane and is released by exocytosis (151, 152).

DENV pathogenesis

DENV can affect people of all ages including infants, children, adults and elderly but the interplay between the virus and host is what determines the clinical outcome. Disease manifestations from DENV infections range from asymptomatic infections, a mild febrile illness known as dengue fever (DF), or the more severe DHF and DSS. During an initial infection, most children experience subclinical infection or mild undifferentiated febrile syndromes (153). In this situation, lifelong immunity against the primary infecting virus occurs. During a secondary infection the pathophysiology of the

disease changes dramatically, specifically if the secondary infection is with a different DENV serotype. Heterotypic secondary infections are the cause of 90% of the DHF cases reported (154). One working hypothesis of dengue pathogenesis during severe disease is the result of ADE during secondary infections (153).

ADE occurs when remaining sub-neutralizing antibodies following a primary DENV infection bind to an infecting viral particle from the secondary heterotypic infection. These antibody-virus complexes then bind to Fc receptors on macrophages and dendritic cells via the FcγR of the antibody (143). The result of ADE is a higher number of infected cells and therefore a heightened immune response to the infection (143). ADE also results when infants are born to dengue immune mothers (155). In this situation antibodies transferred from the mother to the child for the first 4 months postpartum protect children, but after this the antibodies become non-neutralizing and the child becomes at risk for ADE. When the child reaches one year the anti-DENV antibody levels decline and the child is no longer at risk for severe disease (156, 157).

During an initial infection with dengue patients often have no symptoms or they present symptoms such as fever, malaise, headache, body pains, and rash that are characteristic of DF. At this point, clinicians cannot predict if these patients will progress to severe disease (153, 158). However, most of these patients will go on to recover from the disease within a week to ten days of onset (159). Viremia is apparent 1-2 days prior to the onset of symptoms and peaks during the first 2 days of fever. About 5% of the patients that report with symptoms of DF will go on to have DHF or DSS. The critical period in DHF starts at the moment of defervesence but it is possible that haemorrhagic manifestations may occur 24 hours earlier. DHF is characterized by a high fever,

haemorrhagic manifestations, thrombocytopenia (platelet count 100,000/mm^3 or less), haemoconcentration (> 20% difference), and 10 to 100 fold higher viremia than in DF. Petechiae, epistaxis, bleeding at the venipuncture sites, gum bleeding, and haematemesis are also observed in some patients. The most significant pathophysiological even that characterizes severe disease is plasma leakage. Signs of circulatory failure are also apparent in patients with severe disease (irritability, cold extremities, restlessness, flushed face). At this point it is important to closely monitor these people for signs of progression to shock such as intense abdominal pain, persistent vomiting, a weak pulse, and hypotension (160). DSS results during the onset of increased vascular permeability and is characterized by the leakage of plasma fluids into the interstitial spaces inducing hypovolemic shock (161). Once a patient is in shock due to DENV infection it is likely that they will either survive or succumb to infection within 24 hours (162).

Treatment of DENV induced disease currently involves treating the symptoms, specifically rehydration therapy and the use of pain relievers such as acetaminophen. There have been several attempts to make antiviral therapies and vaccines. One problem that has been encountered is the need for a therapy that will protect against all four strains of DENV. Another challenge is the need for treatment early in the course of illness, often before patients have a confirmed dengue infection. At present there remains an unmet need for an effective dengue therapeutic that is able to shorten the duration of the illness, prevent the development into severe disease, and reduce the severity of common symptoms (163). There are a number of institutions, both academic and pharmaceutical, that are currently engaged in the discovery and development of DENV therapeutics (163). One encouraging area of research has been the development of therapeutic anti-

DENV monoclonal antibodies that block viral infection. Although it is unlikely that a single monoclonal antibody will be able to neutralize all four DENV serotypes, a potent neutralizing antibody cocktail is a likely candidate (130, 163). It should be remembered, however, in addition to effective therapeutic intervention we need to focus on an integrated approach that also aims to control the mosquito vector, especially in the case of *Aedes aegypti* that transmits several diseases (133).

Immune response to DENV

The first line of defense against DENV infection is the recognition of the virus by host cell molecules, specifically pattern recognition receptors such as toll-like receptors (TLRs). The TLRs that are primarily involved in dengue viral recognition are TLR-7 and TLR-3, which recognize DENV RNA after endosomal acidification (164). Recognition of viral RNA by TLR-3 results in a series of phosphorylation events that leads to the nuclear translocation of interferon regulatory factor 3 (IRF3), activator protein 1 (AP-1), and NF-κB. This induces the production of IFN-α/β, interferon stimulating genes, and chemokines (165). In addition to TLR-3, DENV is recognized by the cytoplasmic helicases retinoic-acid-inducible gene I (RIG-I) and melanoma differentiation associated gene 5 (MDA-5) to induce IFN-α/β (166). The interferon response to DENV triggers a warning signal to adjacent non-infected cells and an autocrine induction of cellular antiviral responses (167). IFN-α/β binds to its receptors on infected and neighboring cells and activates a signaling pathway that eventually leads to the induction and production of numerous antiviral proteins and pro-inflammatory cytokines (168). Another host cell type that is important during initial DENV infection is the natural killer cell, the main producers of IFN-γ (169). It is important to note that several studies have demonstrated

that DENV viral proteins NS2A, NS4A, NS4B, and NS5 are able to inhibit the IFN-α mediated innate antiviral response by blocking activation of the signal transducers and activators of transcription (STAT) signaling pathway (170-173).

The major target cells for DENV, the monocytes and dendritic cells, are antigen-presenting cells (APCs) that are critical for stimulating cell mediated immunity. Cell mediated immunity is comprised of two major subsets of T cells, CD4 and CD8. Little is known about the role of CD8+ T cells during a DENV infection but it has recently been revealed that the cytolitic effect of these cells is important in controlling a primary DENV infection (174). It has also been reported, however that cellular immunity is not fully activated during acute infections and the memory T cell activation during heterologous secondary DENV infections results in a massive production of cytokines and chemokines (175, 176). CD4+ T cells play a different role in the response to DENV by mediating cytotoxicity and producing cytokines. CD4+ T cells also activate APCs and B cells.

The humoral response to DENV infection usually occurs approximately 5-6 days post infection with DENV, with IgM antibodies typically produced first. While IgM peaks at two weeks after onset, DENV specific IgG is detectible in patients after the first week and continues to rise (153). The antibody response is mainly directed against the E and PrM structural proteins on the surface of the virus (177, 178). There are is also evidence that suggests that antibodies directed at the NS1 protein, which is expressed on the surface of infected cells and is secreted from these cells as a soluble factor (177, 179, 180). It has been demonstrated that these antibodies can activate complement-mediated lysis of DENV-infected cells and protect mice from DENV challenge (181-184). However, anti-NS1 antibodies have been reported to be cross reactive with human

platelets and endothelial cells, leading to increased vascular permeability. Antibodies to all of the defined DENV epitopes have varying degrees of cross-reactivity to different DENV serotypes as well as different neutralization potential.

Antibody neutralization of DENV occurs via multiple mechanisms. Antibodies can bind to the virus and block binding to host cell receptors. Antibodies can also bind to virus and be internalized with the virus, but block fusion with the endosomal membrane. Another possibility is that antibodies specific for DENV virions bind to the incoming virus and enhance its uptake into host cells via FcγR binding, thus is the case during ADE. ADE occurs during secondary heterotypic DENV infections and is characteristic of patients suffering from DHF or DSS. The tropism of dengue virus for monocytes, macrophages and dendritic cells, all FcγR bearing cells, creates the opportunity for DENV specific antibodies to enhance viral entry. During ADE, antibodies that are non-neutralizing or sub-neutralizing against a primary infecting virus remain active and bind to the heterotypic secondary infecting virus or viral particle. Studies with anti-E protein antibodies suggest that when virion opsonization is below the threshold necessary for virus neutralization, these antibodies participate in ADE (185). The formation of a antibody-virus complex occurs and this complex is shuttled to those FcγR bearing cells (158, 185-188). This leads to an increase in the number of cells that are infected and an increase in the number of virus particles produced per infected cell (189). DENV infection through the mechanism of ADE can induce the production of IFN-α, TNF-α, and IL-10 as well as upregulate costimulatory molecules CD40 and CD86 (190). The activation of complement by antibody-virus complexes also contributes to the disease state by activating complement, which induces temporal plasma leakage (191, 192).

In patients with DHF or DSS, ADE is often accompanied by original antigenic sin, a phenomenon wherein sequential exposure to closely a related virus reduces the novel response to the secondary virus and impairs the development of immunological memory. The hallmark of the pathogenesis of these two disease states is the loss of endothelial integrity, which is assumed to be the result of an abnormal immune response to the virus, the "cytokine storm" (143). It is believed by man scientists that original antigenic sin is responsible for the cytokine storm. During a secondary heterotypic infection low avidity memory T cells are re-activated and inefficient in clearing the new viral infection. It has been demonstrated that these T cells have suboptimal degranulation, altered cytokine production and cytolytic activity. These T cells are not only unable to efficiently clear the DENV infection but they cause a massive immune activation (175, 193-195).

Figure 9 shows an integrated model describing the immunopathogenesis of severe DENV infection. It is likely that many immunological processes contribute to DHF and DSS (143).

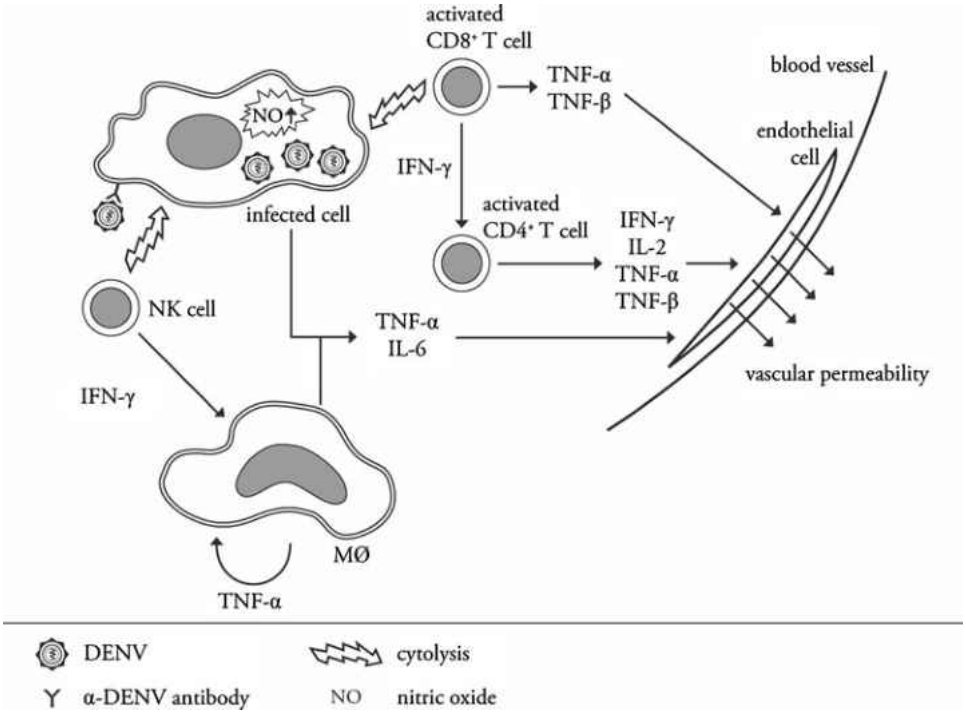


Figure 9. Immunopathogenesis of severe dengue – an integrated model.

The development of passive immunotherapy to treat DENV infections remains an elusive goal of many. In order to treat DENV effectively with antibody therapy it is necessary to determine DENV specific neutralizing epitopes. Current research is primarily focused on elucidating epitopes within the E protein. The E protein is the principal surface component of the dengue virion and is composed of three domains (DI, DII, and DIII) (196). DI is the central domain and contains non-neutralizing epitopes. Several neutralizing epitopes have been found within the conserved fusion loop region in DII and the lateral ridge and fusion loop of DIII (196-207). A smaller body of research suggests that there are protective epitopes within PrM and some of the nonstructural proteins, specifically NS1, NS3, and NS5 (196, 206, 208-213). All of the characterized DENV epitopes have been revealed using either mammalian or murine antibodies, both of which have reactive Fc portions. The problem with this trait is that if used for treatment they have the ability to complex with virus and interact with FcγR on target cells, monocytes, macrophages and dendritic cells, and potentially induce ADE.

In a recent study, Balsitis *et al.* verified using aglycosylated and $F(ab')_2$ DENV specific IgG that the interaction between the Fc portion of the serotype cross-reactive antibodies and the FcγR is required to induce ADE. Additionally they demonstrated that the aglycosylated DENV-specific antibodies were as neutralizing as their naturally glycosylated (or non-modified) variants, were incapable of inducing ADE, and were therapeutic following a lethal, antibody-enhanced infection when administered up to 48 hours later (214). We, therefore, hypothesized that avian IgY, that do not interact with mammalian FcγR, would provide a novel therapy for DENV induced disease.

Preliminary studies with West Nile Virus IgY

There are many advantages to using IgY for the treatment of DENV infections. One possibility is that IgY will be able to neutralize a viral infection in the absence of ADE. IgY is also very well tolerated in humans, and can be produced in large quantities very efficiently. Past research in our lab has exploited geese as a source of IgY. Initial studies aimed to determine if the serum from West Nile Virus (WNV) immune geese could protect against WNV infection in geese. Geese were given immune serum prior to and post WNV infection and mortality was assessed. Of the ~10,000 geese per group there was a ~65% reduction in mortality when administered as a prophylactic and ~62% reduction in mortality when administered as a treatment (unpublished data Schiltz, Petell, & Bradley). These experiments were repeated with purified anti-WNV IgY and similar results were observed. Purified anti-WNV IgY was also tested in WNV infected golden hamsters. All of the hamsters treated with the anti-WNV IgY survived and had viral titers of zero, compared to the sham treated hamsters who had 100% mortality and ~65% viral titer (unpublished data Schiltz, Petell, & Bradley).

DENV2 epitope mapping of goose-derived IgY: IgY and IgYΔFc

The aim of the current research study was to identify anti-DENV2 IgY protective epitopes and determine the ability of these specific antibodies to treat DENV2 infections. Epitope mapping was performed using 4 different viral proteins: E protein, PrM protein, NS1, and NS3 with either anti-DENV2 IgY or anti-DENV2 IgYΔFc. Here we present data suggesting that different populations of IgY recognize different DENV epitopes and IgY recognizes epitopes uncharacterized for mammalian or murine IgG.

Materials and Methods

Geese

Ten geese were vaccinated with 120 µg of Dengue Killed Virus (Dengue Type 2 Antigen, Microbix Biosystems Inc.) on Day 0 and 60 µg boost immunizations on week 2 and 4. Immunizations consisted of 2 x 200 µL subcutaneous injections at the back of the neck in two different injection spots. The eggs were collected starting from week 4 after the first immunization and stored at 4°C till further use.

Purification of IgY and IgY(DFc) from Goose Egg Yolk

Yolks were isolated and rinsed with water and then punctured to drain the contents and diluted 1:10 with cold deionized water, stirred, and acidified to pH 5.0. The diluted yolk was centrifuged at 10,000 x g for 30 minutes, and the supernatant was filtered. In order to separate the full-length IgY from the transcriptionally truncated IgYΔFc a sequential series of 30%, 40% and 50% ammonium sulfate were used. The pellets were suspended in 50 mM Tris HCl pH 8.0. Further purification was achieved via hydrophobic charge induction chromatography on 4-Mercapto-Ethyl-Pyridine-linked (MEP) HyperCel sorbent (Pall Corporation) followed by buffer exchange.

Antibody Detection

The antibody activity of anti-DENV2 was determined by ELISA. Briefly, microtiter plates were coated with 100 ml of the capture antigen (Dengue Type 2 Antigen, Microbix Biosystems Inc) and stored at 4°C overnight. After washing the plates 3 times with wash buffer (1X PBS, 0.05% Tween-20 (Fisher Scientific), they were blocked with 400 mL per well of blocking buffer (0.25% BSA (Fisher Scientific), 0.05% Tween-20 (Fisher Scientific)1X PBS) and incubated for 30 minutes at room temperature.

The wells were washed 3 times with wash buffer and incubated with 100 mL of diluted goose antibody and serially diluted down the plate in blocking buffer and incubated at 37°C for 30 minutes. Proper dengue control and Naïve control antibodies were included as standards on each plate. The plates were washed 3 times with wash buffer and blocked for 10 minutes at room temperature. Next, 100 mL of biotinylated rabbit anti-goose IgY antibody was added to each well and incubated at 37°C for 30 minutes. After washing the plates 3 times with wash buffer the wells were blocked for 10 minutes at room temperature. Following this, 100 mL of diluted strepavidin-HRP antibody in blocking buffer (1:2000) was added to each well and the plates were incubated at 37°C for 30 minutes. The plates were finally washed 3 times before adding 100 mL of prepared OPD color substrate to each well and allowed to develop for 15 minutes at room temperature. The reaction was terminated by adding 50 mL of 1N H_2SO_4, and the absorbance read in BioTek plate reader at A_{490}.

***In Vitro* Viral Neutralization and Antibody Dependent Enhancement studies**

The neutralization and enhancement titers for anti-DENV2 and control purified polyvalent IgY sera against DENV2 D2S10 were determined. D2S10 is a DENV2 strain developed in the lab of Dr. Eva Harris in the Division of Infectious Diseases and Vaccinology, School of Public Health, University of California, Berkeley, Berkeley, CA (215). Both neutralization and enhancement experiments were performed twice, each time in duplicate. In brief, the sera were diluted to a starting concentration of 2.0 mg/mL. Twelve 4-fold dilutions were mixed with equivalent volumes of DENV2 D2S10 for 45 minutes before infecting U937 DC-SIGN cells, a DENV-permissive monocytic cell line. The cells were washed two hours following infection, and then fixed and stained for

DENV E protein 24 hours later. The data was analyzed by flow cytometry, and the dilution yielding 50% neutralization (NT_{50}) was calculated using GraphPad PRISM.

To test for potential enhancement, the serum was diluted and mixed with DENV2 as described above and used to infect K562 cells, an erythroleukemic cell line that is not naturally permissive for DENV infection, but can be infected via surface FcγRIIA when DENV virions are coated with sub-neutralizing concentrations of anti-DENV antibody. The cells were fixed, stained, and analyzed as described above 48 hours following infection.

***In Vivo* anti-DENV2 IgY neutralization**

The therapeutic potential of goose-derived anti-DENV2 IgY was tested using conditions that cause 100% mortality in AG129 mice. Six-eight week old IFN-αβR$^{-/-}$ and IFN-γR$^{-/-}$ (AG129) mice were administered a sub-lethal intravenous infection with D2S10 ($2x10^5$ plaque forming units (PFU)). Twenty-four hours after infection, mice were injected i.p. with the indicated amounts of polyclonal anti-DENV2 IgY or the positive control mouse monoclonal antibody (MAb) E60 N297Q in a volume of 200 μL, or 200 μL of PBS as a negative control. Mice were followed for 10 days and observed for morbidity and mortality twice daily. Anti-DENV2 IgYwas administered as 20, 100, or 500 μg per injection, control naïve IgY was administered as 500μg per injection, and the positive control IgG E60 N297Q was administered as 20μg per injection.

Epitope mapping

Anti-DENV2 IgY epitopes were mapped on the E, PrM, NS1 and NS3 proteins via peptide arrays. Specifically, each protein was covalently attached in 11 amino acid

overlapping 15mer peptides to a microarray slide (JPT Innovative Peptide Solutions, Berlin, Germany). All Pepstar™ microarray protocols were provided by JPT. Briefly, a slide sandwich containing the microarray and a dummy slide was made, separated by spacers, in order to increase and incubation environment. The primary antibody serum was incubated on the slide at 4°C overnight in a moist environment. The slide was rinsed 5 times for 4 minutes each with T-TBS, then 5 times for 4 minutes each with ultra pure water. The slide was incubated in the fluorescently labeled (Cy5) secondary antibody solution (1μg/mL) for 45 minutes, washed 5 times with T-TBS, then 5 times with ultra pure water, and dried using a dust free, oil free, high velocity canned air. Fluorescence was measured at a pixel sized of 10μm using the Genepix™ 4000 microarray reader. The signal intensity mean values were calculated for each sub-array and background corrected values were used for interpretation in Microsoft excel. The microarray experiment was repeated with each antibody type on three separate but identical slides; anti-DENV full length IgY, anti-DENV IgYΔFc, and control naïve IgY.

Purification of DENV2 epitope specific IgY and IgYΔFc

Careful analysis of the epitope mapping data revealed several epitopes that were recognized by either the IgY or IgYΔFc with at least 2x higher MFI than the naïve control IgY. We selected peptides within the NS1 proteins to use for generating a population of peptide/epitope specific IgY. This was carried out using an AminoLink coupling resin Kit (Thermo Scientific). The AminoLink aldehyde activated agarose bead resin was suspended by end over end mixing and then centrifuged within a 15 mL conical at 1000 x g for 1 minute. Following, 2 mL of pH 7.2 coupling buffer was added to the column and centrifuged at 1000 x g for 1 minute, this step was repeated once. The bottom

of the column was capped and 2mL (1mg/mL) of the specific peptide solution was added to the column (peptides were synthesized by GenScript). In a fume hood, 40 µL of sodium cyanoborohydride solution was added to the column. The top cap was replaced and the column was mixed by end over end mixing overnight at room temperature. The next day the caps were removed and the column was centrifuged at 1000 x g for 1 minute to collect the unbound peptide. The flow through was collected to determine coupling efficiency. The column was washed with 2 mL quenching buffer and centrifuged at 1000 x g for 1 minute, this step was repeated once. Following this 2 mL of quenching buffer and 40 µL of sodium cyanoborohydride was added to the column and the column was mixed by end over end rocking for 30 minutes at room temperature to block the remaining active sites. The column was then centrifuged at 1000 x g for 1 minute to remove the quenching buffer. The column was washed 4 times with wash buffer and centrifugation at 1000 x g for 1 minute each time. 2 mL of undiluted IgY or IgYΔFc was added to each of the respective columns and the sample was allowed to enter the resin. 0.2 mL of binding/wash buffer was added to the column and the column mixed by end over end rocking at room temperature for 1 hour. To wash the resin, 2mL of binding/wash buffer was added and the column was centrifuged at 1000 x g for 1 minute, this step was repeated once. The flow through was collected to determine binding efficiency. The column washed 4 times with wash buffer. To elute the antibodies 2 mL of elution buffer was added to the column and centrifuged at 1000 x g for 1 minute, this step was repeated twice. The column was equilibrated with 4 mL wash buffer and stored at 4°C.

Plaque reduction neutralization test (PRNT)

Baby hamster kidney (BHK) cells were obtained from ATCC and grown in DMEM with 5% FBS and 1x pen/strep in a tissue culture flask at 37°C. Cells were plated into 6 well tissue culture plates and incubated at 37°C and 5% CO_2. Cells were allowed to grow to be 95-100% confluent. On the day of the experiment 1:15,000 dilution of DENV2 D2S10 was made and aliquoted in 2mL aliquots into sterile 15 mL conicals. 100 µL of each peptide specific antibody to be tested was added to the respective virus aliquots and incubated at 37°C and 5% CO_2 for one hour. Just prior to this the media in the 6 well plates was aspirated and the cells were washed once with 2 mL/well of growth media. The 2 mL DENV2 D2S10 – antibody mixtures were added to each well. Virus only and media only wells were used for controls. The plate was incubated at 37°C and 5% CO2 for 90 minutes. The media was aspirated and 3 mL of 2% methylcellulose overlay was added to each well. The plate was incubated for 7 days at 37°C and 5% CO2. On day 7 the overlay was aspirated and 1 mL of buffered formalin (1 part 10% buffered formalin to 1 part PBS) was added to each well and allowed to incubate at room temperature for 30 minutes. The formalin was aspirated and crystal violet was added to each well. The crystal violet was removed and the wells were washed with distilled water. Plaques were counted to determine antibody neutralization capacity.

Results

Antibody characterization

Following the purification of anti-DENV2 IgY and ELISA was performed to confirm the presence of DENV2 specific IgY and to determine the antibody titer. Egg yolk titers are indicative of serum titers because antibodies are transferred from the serum

of the laying hen to the egg yolk during embryogenesis. Of the 80 eggs that were measured, the average serum titer was 1:850,000 with the highest titer being 1:3,800,000 (Figure 10).

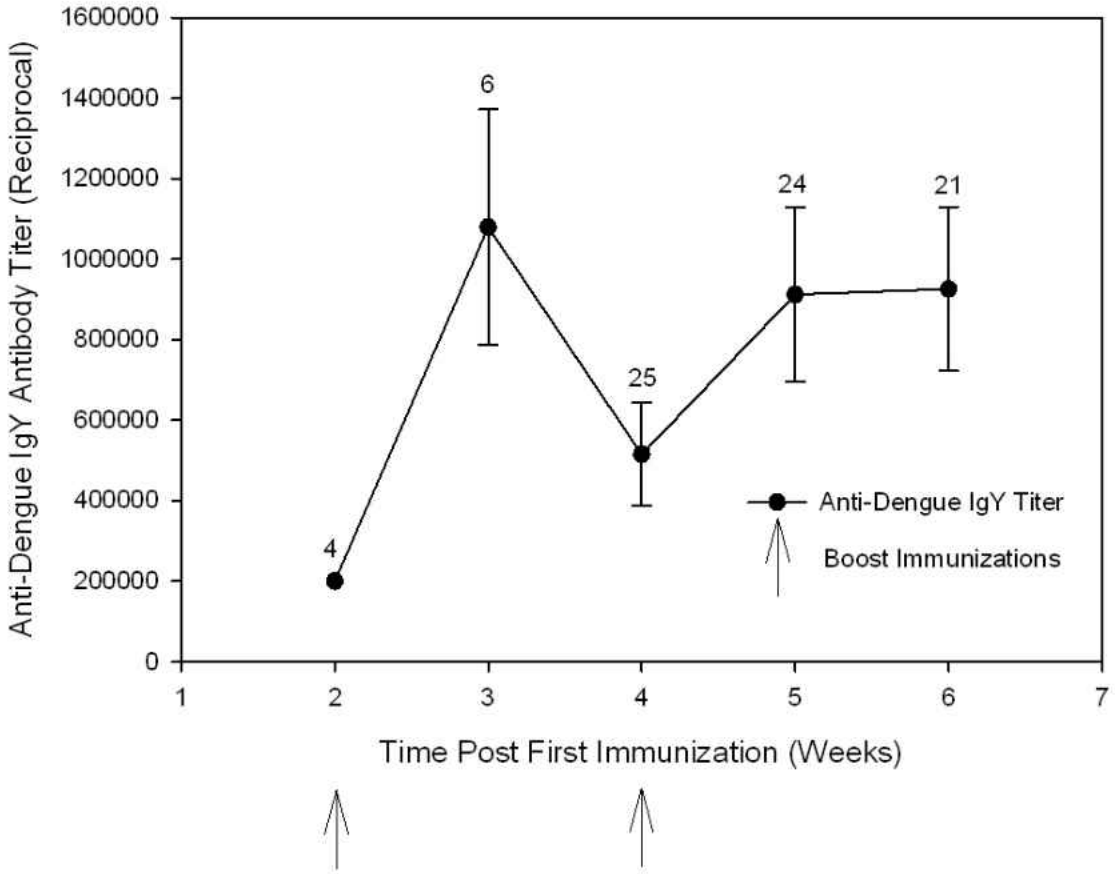
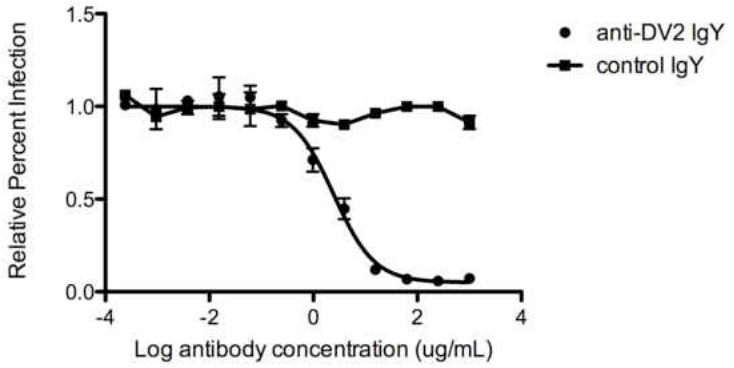


Figure 10. Anti-DENV2 IgY antibody titer in egg yolk. Egg yolks were collected from geese immunized with DENV2 killed virus. Arrows indicate boost immunizations at 2 and 4 weeks post first immunization. Anti-Dengue IgY antibody titer did not differ among weeks (ANOVA on ranks, $p = 0.157$). Data presented as mean ± SE. Sample sizes presented within figure.

In Vitro Viral Neutralization and Antibody Dependent Enhancement

Polyvalent anti-DENV2 IgY were purified from serum of DENV2-immunized geese. These antibodies were tested for potential enhancement and neutralization *in vitro*. Anti-DENV2 IgY was mixed with DENV2 D2S10 and used to infect U937 DC-SIGN (neutralization) or K562 (enhancement) cells. The cells were washed, fixed, stained for DENV E protein. The data was analyzed by flow cytometry. For the neutralization experiment the dilution yielding 50% neutralization (NT_{50}) was calculated using GraphPad PRISM. The anti-DENV2 IgY serum NT_{50} was determined to be 1.0 and 2.6 μg/mL in two independent experiments (Figure 11). The control IgY serum did not yield a measurable NT_{50} titer in either experiment. In the enhancement experiment, while the positive control anti-DENV monoclonal antibody resulted in ~ 15% infection at its peak enhancement titer, neither the anti-DENV IgY nor control sera were enhancing across any dilution tested (figure 11).

A.



B.

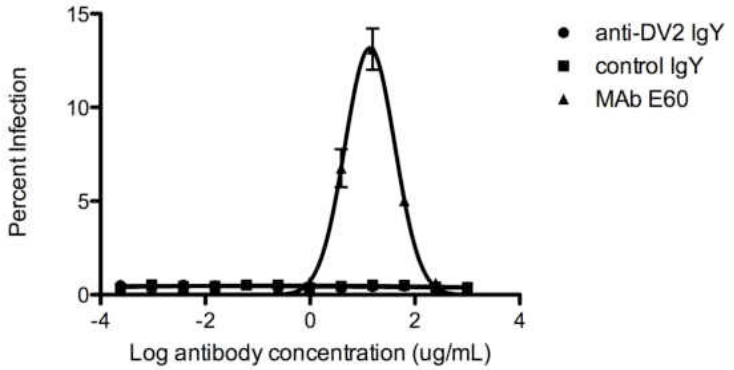


Figure 11. Anti-DENV2 purified IgY neutralizes but does not enhance DENV2 D2S10 *in vitro*. **A)** Anti-DENV2 IgY (NT_{50} 2.6μg/mL), but not control, purified IgY neutralized DENV2 D2S10. Relative percent infection is shown on the y-axis, and log reciprocal antibody concentration on the x-axis. The data are representative of two independent experiments. **B)** Neither anti-DENV2 IgY nor control purified IgY enhanced DENV2 D2S10, whereas control MAb E60 generated ~ 15% enhancement at the peak enhancing titer. Percent infection is shown on the y-axis and log reciprocal antibody concentration on the x-axis. These data are representative of two independent experiments.

In Vivo anti-DENV2 IgY neutralization

AG129 mice were challenged with a lethal dose of D2S10 and the *in vivo* neutralization capacity of anti-DENV2 was determined. In Figure 12, we have combined the results of six different experiments using a lethal dose ($1.0x10^7$ PFU) of DENV2 D2S10. We consistently observed therapeutic efficacy with 2 mg anti-DENV2 IgY (n=13) administered 24 hours post-infection, similar to the 100% therapeutic protection observed with our positive control, 20 mg of MAb E60-N297Q (n=6). However, 2 mg of control IgY (n=16) provided 50% therapeutic protection. Therefore, we tested 1 mg of anti-DENV2 IgY, which also provided 100% protection (n=8); however, administration of 1 mg of control IgY yielded 25% protection (n=4). These results indicate to us that some non-specific protection may be provided by large amounts of goose antibodies regardless of specificity, in this model. The dose of 500 mg anti-DENV2 IgY (n=6) provided 66% protection, and the dose of 50 mg anti-DENV2 IgY (n=6) provided 33% protection, whereas 50 mg of control IgY (n=2) provided no protection. The viral dose was lethal in all the mice that received only PBS 24 hours post-infection (n=8).

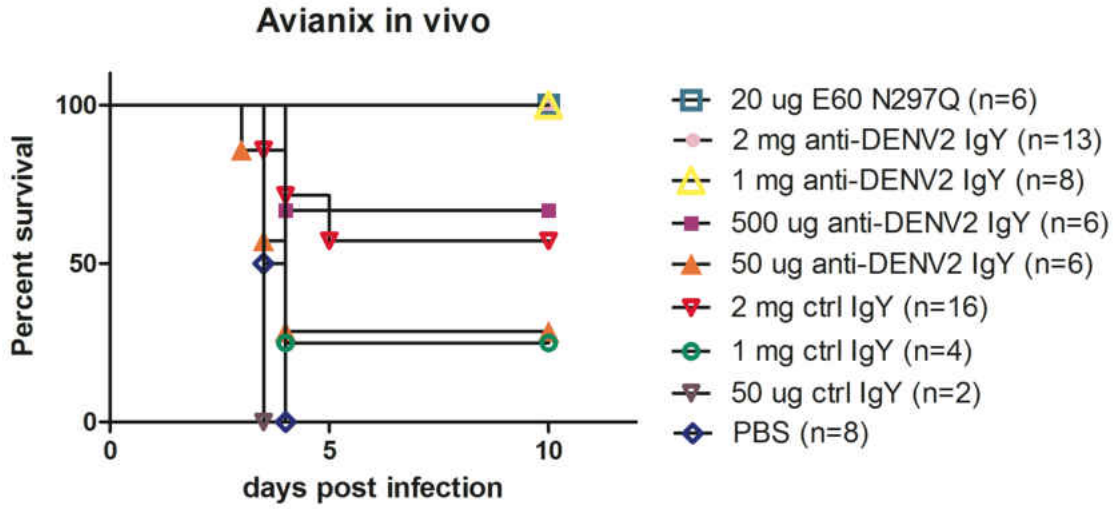


Figure 12. Therapeutic efficacy of anti-DENV2 *in vivo*. 6-8 week old AG129 mice were administered a lethal dose ($1X10^7$ pfu) of DENV2 D2S10 i.v. in a volume of 100 mL. At 24 hours p.i. mice were injected one time i.p. with the indicated amounts of antibody in a volume of 200 mL and 200mL of PBS was administered to a group of mice as a negative control. Mice were followed for 10 days and observed for morbidity and mortality twice daily. Number of mice per group is indicated.

DENV2 IgY epitopes

Anti-DENV2 IgY epitopes were mapped on the E protein, PrM protein, and NS1 and NS3 proteins. Full length anti-DENV2 IgY and alternatively spliced anti-DENV2 IgY epitopes were compared to each other and to previously characterized anti-DENV2 IgG epitopes. Our results suggest that there are both similar and different epitopes recognized by either full length or alternatively spliced anti-DENV2 IgY. Similarly, anti-DENV2 IgY recognized different epitopes than anti-DENV2 IgG. We noticed that naïve IgY recognized several of the same epitopes that both full length and alternatively spliced IgY recognized, however there were epitopes recognized that were unique to either of the anti-DENV2 IgY populations.

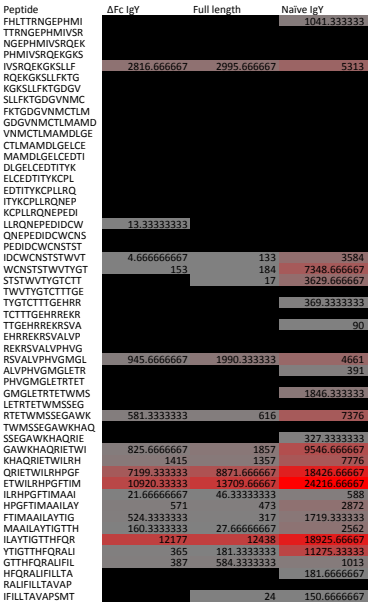
Heat maps were generated to display the epitope mapping data. Heat maps display the MFI in a color gradient. The red color represents strong binding of our antibody to the indicated peptide (Figures 13-16).

Figure 13. Identification of DENV2 NS3 epitopes. Amino acid sequence for NS3 was used to make 15-mer peptides for microarray slide. Slides were incubated with anti-DENV2 IgYΔFc, anti-DENV2 IgY full length, or naïve control IgY. Reactivity is measured based on a spectrum ranging from no reactivity in black to high reactivity in red. Values represent MFI-background.

Peptide	ΔFc IgY	Full length	Naïve IgY
IGISNRDFVEGVS			
SNRDFVEGVSGGS			
DFVEGVSGGSWVD		344.3333333	3316.666667
EGVSGGSWVDIVL	532	527.6666667	1706.666667
SGGSWVDIVLEHG	1835.333333	118.6666667	528
SWVDIVLEHGSCV	163.6666667		81.66666667
DIVLEHGSCVTTM			
LEHGSCVTTMAKN			
GSCVTTMAKNKPT			
VTTMAKNKPTLDF			
MAKNKPTLDFELI		299.6666667	1171.333333
NKPTLDFELIKTE		34	
TLDFELIKTEAKQ	82	43.33333333	726
FELIKTEAKQSAT			
IKTEAKQSATLRK	133.3333333		
EAKQSATLRKYCI	467	375.6666667	2203.333333
QSATLRKYCIEAK	1074.666667	967	1416
TLRKYCIEAKLTN	549.6666667	2469.333333	2711.333333
KYCIEAKLTNTTT		1202.666667	
IEAKLTNTTTESR			
KLTNTTTESRCPT			
NTTTESRCPTQGE			
TESRCPTQGEPSL			
RCPTQGEPSLNEE			
TQGEPSLNEEQDK			
EPSLNEEQDKRFV	612.6666667		
LNEEQDKRFVCKH			
EQDKRFVCKHSMV	2038.666667	421.3333333	5295
KRFVCKHSMVDRG			
VCKHSMVDRGWGN			
HSMVDRGWGNGCG			
VDRGWGNGCGLFG			722
GWGNGCGLFGKGG			
NGCGLFGKGGIVT			1490.666667
GLFGKGGIVTCAM			
GKGGIVTCAMFTC			979.3333333
GIVTCAMFTCKKD			1313.666667
TCAMFTCKKDMKG			
MFTCKKDMKGEVV			
CKKDMKGEVVQPE			
DMKGEVVQPENLE	1649.333333		
GEVVQPENLEYTI	5304.666667	224.3333333	
VQPENLEYTIVIT	749	307.6666667	
ENLEYTIVITPHS	118.3333333	75.33333333	
EYTIVITPHSGEE			
IVITPHSGEEHAV	774.3333333	22	
TPHSGEEHAVGND			
SGEEHAVGNDTGK			
EHAVGNDTGKHGK	52		
VGNDTGKHGKEIK			
DTGKHGKEIKITP			
KHGKEIKITPQSS			
KEIKITPQSSITE			
KITPQSSITEAEL			
PQSSITEAELTGY			
SITEAELTGYGTV			
EAELTGYGTVTME			
LTGYGTVTMECSP			
YGTVTMECSPRTG			
VTMECSPRTGLDF			
ECSPRTGLDFNEM			
PRTGLDFNEMVLL		192.3333333	1698.333333
GLDFNEMVLLQME			173.6666667
FNEMVLLQMENKA			
MVLLQMENKAWLV	837.3333333	462	1886.333333
LQMENKAWLVHRQ	1538.666667	1693.666667	10980
ENKAWLVHRQWFL	12062.33333	12182	20134.66667
AWLVHRQWFLDLP	10184	12098.33333	33207.33333
VHRQWFLDLPLPW	21589.33333	17183	43312.66667
QWFLDLPLPWLPG	931	953.6666667	3694
LDLPLPWLPGADT		26.66666667	1473.666667
PLPWLPGADTQGS			
WLPGADTQGSNWI	31.33333333		1351.666667
GADTQGSNWIQKE			
TQGSNWIQKETLV			
SNWIQKETLVTFK	147	240	1736.666667
IQKETLVTFKNPH			
ETLVTFKNPHAKK			
VTFKNPHAKKQDV			
KNPHAKKQDVVVL	376.6666667	108.6666667	
HAKKQDVVVLGSQ			
KQDVVVLGSQEGA			
VVVLGSQEGAMHT			
LGSQEGAMHTALT			
QEGAMHTALTGAT			
AMHTALTGATEIQ			
TALTGATEIQMSS			
TGATEIQMSSGNL			

Peptide	ΔFc IgY	Full length	Naïve IgY
TEIQMSSGNLLFT			
QMSSGNLLFTGHL	2285.666667	3923.666667	11835
SGNLLFTGHLKCR	7286.666667	5230.666667	14341
LLFTGHLKCRLRM	8181.333333	7540.666667	8165
TGHLKCRLRMDKL	3312.666667	629.3333333	337.6666667
LKCRLRMDKLQLK	736	660	919
RLRMDKLQLKGMS	77	65.66666667	94.66666667
MDKLQLKGMSYSM	2103.666667	1543.666667	4902.666667
LQLKGMSYSMCTG	384.6666667	302.6666667	3306.333333
KGMSYSMCTGKFK	5106.666667	3678.333333	
SYSMCTGKFKVVK	2136.333333	358	267
MCTGKFKVVKEIA			
GKFKVVKEIAETQ			
KVVKEIAETQHGT			
KEIAETQHGTIVI			88.33333333
AETQHGTIVIRVQ			
QHGTIVIRVQYEG	412.6666667		
TIVIRVQYEGDGS			
IRVQYEGDGSPCK			
QYEGDGSPCKIPF			
GDGSPCKIPFEIM			
SPCKIPFEIMDLE			
KIPFEIMDLEKRH			
FEIMDLEKRHVLG			
MDLEKRHVLGRLI	837.3333333	3668.333333	2100.666667
EKRHVLGRLITVN	2990	3651.333333	4237.666667
HVLGRLITVNPIV	156.3333333	708.6666667	2966
GRLITVNPIVTEK			
ITVNPIVTEKDSP			
NPIVTEKDSPVNI		633.6666667	821.6666667
VTEKDSPVNIEAE			
KDSPVNIEAEPPF	163.6666667		
PVNIEAEPPFGDS			
IEAEPPFGDSYII			
EPPFGDSYIIGV	383		3911
FGDSYIIGVEPG		475	3592.666667
SYIIGVEPGQLK			218.6666667
IIGVEPGQLKLNW		1113	3925.666667
VEPGQLKLNWFKK	2735.666667	2675.333333	10662.66667
GQLKLNWFKKGSS	164	42.66666667	614.3333333
KLNWFKKGSSIGQ	537.3333333		52
WFKKGSSIGQMLE	2336.333333	41	
KGSSIGQMLETTM			
SIGQMLETTMRGA			
QMLETTMRGAKRM			
ETTMRGAKRMAIL	586.6666667	24.33333333	501
MRGAKRMAILGDT			
AKRMAILGDTAWD			
MAILGDTAWDFGS		79.33333333	3234.666667
LGDTAWDFGSLGG			
TAWDFGSLGGVFT	168.6666667	1045.333333	12727.66667
DFGSLGGVFTSIG			2520.333333
SLGGVFTSIGKAL			
GVFTSIGKALHQV			777.3333333
TSIGKALHQVFGA	363	187	
GKALHQVFGAIYG	5134.333333	4906.333333	17752.33333
LHQVFGAIYGAAF	813	698.3333333	3921.666667
VFGAIYGAAFSGV	238	195.6666667	2313.333333
AIYGAAFSGVSWT		180	5484
GAAFSGVSWTMKI	87.33333333	197.6666667	2786.666667
FSGVSWTMKILIG	2774	2295.666667	5333.666667
VSWTMKILIGVII	178.6666667	222.6666667	959.6666667
TMKILIGVIITWI	155.3333333	175.3333333	1799.666667
ILIGVIITWIGMN		41.33333333	172.6666667
GVIITWIGMNSRS			
ITWIGMNSRSTSL			
IGMNSRSTSLSVS			
NSRSTSLSVSLVL		49.66666667	29.33333333
STSLSVSLVLVGV	548	735.6666667	1613
LSVSLVLVGVVTL	107	140.3333333	243.6666667
SLVLVGVVTLYLG			
LVGVVTLYLGVMV	904.6666667	427.3333333	1827.666667
GVVTLYLGVMVQA			521

Figure 14. Identification of DENV2 E protein epitopes. Amino acid sequence for E protein was used to make 15-mer peptides for microarray slide. Slides were incubated with anti-DENV2 IgYΔFc, anti-DENV2 IgY full length, or naïve control IgY. Reactivity is measured based on a spectrum ranging from no reactivity in black to high reactivity in red. Values represent MFI-background.



Peptide	ΔFc IgY	Full length	Naïve IgY
FHLTTRNGEPHMI			1041.333333
TTRNGEPHMIVSR			
NGEPHMIVSRQEK			
PHMIVSRQEKGKS			
IVSRQEKGKSLLF	2816.666667	2995.666667	5313
RQEKGKSLLFKTG			
KGKSLLFKTGDGV			
SLLFKTGDGVNMC			
FKTGDGVNMCTLM			
GDGVNMCTLMAMD			
VNMCTLMAMDLGE			
CTLMAMDLGELCE			
MAMDLGELCEDTI			
DLGELCEDTITYK			
ELCEDTITYKCPL			
EDTITYKCPLLRQ			
ITYKCPLLRQNEP			
KCPLLRQNEPEDI			
LLRQNEPEDIDCW	13.33333333		
QNEPEDIDCWCNS			
PEDIDCWCNSTST			
IDCWCNSTSTWVT	4.666666667	133	3584
WCNSTSTWVTYGT	153	184	7348.666667
STSTWVTYGTCTT		17	3629.666667
TWVTYGTCTTTGE			
TYGTCTTTGEHRR			369.3333333
TCTTTGEHRREKR			
TTGEHRREKRSVA			90
EHRREKRSVALVP			
REKRSVALVPHVG			
RSVALVPHVGMGL	945.6666667	1990.333333	4661
ALVPHVGMGLETR			391
PHVGMGLETRTET			
GMGLETRTETWMS			1846.333333
LETRTETWMSSEG			
RTETWMSSEGAWK	581.3333333	616	7376
TWMSSEGAWKHAQ			
SSEGAWKHAQRIE			327.3333333
GAWKHAQRIETWI	825.6666667	1857	9546.666667
KHAQRIETWILRH	1415	1357	7776
QRIETWILRHPGF	7199.333333	8871.666667	18426.66667
ETWILRHPGFTIM	10920.33333	13709.66667	24216.66667
ILRHPGFTIMAAI	21.66666667	46.33333333	588
HPGFTIMAAILAY	571	473	2872
FTIMAAILAYTIG	524.3333333	317	1719.333333
MAAILAYTIGTTH	160.3333333	27.66666667	2562
ILAYTIGTTHFQR	12177	12438	18925.66667
YTIGTTHFQRALI	365	181.3333333	11275.33333
GTTHFQRALIFIL	387	584.3333333	1013
HFQRALIFILLTA			181.6666667
RALIFILLTAVAP			
IFILLTAVAPSMT		24	150.6666667

Figure 15. Identification of DENV2 PrM epitopes. Amino acid sequence for PrM was used to make 15-mer peptides for microarray slide. Slides were incubated with anti-DENV2 IgYΔFc, anti-DENV2 IgY full length, or naïve control IgY. Reactivity is measured based on a spectrum ranging from no reactivity in black to high reactivity in red. Values represent MFI-background.

Figure 16. Identification of DENV2 NS1 epitopes. Amino acid sequence for NS1 was used to make 15-mer peptides for microarray slide. Slides were incubated with anti-DENV2 IgYΔFc, anti-DENV2 IgY full length, or naïve control IgY. Reactivity is measured based on a spectrum ranging from no reactivity in black to high reactivity in red. Values represent MFI-background.

Determination of anti-DENV IgY neutralizing epitopes

We selected peptides where either anti-DENV2 IgY or IgYΔFc binding to that spot generated a MFI that was at least 2x greater than the positive control peptide. These peptides were used in an AminoLink affinity purification column to purify antibodies that specifically recognize that peptide (peptide specific antibody). To test the neutralization potential of these peptide specific IgY antibodies we used a PRNT assay. Preliminary data from these experiments suggest that peptide specific IgY antibodies may be able to neutralize DENV infection in vitro. Further studies will determine if neutralization is greater than the polyclonal combined anti-DENV2 IgY and what epitopes have the greatest neutralization capacity.

Discussion

In this study we demonstrate that anti-DENV2 IgY purified from goose egg yolk is effective in neutralizing DENV2 D2S10 viral infection both *in vitro* and *in vivo*, in the absence of ADE. Vaccination with the DEVN2 antigen induced a strong humoral response in the geese, with titers maintained for over six weeks and reaching as high as 1: 3,800,000 (figure). We consistently observed therapeutic efficacy with 2 mg anti-DENV2 IgY administered 24 hours post infection, similar to the 100% therapeutic protection observed with the MAb E60-N297Q positive control. Our results also indicate some non-specific protection that may be provided by large amounts of naïve goose IgY as indicated by both the *in vivo* challenge data and the epitope mapping. Experiments that were performed prior to epitope mapping were done using the total combined full length IgY and IgYΔFc polyclonal antibody population. Further studies will determine the

neutralization capacity of full length IgY compared to IgYΔFc as well as the neutralization capacity of the epitope specific affinity purified IgY and IgYΔFc.

At present, there are no licensed therapies or vaccines for the protection of DHF or DSS, the severe manifestations of DENV infection. The development of a vaccine has been problematic, in part due to the possible risk of eliciting suboptimal immune responses that will lead to ADE and severe disease following infection with heterologous virulent strains. In the absence of an effective vaccine, passive immunotherapy with neutralizing antibodies may provide an alternative for the treatment of dengue. Our data suggests that ADE does not occur when anti-DENV2 IgY is administered as a treatment for dengue. This characteristic is especially advantageous because it does not require any genetic modification or engineering to prevent enhancement, unlike other non-avian antibody therapies (214). It has been suggested that ADE results in increased viral load, increased activation of cytokines, and the activation of complement. All of these phenomena taking place simultaneous to the immune response to the actual infection increase the likelihood of vascular leakage and tissue damage. When tissue is damaged and the vasculature inadequate, the recruitment of immune mediators is also occurring at these organ sites. This massive increase in cytokines actually becomes detrimental to the patient and may hamper the clearance of the virus. Likewise, activation of the immune system may remain high after viral clearance. The combined immune response to DENV, specifically during heterotypic secondary infections elucidates the need to develop therapeutics that will not induce ADE, such as anti-DENV2 IgY.

Humanized anti-DENV MAbs obtained from mice or non-human primates have been produced to treat dengue, but functionally the majority of these antibodies are

weakly neutralizing and serotype cross reactive (188, 206, 216). Potently neutralizing human MAbs are rare indicating that only a small fraction of the total antibody response during natural infection is responsible for virus neutralization. We have done extensive epitope mapping of three complete DENV proteins; E protein, PrM protein, NS1, and NS3. Our results suggest that naïve IgY and anti-DENV2 IgY recognize similar epitopes, but anti-DENV2 IgY also recognizes unique epitopes. The majority of the unique epitopes were located within the E protein, with some located in the NS3 protein. These data are consistent with the current literature suggesting that the most neutralizing epitopes are located within the E protein. In contrast, the NS3 epitopes presented are uncharacteristic and may be potential neutralizing epitopes that have yet to be exploited by other research groups. It is important to note that all of the unique anti-DENV2 IgY epitopes, both full length IgY and IgYΔFc are epitopes that have yet to be published as neutralizing epitopes for any mammalian or murine MAbs. This confirms that IgY recognizes different DENV2 epitopes than IgG, and therefore the antibody population generated in geese is different from what would be generated for production of human anti-DENV MAbs.

Conclusions

Our results suggest that anti-DENV2 IgY is capable of neutralizing but not enhancing a DENV2 infection. The anti-DENV2 IgY administered to mice post lethal challenge with D2S10 is protective. We have confirmed that the antibody repertoire generated against DENV in geese is different from what occurs in mammals, and thus provides an increase in potential viral neutralization. Future research will focus on

characterizing the neutralization and therapeutic capacity of the affinity purified epitope specific anti-DENV2 IgY.

CHAPTER V

DISCUSSION

The development of bioavailable, passive immunotherapies to treat infectious diseases remains an elusive goal. In recent years, passive immunization with avian derived antibodies has emerged as an attractive alternative to mammalian derived antibodies to treat disease. Most of the polyclonal antibodies used in passive therapeutics are of human origin (4). In order to obtain sufficient antibody titers, pre-screened volunteer human donors are generally immunized with the antigen, and their plasma is then collected. Unfortunately, there are substantial restrictions to this method including the limitations to the types of vaccines used, the number of immunizations permitted, the adjuvant used, and the amount of plasma that can be collected (7). Furthermore, humanized or human derived antibodies are highly susceptible to unwanted interactions with conserved proteins, which in turn leads to unwanted immune mediated pathologies (9). In addition, human-derived antibodies pool are also potentially contaminated with other unwanted infectious agents, putting the recipient of the immunotherapy at risk of potentially life threatening infections. The development of unconventional methods of generating antibodies that are not only suitable for use as therapeutics but also cost and quantity efficient is an area of active research.

IgY, the major serum immunoglobulin in birds, is transferred from the serum to the egg yolk during embryonic development. The natural deposition of IgY into the egg

yolks of immunized birds provides both an excellent source of polyclonal antibodies but also facilitates a less invasive technique for collecting these antibodies (27, 44, 9). In addition, the sustained high titers of IgY in immunized birds provide a long-term supply of substantial amounts of antibody (9).

In an attempt to increase the potential application of IgY therapeutics, one of our research goals was to determine the bioavailability of IgY following oral administration. As stated above, there are currently several disease agents that have been utilized in the production of antigen specific IgY for passive oral therapeutics. For the first time, our research suggests that IgY is bioavailable as soon as 24 hours post oral administration, and under specific biochemical conditions remains in the serum for up to 7 days. These data suggest that IgY is able to cross the epithelial barrier in the intestine and potentially exert its effects outside of the GI tract. Although the ability of orally administered IgY to provide protection against disease agents remains to be determined, these data provide evidence for such possibilities.

There are several physiological, physiochemical, and biopharmaceutical factors that influence the bioavailability of drugs. The mechanism as to how or why IgY is able to seroconvert post oral administration is unknown and under investigation in our lab. One possibility is that IgY is binding to unspecified receptors in the gut facilitating its translocation. It is important to recognize however that the delivery of therapeutics orally, even in the absence of a known mechanism of seroconversion, has many advantages.

Oral therapeutics do not require trained medical professionals for administration, and also do not require additional medical equipment such as sterile needles and syringes. One would argue that this would facilitate better compliance if patients were only required to take an oral medication instead of being injected. Furthermore, oral IgY would be good candidate therapeutics for use in areas where other administration routes are difficult to implement due to a lack of economic and healthcare development.

One organism of interest for the development of antigen specific IgY is *P. falciparum*, the causative agent of falciparum malaria, and more specifically CM. The current treatment protocol for CM is the administration of combination antimalarial drugs, particularly those including artemisinin derivatives. Unfortunately the introduction of drug resistant *Plasmodium* strains has facilitated the need for new drug and therapeutic candidates. In this study we demonstrate that anti-malaria and anti-MSP-1specific IgY is able to increase the time to death following the induction of CM in mice, possibly allowing for the introduction of other antimalarial drugs. We also show that IgY treatment on days 2 and 4 post infection decreases parasitemia. These data suggest that malaria specific IgY may be a candidate therapeutic for the use in combination therapies. Further research is being directed at determining what other antimalarial drugs and formulations of IgY will offer optimal protection.

A second organism of interest for the development of antigen specific IgY is DENV. At present there are no approved vaccines for DENV, due in large part to the difficulty in providing cross-reactive protection for all DENV serotypes. In this study we employ a novel approach to treating DENV induced disease. The advantage of anti-DENV IgY for the treatment of DENV is not only the ability to provide therapeutic

protection, but protection in the absence of ADE. ADE has been suggested to be a leading cause of the severe DHF and DSS seen during secondary heterotypic infection; therefore utilizing an antibody therapy that provides protection but does not enhance infection or stimulate the immune system to dangerous levels is promising. Another advantage of IgY therapy is that the distinction between birds and mammals allows IgY to target epitopes that are non-immunogenic in mammals and possibly cross protective epitopes that will likely not undergo selective pressure to change as rapidly over time.

IgY also has the potential to be used in areas where vaccination to such disease is not a feasible option. It takes years of research and development to move a vaccine into the market, and is a costly endeavor. IgY has the potential to be developed quickly and efficiently and thus could provide more on demand protection, specifically in situations where the onset of infectious disease in a defined area is very rapid. In some cases where vaccinating a population is difficult, the therapeutic administration of IgY could still provide protection for those individuals.

The detailed mechanisms as to how antigen specific IgY (IgY and IgYΔFc) is able to neutralize and clear these infectious particles in the mammalian system remains to be elucidated and is an area of active research in our lab. Furthermore, determining what receptor(s) IgY uses in the mammal, and the downstream effects of receptor activation will allow for a better understating of how IgY works in the absence of enhancing an immune response. With these data we will be able to better modify, and further customize IgY therapeutics to offer optimal protection.

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