EFFECTS OF CYTOSINE-PHOSPHATE-GUANOSINE

OLIGODINUCLEOTIDES (CPG-ODNS) ON ORAL IMMUNIZATION WITH

PROTEIN ANTIGEN OR REPLICATING PARASITE

A Dissertation

by

KEITH ALLEN AMEISS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2005

Major Subject: Poultry Science

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ABSTRACT

Effects of Cytosine-Phosphate-Guanosine Oligodinucleotides (CpG-ODNs) on Oral

Immunization with Protein Antigen or Replicating Parasite. (May 2005)

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The purpose of this research was to investigate selected methods of mucosal immunization for commercial chickens. Induction of mucosal immunity in commercial chickens through the use of orally administered subunit vaccines or through immunomodulation of the host's response to live vaccines may be a viable means to control enteric infections in commercial poultry. In the present investigations we evaluated a means for delivering protein antigen in the drinking water and the use of CpG-ODNs, a recently reported mucosal adjuvant, in order to both improve this response and to modulate the host's immune response when vaccinated with field strains of *Eimeria acervulina* and *Eimeria tenella*.

In order to evaluate the efficacy of immunizing commercial poultry with subunit vaccines through the drinking water we chose the model antigen Bovine Serum Albumin (BSA). Chicks were administered BSA via intraperitoneal (I.P.) injection, oral crop gavage, or orally through the addition of BSA to the drinking water. These experiments demonstrated the efficacy of drinking water administration to induce antibody

production in the serum, intestine, and bile. When BSA was co-administered with CpG-ODNs we observed a modest increase in this response dependent upon dose.

To evaluate the immunomodulation of the host response to live parasite using CpG-ODNs we used three administration models. The first was a single dose of CpG-ODNs with a trickle immunization regime of *Eimeria acervulina*. The second was co-administration of CpG-ODNs with a clinical dose of *Eimeria acervulina* or *tenella*. The third was pre-administration of CpG-ODNs 24 hours prior to the clinical dose of either species. These studies demonstrate that the first and third models were effective in reducing lesions and improving performance.

DEDICATION

I dedicate this manuscript to my wife, Sharon. Without you're love, support, and patience through the entire process, this not only would have been much more difficult, it wouldn't have been half as fun either.

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CHAPTER I

INTRODUCTION

The poultry industry represents one of the most important sectors in all modern animal agriculture within in United States. It is considered to be a $22 billion industry and employs about 240,000 workers (U.S.D.A., 2004) It is one of the fastest growing segments of the animal industry and, as such, involves intensive rearing conditions where large numbers of birds, usually flocks with tens of thousands, are raised inside a closed house (Sharma, 1999). To control disease conditions under these dense and intensive rearing environments, vaccines represent an important and invaluable prophylactic and therapeutic strategy for commercial producers (Sharma, 2003). Vaccines induce protection by stimulating lymphocytes to produce antibodies or cytokines and differentiate into memory cells so that subsequent encounters with the same pathogens will be resolved quickly and efficiently (Abbas, 2003; Sharma, 2003).

The fundamental task of the immune system in such a scenario is to protect the host against invading microorganisms or pathogens through a cooperative approach involving both innate and adaptive immunity. Innate immune responses are typically activated by innate system cells, such as macrophages, heterophils, or dendritic cells, which then act in concert to either kill the invading microbe or augment and trigger an appropriate adaptive immune response (Abbas, 2003). Because innate immune

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This dissertation follows the style and format of Poultry Science.

cells lack the highly specific antigen receptors found on T and B cells, they rely on surface membrane proteins known as pattern recognition receptors (PRRs). PRRs have the ability to detect surface components of microbial pathogens known as pathogen associated molecular patterns (PAMPs). PAMPs recognition by PRRs in this setting is an indication of a threat to the host in the form of potential infection (Teixeira et al., 2002). Such recognition activates appropriate defense pathways in the innate system to either remove the pathogen or stimulate the adaptive system to act upon the pathogen for its ultimate removal. Cytosine-phosphate-guanosine (CpG) motifs naturally present in bacterial DNA represent one of the most recent examples of substances that influence adaptive immune responses by activating the innate immune system. CpGs are 6-8 base nucleotide motifs consisting of an unmethylated CpG dinucleotide that induces cells of the innate immune system to increase phagocytosis, pro-inflammatory cytokine production, activation of NK cells, and increase nitric oxide generation by macrophages (Hemmi et al., 2000; Krieg, 2002).

The most critical line of defense against pathogens in an organism is the mucosal associated lymphoid tissues (MALT), the largest of which, in terms of lymphocyte populations, is the gut associated lymphoid tissues (GALT) (Bar-Shira et al., 2003; Lillehoj and Trout, 1996; Yun et al., 2000b). This system is considered to be the most important with regards to enteric pathogens as it is both the primary route of entry and site of infection, as such it is also an ideal route for vaccine administration (Bar-Shira et al., 2003). The ability of CpG-oligodeoxynucleotides (ODN) to act as vaccine adjuvants has been explored extensively in recent years in mammals but very little research has

been conducted along these lines in commercial poultry. Therefore, the objective of this

investigation was to evaluate the administration of CpG-ODN as an adjuvant to oral

administration of a protein antigen or to oral administration of live parasite.

CHAPTER II

REVIEW OF LITERATURE

BACKGROUND

Basic Concepts of Immunology

The term immunity comes from the Latin word *immunitis* originating from the Roman Empire where it signified freedom from taxes, services, and prosecution (Abbas, 2003). Historically, immunity has been defined as the condition in which an organism can resist disease; however we now know that this resistance to infection also involves a response to non-infectious foreign substances. Thus the modern definition of immunity has been altered to include the reaction to foreign substances such as microbes, as well as macromolecules such as proteins and polysaccharides that don't necessarily have a physiological or pathological consequence (Abbas, 2003). This reaction to foreign substances, including the response to vaccines, is mediated by the immune system.

Taken as a whole, the immune system is the only defense an organism has against infectious disease (Lynn et al., 2003). At the most basic level an immune response is a response to an antigen. Antigens are molecules, such as proteins or sugars, which can react with specific antibodies or antigen receptors on lymphocytes. Antigens that can elicit an antibody response by themselves are termed immunogens (Janeway and Travers, 1997). A whole host of factors can affect this response including the nature of the antigen, how the host is exposed to it, as well as the genetics and general health of the host (Abbas, 2003).

Innate and adaptive immunity are two integrated but distinct forms of immunity which must be able to perform three functions; recognize pathogens, kill pathogens, and not damage host cells (Beutler, 2004). Both the innate and adaptive arms of the immune system perform these functions, but in very different ways. Specifically they differ in how they detect pathogens, respond to pathogens, reaction time, and discrimination of self and non-self (Abbas, 2003). The innate immune system detects conserved molecular patterns of pathogens through receptors that are fixed in the genome and are expressed on cells which are essentially identical. The adaptive immune system uses receptors encoded in gene segments and subject to rearrangement that detect details of molecular structures and are expressed in a clonal fashion. Likewise, the innate immune system responds through expression of co-stimulatory molecules, cytokines, and chemokines, while the adaptive immune system responds through clonal expansion or anergy (non-responsiveness) and the expression of effector cytokines such as IL-2, IL-4 and IFN-γ. Innate immunity also responds more quickly than adaptive and, due to evolutionary selection, does not react with self-antigens. The discrimination of adaptive immunity, however, is done in individual somatic cells and does allow some cross-reactivity (Janeway and Medzhitov, 2002).

Innate Immunity

Innate immunity is the first line of defense against invading pathogens (Zekarias et al., 2002). Physical and chemical barriers, such as skin, feathers, lysozyme, lactoferrin and antimicrobial peptides are generally regarded as part of innate immunity as they play

an important role in preventing the entrance of pathogens, but will not be extensively considered here.

Once a pathogen has entered an organism the first cells to encounter it are the phagocytes. These include macrophages, dendritic cells, and heterophils, all of which play crucial roles in inflammation and the initiation of an immune response (Harmon, 1998; Qureshi, 1998). These cells ingest the pathogen through pinocytosis, phagocytosis, or receptor mediated endocytosis (Qureshi, 1998). The recognition of pathogens by the innate system is generally done by receptors fixed in the genome called pattern recognition receptors (PRRs) (Janeway and Medzhitov, 2002). PRRs recognize pathogen associated molecular patterns (PAMPs). PAMPs are characterized by three primary traits (1) they are expressed by microbes, but not host cells (2) they show little variation among a given class of microorganisms, and (3) they perform some essential function for the microbe (Teixeira et al., 2002). In both mammals and birds PRRs can be secreted, expressed on the cell-surface, or expressed intracellularly. Secreted PRRs encompasses the collectin family of proteins which includes mannose binding lectin (MBL) and pulmonary surfactant proteins, the pentraxin family of proteins which includes C-reactive protein (CRP) and serum amyloid protein (SAP), LPS binding protein (LBP), the secreted form of CD14, and C3b (Beutler, 2004; Janeway and Medzhitov, 2002; Teixeira et al., 2002). Cell surface PRRs include a number of receptors expressed on macrophages and dendritic cells. These include scavenger receptors (SR) such as the scavenger receptor type A (SR-A) family which recognizes a broad spectrum of ligands including double- stranded RNA (dsRNA), LPS and lipoteichoic acid (LTA) (Peiser et

al., 2002). Cell surface PRRs also include the macrophage mannose receptor (MMR), as well as some toll-like receptors (TLRs). Those TLR expressed on the cell surface are TLR1, which with TLR2 bind bacterial lipopeptides and glycophosphoinositol (GPI) anchored proteins from parasites, TLR4, which binds LPS from gram-negative bacteria , TLR5, which binds flagellin, and TLR6, which binds lipoteichoic acid (LTA) from bacteria and zymosan from fungi (Janeway and Medzhitov, 2002). Intracellularly expressed PRRs include the virally induced double-stranded RNA-activated protein kinase, as well as some others TLRs. The TLRs expressed intracellularly are TLR3, which binds double stranded RNA, TLR7 and TLR8, which bind single stranded RNA, and TLR9, which binds unmethylated CpG ODNs from bacteria (Lynn et al., 2003; Teixeira et al., 2002).

The interactions of PAMPs and PRRs have a number of potential outcomes. Those that are secreted generally aid in the internalization of the antigen through opsonization by antibodies or aid in complement-mediated killing (Beutler, 2004). Surface expressed and intracellular PRRs aid in the internalization of the antigen, but may also aid with its enzymatic degradation, further processing and presentation through the MHC class I or II molecules, and by the induction of co-stimulatory signals resulting in the activation of the adaptive immune system by activating naïve T and B cells to proliferate and differentiate (Aderem and Underhill, 1999; Janeway and Medzhitov, 2002; Werling and Jungi, 2003).

Adaptive Immunity

In contrast to innate immunity, adaptive immunity is both slower to respond as well as more specific and effective. This effectiveness stems from the fact that adaptive immunity involves highly specific receptor-antigen interactions, allowing it to focus on specific pathogens, as well as being tailored to deal with pathogens that are both extra and intracellular (Erf, 2004). These two categories are referred to as humoral and cell mediated responses (Abbas, 2003). Humoral immunity consists of the activation of B cells which is responsible for the production of immunoglobulins. Immunoglobulins are antibodies and mainly present in serum, or on cell surfaces, egg yolk, bile, and mucosal secretions (Abbas, 2003). The specificity of antibodies in birds is generated through combinatorial joining of the variable regions during development and, later in life, somatic gene conversion. This differs from most mammals where somatic mutation is responsible for further diversity (Butler, 1998). Antibodies can react to pathogenic organisms in a number of different ways. They can act directly by neutralization, preventing the pathogen from attaching to surface receptors of target cells thereby inhibiting replication and invasion, or indirectly. Indirect actions include opsonization, through attachment to the microbes surface, thereby facilitating their internalization by macrophages and phagocytic cells leading to subsequent destruction of pathogen and activation of complement by the classical pathway. In this pathway antibodies bind to the surface of the pathogen and activates complement proteins, which are bound to phagocytes receptors facilitating their phagocytosis and destruction (Abbas, 2003). There are three main isotypes of immunoglobulins in chickens: IgA, IgG(Y), and IgM.

This differs from mammals in that most mammals have multiple subtypes of IgG as well as two other standard types. These other types are IgD, which is expressed during certain developmental stages, and IgE, which is involved in allergy and immunity to nematodes. (Abbas, 2003; Avery et al., 2004). IgM is found on the surface of most B cells, and is usually the antibody that predominates in a primary immune response. Class switching of an antibody occurs when IgM producing cells stop producing this isotype and "switch" to the production of another isotype like IgG or IgA. The cytokines that stimulate the B cells to induce a class switch are IL-4 and IL-5. IgG is the predominant immunoglobulin circulating in blood and IgA is the primary antibody involved in mucosal immunity (Holmgren et al., 1992; Staeheli et al., 2001).

Cell mediated immunity involves the activation of T lymphocytes or T cells. T cells exist as two different classes of cells; 1) T helper lymphocytes (CD4+ or Th cells), which function to secrete cytokines for regulating immune responses, and 2) T cytotoxic lymphocytes (CD8+ or Tc), which destroy cells infected with intracellular pathogens (Abbas, 2003). T cells recognize antigen through the T-cell receptor (TCR) which binds to a small linear portion of the antigen. However, the TCR can only recognize this peptide when it is associated with self-MHC, this is termed "MHC-restriction". The two types of T-cells are dependent upon different types of MHC, Th cells recognize antigen in the context of MHC class II and Tc cells recognize antigen in the context of MHC class I. Expression of the two MHC classes also differs. MHC class I is expressed on all nucleated cells, whereas MHC class II is only expressed on antigen presenting cells (Erf, 2004).

Antigen presenting cells, such as dendritic cells, B cells, and macrophages, are the cell populations that integrate the innate and adaptive immune systems. In their immature form, these cells phagocytose and degrade pathogens and express the degraded microbial peptides in major histocompatibility complex (MHC) class I or II molecules for presentation to lymphocytes (Abbas, 2003; Janeway and Medzhitov, 2002). After the identification of the pathogen, the non-mature antigen presenting cells of the innate system control the acute innate host response by phagocytosing and then killing the invading microbe with reactive oxygen and nitrogen intermediates (superoxide anion, hydrogen peroxide, chloramines and hydroxyl radicals) and proteolytic enzymes (Abbas, 2003; Werling and Jungi, 2003). The end product of this microbial recognition is the activation of intracellular signaling pathways that initiate maturation of the APC resulting in a number of cellular responses. These include production of inflammatory and effector cytokines such as TNF-α, IL-1β, IL-12 and type I interferons, up regulation of co-stimulatory molecules like CD80 and CD86, and presentation of the antigenic peptides through their expression in MHC class I or II molecules for antigen presentation to the acquired immune system (Swaggerty et al., 2004; Werling and Jungi, 2003).

The most important outcome from the activation of naive T cells through the presentation of the antigen-MHC complex is the proliferation of the antigen-specific T cells and the subsequent differentiation into effector and memory cells (Wagner, 2002). The origin of the pathogen will determine the class of MHC molecule used. Any extracellular antigen or protein, such as bacteria, parasites, and bacterial toxins, will be presented through an MHC class II complex and recognized by a CD4+ T cell. Any

antigen or protein with an endogenous origin or presence in the cytosol, such as a virus

or intracellular bacterium, will be presented through the MHC class I pathway and will

be recognized by CD8+ T cells. T cells become activated and initiate the production of

different cytokines that will function in the effector stages of cell mediated immunity. T

cells will also increase the expression of receptors for different cytokines once activated.

One of the most important cytokines secreted for proliferation of T cells is IL-2, an

autocrine cytokine, which acts as a growth factor (Abbas, 2003; Erf, 2004). Dependent

on the T cell phenotype activated (CD4+ or CD8+) a specific differentiation pathway will

be followed. CD4+ T cells differentiate into one of two, Th1 or Th2, effector Th cell

subsets that are defined by different cytokine profiles and the type of effector functions

they initiate in other cells of the immune system (Erf, 2004). Th1 cells typically secrete

cytokines such as IL-2, IFNγ, and TNF-α, all of which play important roles in promoting

the cell mediated immune response. Th2 cells secrete IL-4, IL-5, IL-6, and IL-10

promoting the humoral immune response (Abbas, 2003; Erf, 2004; Staeheli et al., 2001).

When CD8+ T cells recognize the antigen presented, they differentiate into cytotoxic

lymphocytes (CTLs) which have the capacity to lyse and kill cells expressing foreign

antigen in MHC I molecules through the release of perforins, granzymes, and cytolysins

(Abbas, 2003). The most important function of the CD4+ effector cells is to activate

phagocytic cells and lymphocytes. In addition to cytokine signals, other direct molecular

interactions are also necessary. These costimulators help T lymphocytes to work in

harmony and include the interactions of CD-2 (on the T-cell) with LFA-3, CD40L with

CD40, and CD28 with B-7 (Abbas, 2003).

B lymphocytes require stimulation similar to T cells in order to proliferate and differentiate into antibody producing cells or memory cells (Abbas, 2003). This stimulation can come from two sources dependent on the type of B-cell. The first applies to conventional B cells, also called the B2 subgroup. This form of stimulation occurs when antigen binds to the immunoglobulin on the surface and activation occurs with the help of costimulation by antigen-specific Th2 cells through the interaction of CD40 with its ligand and the secretion of cytokines (Bernasconi et al., 2002). The second applies to the B1 subgroup. This form of stimulation is T-cell independent, occurs in the absence of antigen, produces primarily IgM, and is activated through microbialy derived products such as LPS or CpG-ODN, which stimulate B cells via TLR4 and TLR9 respectively (Bernasconi et al., 2002; Fagarasan and Honjo, 2000). This second form of stimulation may be particularly important in intestinal immunity as many of the IgA producing cells in the intestine are derived from the B1 subtype after nonspecific stimulation by T-cells (Fagarasan and Honjo, 2000).

Cytokines are essential effector molecules of both innate and acquired immunity and have been implicated in actions as diverse as mediating precursor cell development and inhibition, cellular apoptosis (programmed cell death) and enhanced cell survival, tumor rejection and metastasis, pro-inflammatory and anti-inflammatory responses, as well as the induction of either protection against infectious diseases or pathogenesis of infectious diseases (Kogut, 2000). They are soluble low molecular weight polypeptides and glycopeptides produced by different immune cells in order to regulate other cells and can be classified as interferons (IFNs), tumor necrosis factors (TNF), interleukins

(IL), and chemokines (Staeheli et al., 2001). To date most of the cytokines found in mammals have been identified in chickens as well. These include IFNα, IFNβ, IFNγ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, IL-16, IL-17, and IL-18 (Avery et al., 2004; Lillehoj et al., 2004; Staeheli et al., 2001). While their functions seem to be conserved between mammals and birds, their sequence similarity is not. Sequence similarity between mammalian and avian cytokines typically ranges from 30-50% (Hilton et al., 2002). IFNα and IFNβ, also called type I IFNs, are both expressed at elevated levels in response to viral infection, whereas IFNγ, the only type II IFN, is involved in most stages of the immune response and inflammation (Kogut, 2000; Staeheli et al., 2001). IL-1β primarily induces secretion of pro-inflammatory cytokines such as IL-6 and TNF-α. Many of the cytokines are also defined as to the type of T helper response they promote, Th1 or Th2. Homologues for Th1 cytokines have been identified in the chicken and include IFN-γ, IL-2, IL-12, and IL-18 (Davison, 2003; Staeheli et al., 2001). Until recently the only Th2 cytokine homologue identified in the chicken was IL-6, which induces IL-4 production in mammals and is a regulator of immunoglobulin synthesis (Staeheli et al., 2001). Recently, however, a gene cluster encoding IL-3, IL-4, IL-13, and GM-CSF was identified in the chicken and a number of Th2 cytokines were cloned (Avery et al., 2004). Interestingly the avian homologue for IL-5 was also present in the cluster but was a pseudogene. However, another sequence in that same cluster, clone KK34, was separately identified and demonstrates many of the features of IL-5 (Avery et al., 2004; Koskela et al., 2004). In addition, the presence of a Th1 response in chickens has been demonstrated through the use of maleylated proteins, which have been

demonstrated to promote a Th1 response in mammals. The promotion of delayed type hypersensitivity, induction of IFN-γ, and reduction of antibody response were observed when using this method (Vandaveer et al., 2001). However, due to the recent identification of Th2 cytokines in chickens, the Th1/Th2 paradigm has not yet been fully confirmed.

It is well established that certain types of immune responses are restricted to certain areas of the body. Due to the fact that the vast majority of pathogens gain entry into the body through mucosal surfaces, the mucosal associated lymphoid tissues (MALT) are one of the most critical lines of defense against pathogens. The MALT can also be divided into lymphoid tissues of specific areas such as bronchial (BALT), nasal (NALT), and gut associated lymphoid tissue (GALT) (Bar-Shira et al., 2003; Yun et al., 2000b). Of these, the GALT forms the major component, containing more than half of the lymphocytes associated with the MALT (Lillehoj and Lillehoj, 2000). The GALT consists of multiple layers of tissues with the epithelial cells and lymphocytes comprising the outer layer above the basement membrane, beneath which is the lamina propria (LP), lymphocytes, and the submucosa (Yun et al., 2000b). There are also specialized lymphoid organs present in the GALT. In the chicken these include the bursa of Fabricius (BF), cecal tonsils (CT), Meckel's diverticulum, Peyers patches (PP), collections of intraepithelial lymphocytes (IELs) such as T-cells and NK cells, and other localized or diffuse immune cells throughout the lamina propria (Lillehoj and Trout, 1996). All of these cells are known to secrete and respond to cytokines forming a bi-directional communication network that is important for both protective immunity as

well as modulation of the immune responses to innocuous antigens such as food antigens (Klipper et al., 2000; Yun et al., 2000b). This balance between immunity and tolerance becomes very important when vaccination protocols are considered and will be addressed in more detail below.

Vaccination and the Use of Adjuvants

Well before the mechanisms of immunity and disease resistance were known it was observed that recovery from an infectious disease resulted in increased protection from re-infection. In practice, early immunization involved purposely exposing a person to a pathogen in order to obtain a milder form of the disease, which then would protect from more serious sickness. This method was used to protect against smallpox by inhaling dried smallpox scabs, a process known as variolation, as early as 1000 AD in China, and by the early 18th century in Europe (Levine and Lagos, 2004). In the mid 1700s Paul Adámi experimentally vaccinated cattle for rinderpest by implanting threads impregnated with infected animal saliva (Foss and Murtaugh, 2000).

The first publicly demonstrated use of a related or modified organism to vaccinate against infectious disease was not until 1776 by Edward Jenner (Levine and Lagos, 2004). Jenner formalized the practice of inoculating with cowpox, which stemmed from the observation that people working with cowpox infected cattle were protected from smallpox, as "vaccine inoculation" (Levine and Lagos, 2004). It is assumed that Jenner used cowpox, however, the virus used may have actually been horsepox (Taylor, 1993). The first modification of bacteria for use as a vaccine was described by Louis Pasteur in 1879. A culture of *Pasteurella multocida*, the etiological

agent of Fowl Cholera, was accidentally left at room temperature and, although it failed to induce clinical disease, it did cause the birds to be resistant to subsequent challenge with virulent bacteria (Levine and Lagos, 2004). This observation established the principle of vaccination and was used by Pasteur in the preparation of vaccines for both anthrax and rabies. It was also Pasteur who coined the term 'vaccine' in honor of Jenner's use of the vaccinia virus (Foss and Murtaugh, 2000).

To this day, widespread vaccination remains the most successful method in controlling and preventing losses in farm animals due to infectious diseases. These vaccines consist primarily of live attenuated organisms, killed organisms, and inactivated toxin. The use of attenuated vaccines are limited primarily by safety, as attenuated forms will occasionally revert to virulence, thus causing the disease one intended to prevent. Conversely, killed organisms lack the ability to stimulate strong cell mediated immunity (Singh and O'Hagan, 2003). These issues are being addressed through the development of a number of novel types of vaccines. These include, but are not limited, to vectored vaccines, subunit vaccines, peptide vaccines, and polynucleotide vaccines (Babiuk et al., 1999). Vectored vaccines are attenuated pathogens capable of multiplying in the host and containing foreign genes of interest. These can be viruses or bacteria that have either been attenuated or are not pathogenic to the animal being vaccinated (Pastoret, 1999). Subunit vaccines are defined as containing one or more pure or semi-pure antigens. Peptide vaccines are a reduction of subunit vaccines. Instead of the entire protein only individual epitopes, sequences recognized by an antibody, are used. Polynucleotide vaccines, also called genetic or DNA vaccines, are cDNA

sequences which encode for an antigen. These sequences are taken up and expressed by host cells, thus enabling the host to expose itself to the antigen (Babiuk et al., 1999). These issues are further complicated, in the cases of subunit, peptide, and DNA vaccines, by the difficulties in delivering them to mucosal surfaces and the lack of immunogenicity of subunit vaccines (Medina and Guzman, 2000). Some of these difficulties can be overcome through the use of adjuvants.

Adjuvants are compounds which, when combined with an antigen, potentiate the immune response by replacing some property of the living pathogen lost through attenuation or separation into component parts (Foss and Murtaugh, 2000; Medina and Guzman, 2000). Mechanistically the actions of adjuvants can be divided into three categories: persistence, location, and context. Persistence refers to increasing the time of antigen contact. This replaces pathogen replication and reinfection and is done generally by creating a 'depot' through emulsions, or in the context of mucosal immunity continuous exposure to the antigen or encapsulation. Location refers to where the antigen makes contact. This replaces opsonization of some pathogens or the intracellular location of viruses and some bacteria and is done generally by intradermal injections, or in the context of mucosal immunity the uptake of DNA vaccines and the 'targeting' of antigen by molecules such as the Cholera toxin-B (CT-B) subunit. Context refers to enhancement of immune cells directly through cytokine expression or costimulatory molecules. This replaces a pathogens ability to induce cytokines through pathogen associated molecular patterns (PAMPs) and can be done generally through the use of bacterially derived components such as lipopolysacharide (LPS), or in the context of

mucosal immunity through the use of cholera toxin (CT) or unmethylated CpG-ODNs (Eriksson and Holmgren, 2002; Foss and Murtaugh, 2000). Adjuvants can be roughly divided into two types, vaccine delivery systems and immunostimulatory adjuvants, however some are not easily classified. The former group includes emulsions, microparticles, immunostimulatory complexes (ISCOMs) and lipsomes. These function primarily to direct the antigen to APCs. Immunostimulatory adjuvants function primarily to activate APCs and induce cytokines. These include bacterial products such as monophosphoryl lipid A (MPL), CpG-ODNs, and saponins as well as the direct activation of APCs via expression of recombinant cytokines in vectors (Muir et al., 2000; Rankin et al., 2002; Singh and O'Hagan, 2003).

As the intestinal surfaces of commercial poultry are subject to assault by a variety of microorganisms, and the need for mass vaccination necessitates the use of the oral route, two strategies will be discussed in more detail and in the context of poultry immunization. These are oral immunization with protein antigen and the use of CpG-ODNs as mucosal adjuvants.

The primary obstacle to oral immunization with protein antigens, or subunit vaccines, is the induction of tolerance. However, even if a response is mounted, it is often weak and short-lived. Tolerance induction has been addressed more fully than the strength of the response due to the investigation of tolerance mechanisms in both mammals and chickens. Oral tolerance is a condition of unresponsiveness of the immune system to an orally administered antigen (Miller and Cook, 1994). The avian immune system does become tolerant to orally ingested food antigens in a fashion similar to

mammals, however, tolerance in chickens is developmentally dependent. It is usually

only induced when the animal is a neonate, usually during the first week of life (0- 10

days post hatch). After this period fed antigens become immunostimulatory and do not

induce tolerance. Interestingly, this phenomenon occurring at 10 days of age in the

chicken coincides with the appearance of the Peyer's patches (PP), more extensive B and

T cell colonization, and subsequent cytokine expression at mucosal sites, all of which are

required for the induction of humoral responses in the mucosa (Bar-Shira et al., 2003;

Klipper et al., 2001; Miller and Cook, 1994). The ability of an orally administered

antigen to either induce tolerance or immunity depends not only on the age of the

animal, but on the frequency, dose and physical presentation of the antigen given. It has

been shown that solubilized antigen given orally in low concentrations on consecutive

days induce a robust systemic humoral response. When administered in similar

concentration and route of administration, but using a dry or pelletized form of antigen,

tolerance is induced (Klipper et al., 2000,2001). Thus, after the birds become more

immunologically mature the ability of developing oral tolerance to solubilized antigens

is lost. A possible explanation for this difference from mammals is the difference in

evolution. The bird is immediately exposed to food antigens during the period in which

the immune response is immature and can be tolerized. Since the diet is usually the same

at later ages there would be no need for continuing tolerance. In the mammal, however,

the change of diet after weaning exposes it to a completely new set of innocuous food

antigens at a time when the immune system is almost fully mature (Klipper et al., 2001).

Additionally it has been demonstrated that the hyper immunization of breeding hens

with BSA and the subsequent presence of maternal antigen-specific antibodies in progeny inhibit tolerance. These authors suggest this to be mechanisms to describe why some pathogens which are exposed to the neonatal chick do not induce tolerance (Klipper et al., 2004).

The discovery of the immunostimulatory actions of bacterial DNA stemmed from a series of investigations describing reduction of tumor growth after vaccination with bacille Calmette-Guérin (BCG) (Hacker et al., 2002). Later Kreig and co-workers demonstrated both direct and indirect stimulation of B cells, stimulation of NK cells to secrete IFN-γ, and the initiation of anti-tumor activity attributable to CpG-ODNs present in bacterial DNA (Krieg, 1996,1999,2002). In these investigations CpG-ODN were characterized as specific sequences of oligodeoxynucleotides (ODNs) with unmethylated cytosine-phosphate-guanosine (CpG) dinucleotides. CpG-ODNs are 6-8 base nucleotide motifs consisting of CpG dinucleotides linked together by phosphodiester bonds and usually flanked by two 5' purines and two 3' pyrimidines. ODN uptake into the cells requires the participation of a voltage-gated channel due to the fact that they are polyanions and cannot diffuse across cell membranes freely (Krieg, 2002). These motifs are approximately 20 times more common in bacterial than in vertebrate DNA, due to CpG suppression and selective methylation (Cardon et al., 1994). These differences define CpG-ODNs as PAMPs and thus enable vertebrates to protect themselves against invading pathogens (Klinman et al., 1996). CpG DNA is recognized by a member of the Toll-like receptor (TLR) family of PRRs which triggers the host's innate immune system. CpG-ODNs present in bacterial DNA represent one of the most recent examples

of PAMPs that influence adaptive immune responses by activating the innate immune system (Hemmi et al., 2000).

The chemical structure of CpG-ODN greatly influences its ability to stimulate innate system cells and changes in the bases flanking the CpG dinucleotides have a great influence on the response because they influence recognition by the innate immune system. Although CpG-ODN uptake involves the binding to cell surface proteins with a non-specific antigen-receptor, the immunostimulatory effects of CpG-ODN are initiated by binding to an intracellular receptor, TLR9 (Krieg, 2002). Although this is not well understood, it is possible that TLR-9 may require a specific sequence in order to recognize CpG-ODN as a PAMP and bind to it as an antigen dependent response according to the sequences that are surrounding the CpG dinucleotides (Dalpke et al., 2002; Pisetsky, 1999; Zhao et al., 1999). Some research groups have proposed different consensus base sequences for immunostimulatory DNA sequences in different species. The sequence GACGTT has been shown to be the optimal immunostimulatory sequence in mice and rabbits. This sequence is inhibitory for other species however, and distinct from the most stimulatory sequence for humans GTCGTT (Rankin et al., 2001). The immunostimulatory effects of CpG-ODNs have also been examined in both laboratory and domestic animals, including rabbits, sheep, horses, pigs, chickens, dogs, and cats. These investigations have demonstrated that responsiveness to unmethylated bacterial DNA sequences is a phenomenon conserved across many species (Rankin et al., 2001) They found that the main characteristic of ODN stimulating an immune response was the presence of a GTCGTT motif. They reported that three GTCGTT motifs within the

ODNs had a stronger and more consistent stimulatory effect, while ODNs with only one or two motifs did not activate the immune cells as consistently (He et al., 2003; Rankin et al., 2001; Vleugels et al., 2002). Most of the immune enhancing potential in different species is strictly CpG dependent and inversions to GpC abolish this activation (Ahmad-Nejad et al., 2002).

Modifications made in the natural backbone chemistry of the CpG may also affect the immunostimulatory effects of the bacterial DNA. Natural ODNs are linked by phosphodiester bonds which make them unstable and susceptible to endogenous nucleases which hydrolyze the phosphodiester bonds. To improve stability necessary for both investigation and practical use, synthetic CpG-ODNs have been designed. These analogs differ from the native forms by the substitution of a molecule of oxygen (O-) at the phosphate group in the nucleotide, thus making them more resistant to nucleases. Reported analogs include methylphosphonate ODNs, phosphoramidate ODN, phosphorothioate ODN, and (N3'---- P5') phosphoramidate (Gallo et al., 2003). The most common ODN recently reviewed in the literature contained the phosphorothioate backbone due to their ability to increased nonspecific binding to different proteins, increased efficiency of binding to cell membranes, and a higher degree of cellular uptake. ODN with this backbone are taken up much more efficiently than ODN with the phosphodiester backbone and are more efficiently endocytosed than ODN with a methylphosphonate, in addition to having an *in vivo* half life of 48 hours as compared to the 5 minutes of the phosphodiester backbone (Gallo et al., 2003; Pisetsky, 1999). The ability to induce proliferation of B cells, activate macrophages, NK cells or other

immune cells, will be either enhanced or decreased according to the susceptibility to degradation of the backbone (Krieg, 2002; Pisetsky, 1999; Zhao et al., 2000).

The proposed processes of CpG-ODN stimulation is as follows. CpGs cross the cell membrane via sequence-non-specific receptor-mediated, clatherin dependent endocytosis. Endosomal maturation occurs and the ODN is recognized by TLR-9. Recognition through TLR-9 will induce an immune response involving both innate and adaptive immune system cells (He and Kogut, 2003; Krieg, 1999). The cellular activation by TLR-9 binding proceeds through a signaling cascade involving myeloid differentiation marker 88 (MyD88), IL-1R-associated kinase (IRAK), tumor necrosis factor receptor associated factor 6 (TRAF 6), nuclear factor kappa B (NF- κB) translocation, and up-regulation of genes involved in host defense. After this initial response, the triggering of TLR by specific CpG-ODN, results in TLR-9 specific immune responses (Ashkar and Rosenthal, 2002; Dalpke and Heeg, 2004; Krieg, 1999). CpG-ODN can also act directly on B cells triggering cytokine production (IL-6, and IL-10), and immunoglobulin secretion. They've also been shown to activate dendritic cells to produce pro-inflammatory cytokines like IL-12 and stimulate antigen-specific T-cells, and proliferation of B cells (Krieg, 2002; Medzhitov, 2001).

Chicken Coccidiosis

The etiological agents of chicken coccidiosis are a number of *Eimeria* species including, but not limited to, *E. acervulina*, *E. tenella* and *E. maxima*. Each species has characteristic prevalence, site of infection, and pathogenicity. All species, however, parasitize the epithelial cells of the intestinal lining and cause pathological changes

ranging from local destruction of the mucosa to systemic effects such as blood loss, shock, and death (Vermeulen et al., 2001). These changes lead to economic losses resulting primarily from decreased performance, such as feed conversion and weight gain. In a recent review (Allen and Fetterer, 2002), coccidial infections in poultry were calculated to cost the U.S. poultry producing industry in excess of $450 million annually. When worldwide production was taken into account this increased to over $800 million per year. While these costs take into account the cost of prophylactic medication and losses due to morbidity, mortality and poor feed conversion, it was further estimated that less than 20% of these losses were due to prophylaxis, with the other 80% due to decreased weight gain and poor feed conversion (Vermeulen et al., 2001). Coccidial infections may be classified in one of three ways: (1) as clinical coccidiosis, characterized by mortality, morbidity, diarrhea, or bloody feces, as well as by adverse effects on economical performance; (2) as subclinical coccidiosis, by definition not immediately obvious, but causing reductions in weight gain and feed conversion efficiency of the host, without frank signs of disease; or (3) as coccidiasis, a mild infection causing no adverse effects on the host (Williams, 2002).

Avian *Eimeria* spp. are a member of the phylum Apicomplexa. This phylum contains over 4600 species, many of which have medical and veterinary importance, including the etiological agents of malaria, babesiosis, and cryptosporidiosis. All species of this phylum are protozoan parasites which share an obligate parasitic life cycle and a unique complex of organelles involved in movement and invasion of host cells called the apical complex (Smith et al., 2002). The apical complex generally consists of three

organelles: micronemes, rhoptries, and dense granules (Tomley et al., 2001). The causative agents of chicken coccidiosis belong to the genus *Eimeria*, of the family *Eimeriidae*, of the order *Eimeriida*, of the class Coccidia. There are generally accepted to be seven species responsible for chicken coccidiosis: *Eimeria acervulina*, *E. brunette*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox*, and *E. tenella*. These species are differentiated based upon their site of development, morphological appearance, prepatent and patent times, immunological specificity and reproductive isolation. The species *E. acervulina*, *E. maxima,* and *E. tenella* are considered to be the most important to the poultry industry due to their ubiquity and pathogenicity (Schnitzler and Shirley, 1999).

All *Eimeria* species have homoxenous life cycles, which have been well described by Fernando (Fernando, 1990). In general, environmentally resistant oocysts shed in feces undergo sporogony in the external environment. Sporogeny is a meiotic process requiring oxygen and taking about 24 hours in which a one-celled zygote within the oocyst wall undergoes a series of divisions to form sporozoites. Sporozoites are contained within sporocysts, with two sporozoites per sporocyst and four sporocysts per oocyst (Allen and Fetterer, 2002; Jeurissen et al., 1996). When ingested by a suitable host the oocyst undergoes excystation, the process of releasing infective sporozoites. During this process the oocyst wall is broken by the grinding action in the gizzard, releasing the sporocysts, and, through the actions of trypsin, bile, and CO_2, the sporozoites are released. Upon release the sporozoites actively penetrate the intestinal epithelial cells through the action of materials released from the apical complex. This entry is initiated by contact between the apex of the parasite and the host cell surface.

Progressive internalization follows proceeding from the anterior of the parasite to the posterior eventually enclosing the parasite within a vacuole within the host cell (Dubremetz et al., 1998). While the details of these actions are not known, invasion is dependent upon gliding motility of the parasite, which is dependent upon release of proteins from the microneme (Tomley et al., 2001). Rhoptry proteins may also be involved in invasion as they are found in the vacuole membrane early on, and dense granule proteins are found in the vacuole shortly after it closes (Dubremetz et al., 1998). Sporozoites of some species (*E. brunette* and *E. praecox*) develop within cells at the site of penetration, the villus tips, while sporozoites of other species (*E. acervulina, E. maxima, E. necatrix,* and *E. tenella*) are transported to the crypt epithelium, where they undergo development (Allen and Fetterer, 2002). Within the host cells, sporozoites become rounded and transform into trophozoites which then enter an asexual proliferation phase. This involves schizogony, or merogony, in which nuclear division is followed by cytoplasmic differentiation, resulting in merozoites that are released into the intestinal lumen and reinfect other epithelial cells close to where they were released. After two to five merogonic generations, the sexual reproduction phase or gametogeny, occurs. Merozoites enter host cells and develop into either male microgametocytes or female macrogametocytes. A macrogametocyte is fertilized by a microgametocyte to form a zygote, which is then enveloped in an environmentally resistant oocyst wall. The oocysts are then released from the host cell and shed in the feces. Prepatent periods generally range from 4 to 5 days postinfection and maximum oocyst output ranges from 6 to 9 days postinfection (Allen and Fetterer, 2002; Jeurissen et al., 1996).

Pathological changes in the intestine have been well characterized by a number of investigators. These are comprised of morphological and physiological changes, both of which contribute to an inability to normally absorb nutrients. As different species parasitize defined regions of the intestine these changes will be considered according to the region of the intestine affected.

Eimeria acervulina is the primary species that affects the upper part of the small intestine. There have been several reports indicating the changes in gut physiology during an *E. acervulina* infection and periods of malabsorption seen in chickens are generally attributed to epithelial destruction (Allen, 1987). Normal morphology of the duodenal villi ranges from fingerlike to spatulate-shape. *E. acervulina* infection was found to result in acutely truncated villi with no distinct tip as in uninfected intestines (Witlock and Ruff, 1977). Reduction of the mucosal layer in infected tissue has also been observed. This reduction became progressively more evident from 4-6 day post infection as fewer and fewer cells could be scraped off without disturbing the submucosal layers. However, by day 14 the intestine had recovered and appeared normal compared to uninfected birds (Allen and Danforth, 1984). *E. acervulina* infection also decreased total alkaline phosphatase and sucrase activity in the upper and middle segments compared to controls at day 3, 5, and 7 postinfection while sucrase activity was significantly increased in the lower segment on day 7 postinfection (Allen, 1987).

E. acervulina infections were also observed to affect zinc, oleic acid, and calcium absorptions while effects on protein and amino acid absorption were minimal (Turk and Stephens, 1967). An initial increase in absorption of zinc and oleic acid was observed at

the beginning of the infection but as the infection reached its acute phase zinc and oleic

acid absorption levels were reduced by 25-40% as compared to uninfected birds. The

maximal reduction occurred at the time of the most severe intestinal damage, however

by the time visible intestinal damage was repaired the absorption rates for zinc were

approximately double that of uninfected birds and those for oleic acid about 25% above

uninfected birds (Turk, 1974) *E. acervulina* infection also slows the rate at which

nutrients enter the bloodstream through the lumen of the intestine (Turk and Stephens,

1967). Infection increased the time to maximal levels of oleic acid in the bloodstream

from 1-2 hours in non infected birds to approximately 8 hr in challenged birds. A

decrease in gut motility may be responsible for this delay in absorption as well as the

initial increase observed for zinc and oleic acid as it was hypothesized that a slow

digestive tract rate would permit the nutrients to be in contact with the intestinal mucosa

for a longer period of time. (Aylott et al., 1968; Turk, 1974). Reduced rates of glucose

and octanoic acid oxidation were also observed in birds infected with *E. acervulina* and

were linked to the observation that mitochondria isolated from infected birds oxidized

both octanoic and α-ketoglutaric acids at a lower rate than mitochondria from control

chickens (Allen and Danforth, 1984). A shift in metabolism away from mitochrondrial

oxidation towards the oxidation of glucose through the pentose phosphate pathway due

to *E. acervulina* infection was also observed in the duodenum. This was indicated by a

four-fold increase in the ratio at day 4 postinfection of metabolism of glucose through

the glycolytic pathway versus the tricarboxylic pathway. These findings indicate a shift

in metabolism away from mitochondrial oxidation towards oxidation of glucose through the pentose phosphate pathway (Allen and Danforth, 1984; Bloom and Stetton, 1953).

Eimeria maxima is the primary species that infects the midgut on either side of Meckel's diverticulum. Gross signs include a salmon exudate, thickened intestinal walls, petechial lesions, and hemorraging. Infection can be observed into the duodenum and as low as the intercecal junction (Schnitzler and Shirley, 1999). Normal villi in this region are shorter, broader, and generally apically squared off versus villi of the duodenum. Damage caused by *E. maxima* in the jejunum can include shortened villi, isolated patches of exposed connective tissue of the lamina propria, and epithelial sloughing, as oocysts formation occurs underneath the epithelium (Witlock and Ruff, 1977). *E. maxima* infections can cause decreased absorption of nutrients as well as the above physiological changes in the jejunum. Patterns of zinc and protein digestion and absorption patterns were similar to patterns observed in the duodenum throughout the infection, with increased zinc absorption during the first three days postinfection and returning to normal after recovery. Protein absorption remained equal to uninfected birds for the first three days and dropped to 58% of controls, but returned to levels significantly higher than uninfected birds upon recovery resulting in compensatory gain. Oleic acid and calcium absorption was reduced to 65% of the controls (Turk, 1974).

Eimeria tenella is the primary species that infects the ceca. Uninfected villi of the large intestine are blunter, wider, and thicker as compared to those of the ileum (Witlock and Ruff, 1977). (Whitlock and Ruff, 1977). The proliferation of *E. tenella* destroys the intestinal epithelium, causing the intestinal wall to become swollen, leading to bloody

diarrhea and fibrin clots in the feces (Jeurissen et al., 1996). Only minute changes were seen in absorption rates of calcium, oleic acid, zinc, and amino acids due to infection, thus leading to the conclusion that *E. tenella* infections have little effect on most nutrient absorption (Turk, 1974). Although another investigator observed that infection with *E. tenella* significantly reduces plasma carotenoids, lipids, and proteins as well as body weight gain and feed efficiency, this may be due to a mechanism other than nutrient absorption such as anorexia or bleeding through the gross lesions associated with *E. tenella* infections (Conway et al., 1993).

In addition to pathological changes and subsequent malabsorption of nutrients in the intestine, reduced weight gain and feed conversion due to anorexia is also involved. This has been attributed to a number of mediators including IL-1 and leptin (Klasing et al., 1987; Min et al., 2004). Other nonspecific host responses, not necessarily linked to decreased performance, have also been observed in experimental infections. Modulation of oxidative stress by generation of free radicals such as superoxide and nitric oxide (NO) has been reported to be an important regulator of both host immunity and host-parasite interactions and has been reported to occur in *Eimeria* infections (Allen, 1997a,b). Alterations in whole body thiol balance, specifically reduced blood levels of glutathione during infections, and production of acute phase proteins such as alpha-1-acid glycoprotein, hemopexin, and ovotransferrin, as well as metal binding proteins, have also been observed in infections with *Eimeri*a, although the role these substances have in infection and immunity are not currently known (Min et al., 2004).

The most extensively studied responses to infection with *Eimeria* have been those of the immune response. There are three key points in the life cycle of *Eimeria* where the host immune system can inhibit the parasite. These are the interaction of parasites with host intestinal epithelium cells, within the epithelium in proximity to IELs, and during passage through the lamina propria to the crypt epithelium (Jeurissen et al., 1996). Likewise, the GALT of the chicken serves three functions in defense against enteric pathogens. These are processing and presentation of antigens, production of intestinal antibodies, and activation of cell mediated immunity (CMI) (Min et al., 2004). Both recent and past investigations have elucidated the relative importance of both humoral and cell mediated immunity on *Eimeria* infection.

It is well established that infection with the three major *Eimeria* species results in parasite specific serum IgG(Y), IgA and IgM levels that are generally detectable within a week of infection and reach maximum levels at about two weeks (Galmes et al., 1991; Rose, 1971). Intestinal antibodies have also been detected both at the site of infections and distal areas, with levels of IgM and IgA at the site of parasite replication being greater than those at distal sites but those of IgG not differing. The pattern of antibody production was observed to be the same in the intestine as in the serum with the exception of a transitory rise in IgA in the serum in *E. acervulina* infections and a lack of intestinal IgA in the first week in *E. tenella* infections (Girard et al., 1997). The role of antibodies in *Eimeria* infections is likely not direct. There is evidence that serum transfer from *E. maxima* infected birds can reduce oocyst production in chicks infected with *E. maxima* (Rose, 1971). Immunization of hens with gametocyte antigens from *E*

maxima was also observed to decrease oocyst production chicks infected with *E. maxima, E. acervulina* and *E. tenella* (Wallach et al., 1995). A monoclonal antibody to an *E. acervulina* conoid antigen was also described that inhibited invasion of multiple species of *Eimeria in vitro* (Sasai et al., 1998; Sasai et al., 1996). In contrast there have been studies that found that immunity to infection was not reduced in bursectomized chickens and that antibody levels did not correlate to oocyst output (Lillehoj, 1987; Talebi and Mulcahy, 1995). Therefore, considering the above information, it is thought that the role of parasite-specific antibodies is that of reducing the effective dose through parasite agglutination, neutralization, steric hinderance, and reduced motility as opposed to direct antibody mediated killing (Lillehoj and Lillehoj, 2000).

There is considerable evidence for the importance of CMI in the protection from *Eimeria* as mediated by antigen specific and non-specific activation of T-cells, natural killer (NK) cells, and macrophages. T-cells in particular are important in protection from *Eimeria* infections both from species specific for the chicken and foreign-host species (Lillehoj et al., 2000). A number of studies investigated the effects of cyclosporine-A (Cs-A), betamethasone, and dexamethasone on infection with *Eimeria tenella*. Cs-A enhanced resistance to primary infection when given with the innoculum, but when given before or prior to secondary infection decreased or eliminated protective immunity (Lillehoj, 1987). Additionally this same suppressive effect was observed when Cs-A was given before inoculation with turkey specific *Eimeria* (Kogut and Eirmann, 1991). Administration of both betamethasone and dexamethasone enhanced the severity of *Eimeria* infections and dexamethasone was observed to decrease T-cell proliferation

with no changes in antibody production and allow the development of turkey coccidia in chickens (Isobe and Lillehoj, 1993; Long and Rose, 1970; McLoughlin, 1969). Other studies investigated the relative contribution of CD4+ and CD8+ cells using monoclonal antibody-activated depletion. CD8+ depleted animals produced significantly fewer oocysts after a primary infection but significantly more after a secondary infection with either *E. acervulina* or *E. tenella*. In addition CD4+ depleted chickens produced significantly more oocysts after a primary infection with *E. tenella*, but not *E. acervulina* (Trout and Lillehoj, 1996). While CD4+ cells represent a minor population of the chicken IEL, increases were observed in the duodenum 4 to 8 days after infection with *E. acervulina* and in the ceca 8 days after infections with *E. tenella* (Bessay et al., 1996). It is thought that the role of CD4+ T cells in coccidiosis may involve the production of soluble cytokines such as IFN-γ (Yun et al., 2000b).

Another line of investigation involved the use of two inbred strains of chickens displaying different levels of susceptibility to coccidiosis with the SC strain being more resistant than the TK strain. These strains were shown differ in their T-cell sub-populations, particularly the γδ TCR+CD8+ cell in the intestine after infection with *Eimeria acervulina* (Lillehoj, 1994). Specifically in *Eimeria acervulina* infections, an increased percentage of γδ T cells were observed in the duodenum after post primary and secondary infections (Choi and Lillehoj, 2000). When the SC and TK lines were compared, this increase was observed to occur four days earlier in the SC line as compared to the TK line. An increase in αβ T cells was also observed in both lines by six days after the secondary infection (Choi et al., 1999).The importance of CD8+ T

cells was also evidenced by the significant increases in CD 8+ T ells after *E. acervulina* infection four days post infection, with a higher increase for the SC line as compared to the TK line (Lillehoj et al., 2004).

Macrophages have also been implicated in immunity to coccidiosis. Significant numbers of sporozoites have been found within macrophages after primary and secondary infection with *Eimeria acervulina* (Trout and Lillehoj, 1995). This observation and the fact that macrophages produce a significant amount of IFN-γ following stimulation with both sporozoites and merozoites, lead investigators to believe that macrophage involvement in immunity is through modulation of host response (Zhang et al., 1995). Additionally, NK cell activity was increased in chickens after both a primary and secondary infection with *Eimeria*. NK cell activity in two inbred strains was positively correlated with resistance (Chai and Lillehoj, 1988; Lillehoj, 1989).

As cytokines play a major role in the regulation of immune responses, their role in *Eimeria* infections has also been investigated. However, due to the fact that identification of many chicken cytokines is recent, only a few have been investigated in this capacity. Interferon-γ is the most extensively characterized cytokine in regards to *Eimeria* infection. It has been examined through both quantitative RT-PCR and gene expression profiling (Choi et al., 1999; Laurent et al., 2001; Min et al., 2003; Yun et al., 2000a). IFN-γ mRNA was detected in the cecal tonsils and spleen, but not the duodenum, of SC strain chickens challenged with *E. acervulina* and was increased over 200-fold in the cecum and jejunum of specific pathogen free (SPF) chickens challenged with either *E. acervulina* or *E. maxima*(Choi et al., 1999; Laurent et al., 2001). After

both primary and secondary *E. tenella* infection, a marked increase in IFN-γ was

reported in the spleen, cecal tonsils am IELs (Lillehoj et al., 2004).The role of IFN-γ was

directly assayed by injecting recombinant cytokine into *E. acervulina* infected SC and

TK chickens, resulting in significant decreases in both body weight loss and oocyst

shedding. It was also observed that *E. tenella* sporozoites were inhibited from

undergoing development in a cell line stably transfected with chicken IFN-γ (Lillehoj

and Choi, 1998). These results demonstrate the inhibitory effect of IFN-γ against

Eimeria, but the precise mechanisms have yet to be elucidated.

Interleukin-2 (IL-2) is a potent growth factor involved in T and B cell

differentiation and NK cell activation. Increases in IL-2 mRNA expression in the spleen

and intestine were observed after both primary and secondary infection with *Eimeria*

acervulina (Choi and Lillehoj, 2000). Tumor necrosis factor alpha (TNF-α) is a potent

inflammatory cytokine with pleiotropic functions including NO release and induction of

MHC class II on macrophages (Staeheli et al., 2001). A TNF-like factor has been

experimentally produced following infections with both *E. maxima* and *E. tenella* in a

biphasic fashion. The first peak was associated with pathogenesis and the second with

induction of immunity (Byrnes et al., 1993). Additionally treatment of chickens with

antibodies raised against TNF resulted in partial reduction of weight loss induced by *E.*

tenella infection (Zhang et al., 1995). Transforming growth factor beta (TGF-β) is a

pleiotropic anti-inflammatory cytokine that stimulates repair of damaged mucosal

epithelium and four isoforms have been identified in the chicken (Staeheli et al., 2001).

After *E. acervulina* infection TGF-β4 expression increased 5 to 8 fold in the intestinal

IEL and 2.5 fold in the spleen, whereas TGF-β2 and TGF-β3 remained the same

(Jakowlew et al., 1997). Interleukin-6 is a pleiotropic lymphokine originally described as

a T-cell derived lymphokine and induces the final maturation of B cell into antibody

producing cells (Staeheli et al., 2001). An IL-6 like factor was detected in serum from *E.

tenella* infected chickens during the first few hours indicating a possible role in the

development of acquired immunity (Lynagh et al., 2000). Interleukin-1 (IL-1) is

involved in T cell commitment, induction of fever, activation of the hypothalamic-

pituitary axis and glucocorticoid secretion, triggering of the acute-phase response, and

activation of the vascular endothelium (Staeheli et al., 2001). *E. tenella* and *E. maxima*

infections induce a 27-80 fold increase in production of IL-1β mRNA in the jejunum or

cecum at seven days post infection (Laurent et al., 2001). IL-10 is primarily involved in

modifying immune responses through direct effects on T cells, B cells, APCs and NK

cells by promoting the development of Th2 responses. In *E. maxima* infections a

significant increase in IL-10 mRNA was observed in both the small intestine and spleen

of a susceptible line of chickens (line 15I) as compared to a resistant line (line C.B12).

However, the fact that levels of IFN-γ mRNA expression were the same between the two

lines, indicates that the susceptibility may be due to a higher constitutive expression of

IL-10 (Laurent et al., 2001).

Methods of Control of Coccidiosis

Considering the importance of poultry production and the adverse effects of

coccidiosis on commercially raised poultry, control of this disease is very important.

Biosecurity plays a large part but, due to the ubiquity of coccidial species, is not an

adequate control. Management strategies differ between the United States and Europe. Operations in Europe and Canada do a thorough clean out between flocks, while U.S. operations generally remove caked litter, let the house air out, and then top dress with fresh litter. As the use of live vaccines and the incidence of drug resistance increases in the U.S., it is thought that we may see changes in these practices (Allen and Fetterer, 2002).

Since the introduction of the first true anticoccidial drugs in the 1940s, new anticoccidals have been introduced in increasing numbers, and the judicious use of these drugs has allowed the increased availability of high quality affordable poultry products to the consumer (Allen and Fetterer, 2002; Stephen et al., 1997). Despite the fact that in the 1960s it was stated that resistance did not represent any serious problem in the control of coccidiosis, this clearly is no longer the case, as resistance to every anticoccidial available has been observed (Chapman, 1993). Anticoccidial drugs can be roughly divided into two categories. The first include chemicals which have specific modes of action against parasite metabolism, such as amprolium, clopidol decoquinate, and halofuginone. The second category act through general mechanisms of altering ion transport and disrupting osmotoic balance and are the polyether ionophores such as monensin, lasalocid, salinomycin, narasin, and maduramycin. Despite the success of these compounds, some resistance has developed to all these chemotherapeutic agents (Allen and Fetterer, 2002). This is probably due to the fact that most drugs target the asexual stages, the first or second generation meronts. Since the asexual stages are haploid, the complexities of selection present in diploid organisms are absent. This

means that resistant mutants will be immediately selected for and quickly dominate over non-resistant forms (Chapman, 1993). This fact, along with consumer concern about drug residues in food, has promoted the development of alternative forms of control such as live oocyst vaccination (McEvoy, 2001).

The only current alternative to the use of anti-coccidial drugs is live oocyst vaccination (Chapman et al., 2002). The basis for the use of live oocyst vaccines is the fact that, after an infection, the host is immune to subsequent infections by the same species (Yun et al., 2000b). This immunity is species specific, therefore vaccines are formulated with multiple species. In addition, the use of low numbers of parasite administered multiple times, or trickle immunization, also results in species specific protective immunity (Vermeulen et al., 2001). Live vaccines have been used by the poultry industry for over 50 years, primarily for broiler breeders and replacement layer stock (Chapman et al., 2002). The reluctance in using live vaccines in broilers stems from decreased weight gain and feed conversion due to vaccination as compared to medicated birds, as well as the possibility of introducing new species into the rearing house. Both the development of attenuated vaccines and improved application methods have alleviated some of the concern and led to an increased in use in broilers (Allen and Fetterer, 2002; Chapman et al., 2002).

There are currently a number of commercially available live vaccines for coccidiosis that fall into two classifications, live virulent and live attenuated (Vermeulen et al., 2001). Virulent vaccines are wild type field strains that have been isolated from commercial poultry operations, presumably before the emergence of drug resistance.

Attenuated vaccines are obtained either by selection for early maturation (pre-cociousness) or through serial passage through embryonated eggs (TA lines). The four dominant commercial vaccines used by the poultry industry go by the trade names "Coccivac", "Immucox", "Livacox", and "Paracox". Of these the "Coccivac" and "Immucox" are live virulent vaccines, "Paracox" is precocious, and LivaCox is precocious or TA line. They all incorporate between two and eight species of *Eimeria* dependent upon the market they are targeted to and all provide solid immunity to coccidial infection when applied carefully and under good management conditions (Danforth, 1998; Vermeulen et al., 2001).

As mentioned above efficient delivery of vaccine is crucial to development of protective immunity. Delivery in the drinking water has for the most part been replaced by spraying the vaccine on the feed or the use of an edible colored gel containing the oocysts. While these are generally considered to result in uniform exposure, widespread use of spray cabinets for viral vaccination has prompted the use of this method. This procedure is automated and through the use of red dye in the vaccine it has been observed that between 90 and 95% of the chicks receive the vaccine. It is then further spread through preening behavior (Chapman et al., 2002). Despite the ease of use with spray cabinets, a series of field trials compared spray cabinet, gavage, slurry and gel delivery and concluded that gel delivery gave the best protection to *E. maxima* infection (Danforth, 1998). Another attractive method of vaccination is *in ovo* delivery, direct injection of a vaccine into an 18 day old embryo. This is attractive due to the fact that it is already widely used for vaccination to viral diseases and therefore is already in place.

Recent studies have demonstrated that injection of infective stages of *Eimeria* both cause

an infection in the chick and can contribute to immunity (Weber and Evans, 2003;

Weber et al., 2004).

As stated above in some cases live oocyst vaccination has been observed to lead

to decreased weight gain. One possible solution to this is the use of adjuvants to improve

immunity to the vaccine and possibly provide some protection to the chick during

vaccination. The recently described adjuvant CpG-ODNs, with its immunostimulatory

properties, would seem to be an excellent candidate for this application. A recent study

using SC and TK lines of chickens examined the effects of different delivery methods of

CpG-ODNs twenty-four hours before an infection with *E. acervulina* on weight gain,

oocyst production, and production of antibodies to the antigen 3-1E. Weight gain was

increased when CpG-ODNs were given intravenously (I.V.) to day old TK chicks, but

not orally to either. I.V. injection also resulted in an increase in weight gain dependent

upon the backbone used and the dose given. Oocyst shedding was decreased in only the

TK line of chickens and no differences in antibody responses were observed (Dalloul et

al., 2004). These results suggest that immunostimulatory adjuvants may be useful in

reducing reduction in body weight gain due to live parasite vaccination.

SUMMARY AND CONCLUSIONS

A major research interest in our laboratory at present is the investigation of

immune responses to enteric pathogens in poultry, the response to vaccination, and the

subsequent improvement of vaccination strategies. This is being done through

investigation of alternate modes of vaccine delivery and immunomodulation of the host

immune response through the use of the recently described mucosal adjuvant CpG-ODNs. In the present investigations, we evaluated a means for delivering protein antigen in the drinking water and the use of CpG-ODNs in order to improve this response. We then investigated three methods of modulating the host's immune response with CpG-ODNS when vaccinated with a field strain of *E. acervulina* or challenged with field strains of *E. acervulina* or *E. tenella*.

CHAPTER III

IMMUNOGENICITY OF AD LIBITUM DRINKING WATER

ADMINISTRATION OF BSA IN LEGHORN CHICKENS[*]

INTRODUCTION

Oral administration of solubilized non-replicating protein antigen has been reported to lead to oral tolerance in many mammals resulting in transitory induction of secretory IgA (sIgA) and suppression of systemic IgG, whereas other mammals, such as the guinea pig, are anaphylactically sensitized by administration of oral antigen (Challacombe and Tomasi, 1980; Miller and Cook, 1994). In chickens, however, protein antigen administered orally to chicks older than ten days of age induces a strong humoral immune response involving elevated serum titers of both IgG and IgA isotypes and does not sensitize them to delayed type hypersensitivity after repeated antigen exposure (Klipper et al., 2001; Miller and Cook, 1994). Since resistance to some pathogens correlates with sIgA secretion at mucosal sites, rather than systemic IgG or IgM circulating antibody levels, this difference may have important implications for subunit vaccine delivery in commercial poultry production (Davis et al., 1978; Tomasi and Bienenstock, 1968).

Previous studies have determined a number of factors that influence the response to orally administered protein antigen in chickens. These factors include the form in

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which it is given, the time at which it is administered, the route, and concentration of antigen administered. When BSA was administered in a powdered form to chicks older than 10 days of age, it neither induced a systemic immune response nor increased the response to subsequent immunizations. This is in direct contrast to the responses observed in similar animals to dissolved BSA fed orally, which included a systemic antibody response. However, chicks younger than 8 to10 days of age are not responsive to orally administered dissolved BSA and develop tolerance to it dependent upon the initial dose (Klipper et al., 2001). This responsiveness to antigen correlates with the colonization of the gut mucosa with both B and T lymphocytes and subsequent cytokine expression and production necessary for regulating humoral responses at mucosal sites (Bar-Shira et al., 2003). The route of administration has also been demonstrated to be a factor. Three administrations of BSA via oral gavage one week apart were shown to induce low and inconsistent IgG levels without adjuvant (Hoshi et al., 1999);(Hoshi et al., 1998), whereas when the same solution was fed in trickle fashion via syringe resulted in a strong and consistent IgG response. When dosage or concentration of this type of antigen administration was investigated for optimal responsiveness, it was determined that as little as 2 mg fed for six days consecutively was fully immunogenic (Klipper et al., 2000).

Given the evidence of robust humoral responses elicited to orally administered solubilized BSA and the fact that tolerance seems to be an age dependent event in chickens, oral administration of protein antigen may be practical in a commercial setting for mass vaccination. In order for this approach to vaccination to be practical, mass

immunization strategies must be investigated. The most efficient method for oral administration would be via the drinking water. It was the purpose of this study to evaluate the efficacy of low dose, ad libitum administration of a protein antigen in the drinking water to immunologically mature chickens for eliciting systemic and local intestinal antibody responses. This route of administration was compared to both oral gavage and i.p. routes of administration.

MATERIALS AND METHODS

Experimental Animals

Single-comb-white-Leghorn (SCWL) chicks were obtained from a local commercial hatchery (Hyline International, College Station, TX) on day of hatch and placed in floor pens at an initial density of 18 birds per m^2 with pine shavings as litter. They were maintained at age appropriate temperature and fed a corn-soy chick starter ration, lacking animal fat or protein, and formulated at or above NRC recommendations (National Research Council, 1994) for poultry and water ad libitum.

Experimental Design and Immunization

In Experiments 1 and 2, day of hatch chicks were divided into the following five experimental groups: negative control, i.p. alum control, i.p. BSA, BSA gavage, and BSA in drinking water. To immunize chickens to BSA by i.p. administration, two successive i.p. injections consisting of 5 µg precipitated BSA in 5 mg alum ($AlK(SO_4)_2$) were administered to chickens on days 12 and 18 of each experiment. Non-immunized alum control chickens received i.p. injections of 5 mg alum only at the same time points of each experiment. Chickens in gavage groups received a *per os* gavage once daily

with 12.5 mg of BSA in 0.5 ml water on days 12 through 17 of each experiment.

Chickens in the drinking water group were given ad libitum access to a bell drinker with

water containing 1.4 mg/ml BSA on days 12 through 17 of each experiment.

Sampling

On day twenty-five of life, seven days following the final i.p. immunization and

eight days following the last oral administration, 1 ml of blood was drawn from the wing

(bicipital) vein and allowed to clot for serum collection. Serum was transferred to a clean

microcentrifuge tube and then frozen at $-20°C$ until assayed by indirect ELISA. The

chickens were then euthanized, and, in Experiment 2 only, intestinal sections were

collected to assay local intestinal antibody production stimulated by the evaluated routes

of immunization.

Ex vivo Culture of Intestinal Tissue

To measure antigen-specific IgG or IgA production in intestinal sections

following immunization in Experiment 2, we adopted the method described by

Zigterman and co-workers (Zigterman et al., 1993) with slight modification.

Immediately following euthanasia, intestinal tissue corresponding to the distal ileum,

cecal tonsils, and the distal cecum were removed and immersed in ice cold Hank's

Balanced Salt Solution (HBSS) containing 500 IU/ml of penicillin and 500 μg/ml of

streptomycin. Gut contents were removed by gently flushing each tissue section with

HBSS. Tissues were then cut into pieces weighing 0.3 g each, and each tissue was then

cut into three smaller pieces and again washed with HBSS containing penicillin and

streptomycin. Tissues, respective for each animal sampled within each experimental

group, were then suspended in 6 ml of RPMI 1640 supplemented with 100 IU/ml

penicillin, 100 μg/ml streptomycin, 100 μg/ml gentamicin, and 40 mM HEPES.

Suspensions were centrifuged (5 min, 300g), and 800 μl aliquots of supernatant (ileum,

cecal tonsil, or distal cecum supernatant; labeled t = 0) were taken. The tissues were

resuspended and incubated at 41°C, 5% CO_2, 95% air in twelve well culture plates for

16 hours. After 16 h culture, aliquots were removed and labeled as t = 16 for each

respective sample. BSA-specific IgG, IgM, and IgA titers measured during *ex vivo*

intestinal culture for each experimental animal were determined using indirect ELISA by

subtracting the t=0 values from t=16.

Absorption of Cross-reactive Antibodies and ELISA

Serum or collected medium from intestinal tissue culture, respective for each

experimental animal evaluated, was pre-incubated overnight at 4°C with 40 mg of

chicken albumin per ml and diluted to 1:320, 1:640 and 1:1280 for serum samples and

1:5, 1:10 and 1:20 for intestinal media samples to eliminate the presence of any potential

cross-reactive antibodies. Indirect ELISA was performed on all serum or intestinal

tissue culture samples using 96 well plates coated with 5 μg of BSA per well. Following

overnight incubation, plates were rinsed with PBS-T (pH 7.4, 0.5% Tween 20), 150 μl of

serum or media was added, and plates were agitated for an additional 2 hours. Plates

were again rinsed, goat anti-chicken IgG, IgM, or IgA (Bethyl Laboratories,

Montgomery, TX) was added to each well, and plates were agitated for 1 hour. Plates

were rinsed, and a substrate solution containing 100μl dimethyl sulfoxide (DMSO) with

1 mg tetramethlybenzidine (TMB), and 10 μl of hydrogen peroxide (H2O2) in 10ml of

sodium acetate buffer (pH 5.5) was added. After 15 minutes the reaction was stopped by adding 50µl of 1 M sulfuric acid. Absorbance was read by a multi-well plate reader at a wavelength of 450 nm.

Statistical Analysis

Samples were read in duplicate and normalized for plate effects by dividing by a positive standard. The mean absorbance of samples was log transformed to normalize variance and analyzed using the Univariate General Linear Model procedure for analysis of variance (SPSS 11.01, Chicago, IL). Statistically different means ($P < .05$) were further separated using Duncan's Multiple Range Test (SPSS, v. 11.0)

RESULTS AND DISCUSSION

It has been well established that oral administration of protein in solution is immunogenic and not tolerogenic in domestic chickens. (Klipper et al., 2000; Miller and Cook, 1994). This has been demonstrated by feeding discrete amounts of solubilized antigen to chickens of an immunocompetent age for six to 14 consecutive days (Klipper et al., 2000; Miller and Cook, 1994). Following these regimes, specific antibody production has been reported in both the serum and the bile. Alternative approaches of antigen administration, such as once a week feedings, do not induce an humoral response in the absence of adjuvant (Hoshi et al., 1998; Hoshi et al., 1999).

In the present study, serum IgG responses measured in Experiments 1 and 2 are shown in Tables 1 and 2. Although the background absorbance varied between experiments, trends and observed significance between experimental groups in both experiments were observed to be the same. BSA administered ad libitum in the drinking

water resulted in the generation of greater antibody titers ($P < 0.001$) when compared to titers in chickens in negative control and oral gavage experimental groups. Similarly, IgG titers in chickens in the i.p. BSA group were significantly greater ($P < 0.001$) than all other experimental groups excluding the drinking water BSA group, indicating oral drinking water antigen administration was as efficacious as i.p. administration with adjuvant.

Billiary IgA responses, as shown in Tables 1 and 2, revealed a different trend. Although the background was high in both experiments, BSA administered to chickens in the drinking water induced higher ($P < 0.001$) levels of IgA than i.p., gavage, or either of the control groups. Intestinal IgG responses (Table 2) were similar to responses measured in the serum. In the distal ileum, cecal tonsils, and cecum, IgG levels were higher ($P < 0.05$) in chickens in the i.p. BSA and drinking water experimental groups than levels observed in both control groups as well as the oral gavage group. Intestinal IgA responses, however, did not differ significantly between the groups in any of the intestinal regions evaluated (data not shown).

These data confirm that oral administration of solubilized BSA induces both serum and bile antibody responses and also establishes that a local intestinal antibody response is measurable seven days following the last time of immunization, indicating specific antibodies were produced by lymphocytes in the distal ileum, cecal tonsils, and distal cecum in response to oral BSA immunization. Although measured responses consisted of primarily IgG secretion, it bears mentioning that only one time point during this evaluation of intestinal antibody secretion was evaluated. The fact that sIgA levels

were observed not to differ at seven days after the last immunization, where IgG

secretions did, is not a unique observation. Other studies have reported similar findings

in response

TABLE 1: BSA-specific serum IgG and bile IgA responses in chickens immunized with BSA by intraperitoneal or oral routes of administration in Experiment 1

Group	N=	Serum	Distal Ileum	Cecal Tonsils	Distal Ceca	Bile
Control	4	0.07 ± 0.10[B]	ND	ND	ND	0.68 ± 0.16[B]
BSA – Water	10	0.47 ± 0.07[A]	ND	ND	ND	1.35 ± 0.10[A]
BSA – Gavage	9	0.15 ± 0.07[B]	ND	ND	ND	0.41 ± 0.11[B]
i.p. – Vehicle	3	0.02 ± 0.12[B]	ND	ND	ND	0.74 ± 0.18[B]
i.p. - BSA	9	0.59 ± 0.07[A]	ND	ND	ND	0.89 ± 0.11[B]

Data represent mean +/- S.E. of peak absorbance values determined by indirect ELISA. Serum samples were diluted 1:640, bile sample were diluted 1:80, and media from intestinal culture were diluted 1:10. Means ± S.E. within columns with no common superscript differ significantly at the following levels of significance: [A]P<0.001. ND: Not determined.

to challenge with *Eimeria* species or a purified recombinant antigen from *E. tenella*

(Girard et al., 1997; Girard et al., 1999). It is indeed likely that if measured at a later

day, IgA production may have been elevated in the intestinal sections tested, as seen in

the studies previously mentioned.

TABLE 2: BSA-specific serum IgG, intestinal IgG, and bile IgA responses in chickens immunized with BSA by intraperitoneal or oral routes of administration in Experiment 2

Group	N =	Serum	Ileum	Cecal Tonsils	Distal Ceca	Bile
Control	5	0.16 ± 0.08^B	0.02 ± 0.01^b	0.03 ± 0.01^b	0.03 ± 0.01^b	0.73 ± 0.14^B
BSA – Water	5	0.55 ± 0.07^A	0.13 ± 0.03^a	0.12 ± 0.03^a	0.12 ± 0.03^a	1.11 ± 0.09^A
BSA – Gavage	5	0.20 ± 0.07^B	0.03 ± 0.01^b	0.05 ± 0.02^b	0.05 ± 0.02^b	0.42 ± 0.09^B
i.p. – Vehicle	5	0.14 ± 0.08^B	0.01 ± 0.01^b	0.01 ± 0.01^b	0.01 ± 0.01^b	0.43 ± 0.16^B
i.p. - BSA	5	0.45 ± 0.07^A	0.18 ± 0.10^a	0.12 ± 0.04^a	0.12 ± 0.04^a	0.69 ± 0.09^B

Data represent mean +/- S.E. of peak absorbance values determined by indirect ELISA. Serum samples were diluted 1:640, bile sample were diluted 1:80, and media from intestinal culture were diluted 1:10. Means ± S.E. within columns with no common superscript differ significantly at the following levels of significance: [a]$P<0.05$ or [A]$P<0.001$. ND: Not determined.

As stated above, ad libitum, oral administration of solubilized protein antigen elicits an immune response in commercial lines of SCWL chickens. While this study was limited to SCWL chickens, other studies have reported a similar responsiveness to oral BSA administration in broiler chickens (Klipper et al., 2000). When compared to negative controls and crop-gavaged chickens, animals receiving BSA by drinking water administration produced higher levels of antigen specific antibodies. It is worthwhile to note that IgG levels in those chickens were not significantly different than levels in chickens given two i.p. injections of BSA with alum as an adjuvant.

Observations of drinking water administration being more efficacious in eliciting humoral immune responsiveness were also seen by Klipper and co-workers (Klipper et al., 2001). One possible explanation may involve crop distention associated with the single bolus *per os* administration. Crop distention, in this scenario, may likely result in the release of proteolytic pepsinogen and HCl by the proventriculus, which might result in the degradation of the protein before adequate amounts could reach lymphoid tissue at distal sites in the intestine. The findings of Klipper *et al*. (2000), indicating that feeding powdered forms of BSA to mature chicks induced neither an immune response or tolerance, and Hoshi *et al*. (1998) by demonstrating that gavage administration did not induce immunity and may induce tolerance, adds support to this speculation.

Regardless of the mechanisms involved in the observed responsiveness to oral antigen in the chicken, the present investigation clearly confirms that solubilized oral protein administration induces both systemic and local humoral immune responses. These findings suggest that subunit protein vaccines, administered by a drinking water route of delivery, may be a feasible option for commercial productions or smaller breeder flocks. While additional investigation into such a route of mass vaccination is clearly needed to support such a claim, the ease of administration to large numbers of animals by this approach represents a distinct advantage, even more so than what exists for many live and attenuated vaccines for enteric pathogens, which are presently given orally. Additional research should also include investigation into variability in immunogenicity of antigens isolated from specific pathogens, as well as the level of

protection induced by these antigens. The duration of immunity elicited by oral protein

antigen administration with and without adjuvant also needs to be studied.

CHAPTER IV

EFFICACY OF ORAL CPG-ODNS AS MUCOSAL ADJUVANTS WHEN CO-ADMINISTERED WITH PROTEIN ANTIGEN IN CHICKENS

INTRODUCTION

In all commercial poultry operations, the overwhelming majority of infectious pathogens access the host via the mucosal surfaces of the respiratory, genitourinary and gastrointestinal tracts (Staats et al., 1994). While many live and attenuated vaccines used in production operations involve oral administration or delivery, their efficacy following administration by this route is often variable (Vermeulen et al., 2001). Since oral vaccination appears to be one of the most effective and feasible ways to induce mucosal immunity in commercial operations, it stands to reason that killed or subunit vaccines may also be administered by this route. However, killed and subunit vaccines are often not immunogenic or require multiple boosts to be effective (Husband, 1993; McGhee and Kiyono, 1993), therefore, an adjuvant that can be administered orally and is capable of improving overall immunogenicity is needed.

Bacterial toxins such as cholera toxin (CT) and the structurally related *Escherichia coli* heat-labile enterotoxin (LT) are both potent mucosal adjuvants that have been successfully used in several animal models. Orally administered CT induces a strong mucosal response in both mice and layer type chickens when given with non-replicating antigen (Girard et al., 1999; Lycke and Holmgren, 1987). Similar use of LT has been less successful in both mammals and birds (Lee et al., 1999; Rice et al., 1997).

Despite the reported efficacy of both adjuvants, both their potential for toxicity and present high cost make them impractical for commercial use.

Immunostimulatory DNA sequences (ISS) represent a potential new class of mucosal adjuvants. ISS are DNA sequences containing unmethylated CpG (cytosine-phosphate-guanosine) dinucleotides in a specific base context, or motif. They are most commonly given in the form of synthetic oligodeoxynucleotides (ODN) made with a nuclease resistant phosphorothioate backbone (McCluskie et al., 2000). Several characteristics of CpG-ODNs contribute to its potential usefulness as a mucosal adjuvant. CpG-ODNs have been reported to stimulate B cells to proliferate, secrete immunoglobulins, and secrete IL-6 and IL-12, in addition to protecting them from undergoing apoptosis (Krieg, 2001). Additionally, CpG-ODNs have been shown to enhance expression of class II MHC and B7 costimulatory molecules on antigen presenting cells (APCs) which leads to enhanced antigen presentation to lymphocytes. Activation of monocytes, macrophages, and dendritic cells to secrete select cytokines and chemokines which increase T-helper functions has also been reported (Krieg, 2002).

Stimulatory CpG motifs generally have an ApA, GpA or GpT dinucleotide on the 5' side with two pyrimidines, typically TpT, on the 3' side (Yi et al., 1996). A large degree of conservation among species in term of actual motif recognition apparently exists. While *in vitro* assays determined that GA<u>CG</u>TT was optimal for stimulation of inbred laboratory murine and rabbit lymphocytes, most other species of veterinary importance, including chicken, responded optimally to the GT<u>CG</u>TT motif (Rankin et

al., 2001). In addition to the base sequence, the inclusion of spacers between multiple copies of the motif also influenced responses (Rankin et al., 2001).

The majority of work performed to date using CpG-ODN as adjuvants has involved parenteral immunization of mice, non-human primates and, in one case, sheep (Chace et al., 1997; Davis et al., 2000; Rankin et al., 2001). More recently it has been demonstrated that immunization with CpG-ODNs intranasally, intrarectally, and orally is immunostimulatory in mice, with intranasal and oral being the most effective routes of administration (McCluskie and Davis, 2000). Investigations of the effectiveness of CpG-ODNs as in chickens can be divided into two types. The first type evaluated antibody response to a protein antigen. This includes administration of BSA, incomplete Freunds adjuvant (IFA), and CpG-ODN subcutaneously (SQ) and subsequent measurement of the antibody response over thirty-five days (Vleugels et al., 2002). The second type evaluated protection from a pathogen. Gomis (Gomis et al., 2003) used CpG-ODNs as an immunostimulant to protect from *Escherichia coli* infections. Wang et. al. (Wang et al., 2003) evaluated the usefulness of CpG-ODNs as an adjuvant to IM DNA vaccination against infectious bursal disease virus (IBDV). Recently CpG-ODNs were also used in the context of infection with *Eimeria acervulina* (Dalloul et al., 2004). All three studies observed a decrease in pathogenesis when CpG-ODNs were administered I.V. Dalloul et. al. (Dalloul et al., 2004) observed no increase in body weight gain when given orally before an infectious dose of *Eimeria acervulina,* nor did they report an increase in antibody production. However, a previous study using CT as an oral adjuvant for *Eimeria tenella* or the recombinant antigen 1PE1 observed that antibody levels were

increased in the latter but not the former (Girard et al., 1999).Thus, the objective of this study was to determine whether a single oral administration of CpG-ODN could alter the systemic or mucosal antibody response to BSA when administered to chickens by a drinking water route of delivery.

MATERIALS AND METHODS

Experimental Animals

In both Experiment 1 (n=100) and Experiment 2 (n=80), male single comb white Leghorn (SCWL) chicks were obtained from a local commercial hatchery (Hyline International, College Station, TX) on day of hatch. All chicks were randomized and placed in open floor pens with pine shavings as litter material, at a rearing density of 15 birds per m^2. Chicks were maintained at age appropriate temperatures and given *ad libitum* access to water and a complete chick starter ration devoid of animal fat, formulated to meet or exceed current NRC recommendations for poultry (National Research Council, 1994).

Synthetic CpG-ODN

Both CpG-ODN (Gomis et al., 2003) (5'-TCGTCGTTGTCGTTTTGTCGTT-3') and non-CpG-ODN (5'-CTGGTCTTTCTGGTTTTTTTCTGG-3') were obtained from Integrated DNA Technologies, Inc (Coralville, IA). The ODNs were prepared with a phosphorothioate backbone and reconstituted in pyrogen free PBS immediately before administration.

Experimental Design

In Experiment 1, day of hatch chicks were divided into four groups; negative control, BSA only, BSA with non-CpG-ODN, and BSA with CpG-ODN. At 15 days of age, chicks in respective experimental groups received 0.5 ml of 1.4 mg/ml BSA alone, 0.5 ml of BSA with 75 μg of non-CpG-ODN, or 0.5 ml of BSA with 75 μg CpG-ODN orally via syringe and a feeding needle. Care was taken to allow the birds to drink the solution as opposed to delivering antigen via bolus delivery. Negative control birds received the same volume of water in identical fashion. In Experiment 2 experimental groups consisted of chicks receiving 0.5 ml of 1.4 mg/ml BSA with 0, 5, 50, or 100 μg of the CpG-ODN, administered as described above. After the initial administration of antigen, chicks receiving BSA, in both experiments, were provided with *ad libitum* access to a 1.4 mg/ml BSA solution in bell waterers for six consecutive days, while the negative control chicks were provided water in identical fashion. At twenty-six days after the first BSA administration all experimental groups in both experiments that received BSA were given *ad libitum* access to the BSA solution for 24 hours as a booster immunization.

Sampling

In Experiment 1, five chickens from each experimental group were randomly selected at zero, two, five, twelve, nineteen, and thirty-three days post-first immunization (dpi). After collecting approximately 1 ml of blood from each animal, all chickens were euthanized and the distal ileum, cecal tonsils and distal ceca were collected and cultured as described below. In Experiment 2, approximately 1 ml blood

was collected from all chickens in each of the four experimental groups on zero, two

five, twelve, nineteen, twenty-six, and thirty-three dpi. In both experiments, all blood

was allowed to clot as room temperature, centrifuged, and the serum was transferred to a

sterile microcentrifuge tube and frozen at –20°C until assayed by ELISA.

Ex vivo Culture of Intestinal Tissue

In Experiment 1 only, intestinal specific antibodies to BSA were determined

according to a previously published *ex vivo* tissue culture procedure (Zigterman et al.,

1993), with slight modification. Briefly, after cervical dislocation was performed on

experimental animals, intestinal tissue samples comprising the distal ileum, cecal tonsil,

or the distal cecum were removed and immersed in Hank's balanced salt solution

(HBSS) containing 500 IU / ml of penicillin and 500 μg/ml of streptomycin. Gut

contents were removed by flushing with HBSS. The tissues were then cut into segments

weighing 0.3 g each. Each intestinal tissue segment was then cut into three smaller

pieces and further washed with HBSS containing 500 IU / ml of penicillin and 500

μg/ml of streptomycin. Segments were then suspended in 6 ml of RPMI 1640

supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml

gentamicin, and 40 mM Hepes buffer. The suspensions were centrifuged (5 min, 300 X

g) and 800 μl aliquots of supernatant (specific for tissue type and respective to

experimental group) were removed and labeled as time zero (t = 0) samples. All tissues

were resuspended in fresh RPMI medium and incubated at 41°C in an environment of

saturated humidity and 5% CO_2 in twelve-well culture plates for 16 hours. Following 16

h tissue culture, aliquots were removed as described above and marked as time 16 (t=16)

samples. Immunoglobulin levels for intestinal tissue culture media samples were determined by indirect ELISA, as described below. Levels of intestinal specific immunoglobulin were determined by subtracting the t=0 absorbance values from t=16 values.

Absorption of Cross-reactive Antibodies and Indirect ELISA

In order to minimize background due to the degree of sequence homology between BSA and chicken serum albumin (CSA), an absorption step was performed on all samples in experiment one. Serum or media from intestinal cultures were pre-incubated overnight at 4°C with 40 mg of chicken albumin (Sigma, St. Louis, MO) per ml and diluted to 1:320, 1:640 and 1:1280 for serum samples and 1:5, 1:10 and 1:20 for intestinal media samples. As pre-immunization samples were taken for Experiment 2 and a covariate analysis applied in order to control for both bird to bird variation and non-specific binding serum from those birds were assayed at the same dilution without pre-absorption.

Indirect ELISA was performed using medium binding flat-bottomed 96 well plates (Nalge Nunc International, Rochester, NY). Prior to addition of sample antiserum, plates were coated with 5 μg of BSA per well and agitated overnight. Plates were rinsed with PBS-T and 150 μl of serum or media was added and agitated for 2 hours. Plates were rinsed and 150 μl of horseradish peroxidase (HRPO) conjugated goat anti-chicken IgG, IgM, or IgA (Bethyl, Montgomery TX) diluted 1:10,000, was added to the plates for an additional hour of incubation. Plates were again rinsed with PBS-T and a substrate solution containing 100μl DMSO, 1mg TMB, and 20 μl of H_2O_2 in 10ml of sodium

acetate buffer (pH 5.5) was added. The reaction was stopped after 15 min by adding

50µl of 1 M sulfuric acid. Plates were read in a Sunrise multi-well plate reader (Tecan,

Austria) at a wavelength of 450 nm.

Statistical Analysis

All samples were run in duplicate. Absorbance was corrected for plate effect by

dividing by a positive standard and the mean for each bird was log transformed to

normalize variance. Experiment 1 data was analyzed using the Univariate General

Linear Model procedure for Analysis of Variance (SPSS, v. 11.0). Two-way ANOVA

was performed for each isotype using both day and treatment as factors. In all cases

there was a significant day*treatment interaction (P<0.01) Therefore each day was

analyzed for treatment effect using a One-way ANOVA. Statistically different means (P

< .05) were further separated using Duncan's Multiple Range Test (SPSS, v. 11.0).

Experiment two data was analyzed using the Multivariate General Linear Model for

Analysis of Covariance (SPSS v. 11.0) and means statistically different (P<0.05) from

the BSA only group were determined using the LSD method (SPSS, v. 11.0).

RESULTS

Serum antibody response

To determine whether CpG-ODNs were stimulatory when administered orally, a

dose of 75 µg per chick was chosen for Experiment 1. This dose was selected as it was at

the high end of the stimulatory range reported in previous *in vivo* experiments. As such it

was chosen to potentially overcome any presumptive loss of efficacy associated with

oral administration due to enzymatic degradation within the GI tract. Data from this

initial experiment (Table 3), although indicative of an early increase at 5 dpi, showed no

differences (P>0.05) in levels among any of the measured isotypes in the serum. By 19

dpi, differences in levels of IgG, IgM, and IgA (P<0.001, P< 0.05 and P< 0.05,

respectively) as compared to the negative control and BSA only group were observed.

On 33 dpi, seven days following the booster immunization, while no differences

(P>0.05) in IgG and IgA levels between chickens within any groups receiving BSA were

observed, IgM levels were higher (P<0.05) in chickens within the BSA with CpG group

as compared to either the negative or BSA alone control groups. Administration of non-

CpG-ODNs resulted in overall lower antibody responses than CpG-ODNs in the serum

and most often similar to the BSA only group. They were never significantly greater

than the BSA alone group.

In Experiment 2, where anti-BSA antibodies were measured in individual

chickens through 33 dpi, similar data were collected with only a few notable exceptions.

Again, although there were indications of an early increase by 12 dpi (Figures 1-3), no

difference (P>0.05) in level among any measured isotypes were observed among the

experimental groups. By 19 dpi significantly higher (P<0.05) IgG and IgA levels were

observed for the 50 ug group as compared to BSA alone while IgM levels differed

(P<0.05) for both the 50 and 100 μg groups. At 26 dpi, the day of booster administration,

no differences (P>0.05) in IgG levels were observed. In contrast, significantly higher

IgA and IgM levels were observed for the 5 and 50 μg and 5, 50, and 100 μg groups,

respectively, as compared to the BSA control. After the booster administration on day 33

TABLE 3: Antibody levels in serum of chickens orally immunized with BSA with and without CpG-ODN or a non-CpG-ODN control

| Isotype	Experimental Group	Day 2	Day 5	Day 12	Day 19	Day 33
IgG	Control	0.01 ± 0.004	0.01 ± 0.001	0.00 ± 0.001^B	0.00 ± 0.002^C	0.01 ± 0.004^C
	BSA	0.02 ± 0.009	0.03 ± 0.015	0.69 ± 0.093^A	0.63 ± 0.061^B	0.94 ± 0.011^A
	Non-CpG	0.02 ± 0.008	0.03 ± 0.019	0.46 ± 0.054^A	0.73 ± 0.078^{AB}	0.87 ± 0.018^B
	CpG	0.05 ± 0.036	0.14 ± 0.085	0.27 ± 0.120^A	0.87 ± 0.062^A	0.90 ± 0.034^{AB}
IgM	Control	0.00 ± 0.001	0.01 ± 0.002	0.00 ± 0.001^b	0.03 ± 0.011^c	0.06 ± 0.005^c
	BSA	0.01 ± 0.012	0.01 ± 0.005	0.09 ± 0.023^{ab}	0.16 ± 0.037^b	0.12 ± 0.026^{bc}
	Non-CpG	0.00 ± 0.000	0.02 ± 0.012	0.14 ± 0.040^a	0.10 ± 0.008^{bc}	0.19 ± 0.033^{ab}
	CpG	0.00 ± 0.002	0.14 ± 0.082	0.16 ± 0.046^a	0.32 ± 0.080^a	0.25 ± 0.068^a
IgA	Control	-0.01 ± 0.001	0.03 ± 0.007	0.00 ± 0.001^b	-0.02 ± 0.006^c	-0.02 ± 0.005^b
	BSA	0.00 ± 0.004	0.03 ± 0.010	0.13 ± 0.034^a	0.12 ± 0.019^b	0.59 ± 0.181^a
	Non-CpG	-0.02 ± 0.002	0.04 ± 0.014	0.14 ± 0.019^a	0.16 ± 0.027^b	0.54 ± 0.101^a
	CpG	0.00 ± 0.005	0.13 ± 0.074	0.14 ± 0.042^a	0.35 ± 0.105^a	0.61 ± 0.134^a

Data represent mean +/- S.E. of peak absorbance values determined by indirect ELISA. Serum samples were diluted 1:640, and media from intestinal culture were diluted 1:10. Means ± S.E. with no common superscript differ significantly at the following levels of significance: [a]P<0.05 or [A]P<0.001.

there were no differences in IgG levels observed, but IgA and IgM levels were again significantly ($P<0.05$) greater for both the 5 and 50 μg CpG groups as compared to the BSA control.

Intestinal Antibody Response

Intestinal antibody levels from Experiment 1 showed a less pronounced effect due to CpG-ODNs administration than those observed in the serum. At 12 dpi, significantly higher ($P<0.05$) IgG levels were observed in the distal ileum (Table 4) for the CpG treated group as compared to the negative control group. By 19 dpi, IgG levels for all treatment groups differed ($P<0.05$) from the negative control and persisted through day 33 dpi. Differences in IgM levels were observed between the CpG and BSA groups ($P<0.05$), as well as BSA and negative control groups ($P<0.05$) by 19 dpi. At 33 dpi, differences in IgM levels were observed in only the CpG and non-CpG groups ($P<0.05$) when compared to the negative control group. Differences in IgA levels followed the same general pattern. At 19 dpi differences were observed ($P<0.05$) for the CpG treated group as compared to the BSA and negative control groups, but by 33 dpi all treated groups differed from the negative control ($P<0.05$), but not each other.

In the cecal tonsils (Table 5) all treated groups differed ($P<0.05$) from the negative control group by 19 dpi. These differences persisted through 33 dpi for all three isotypes. However, despite slight elevations in level for the CpG treated group, none differed significantly from the BSA alone group
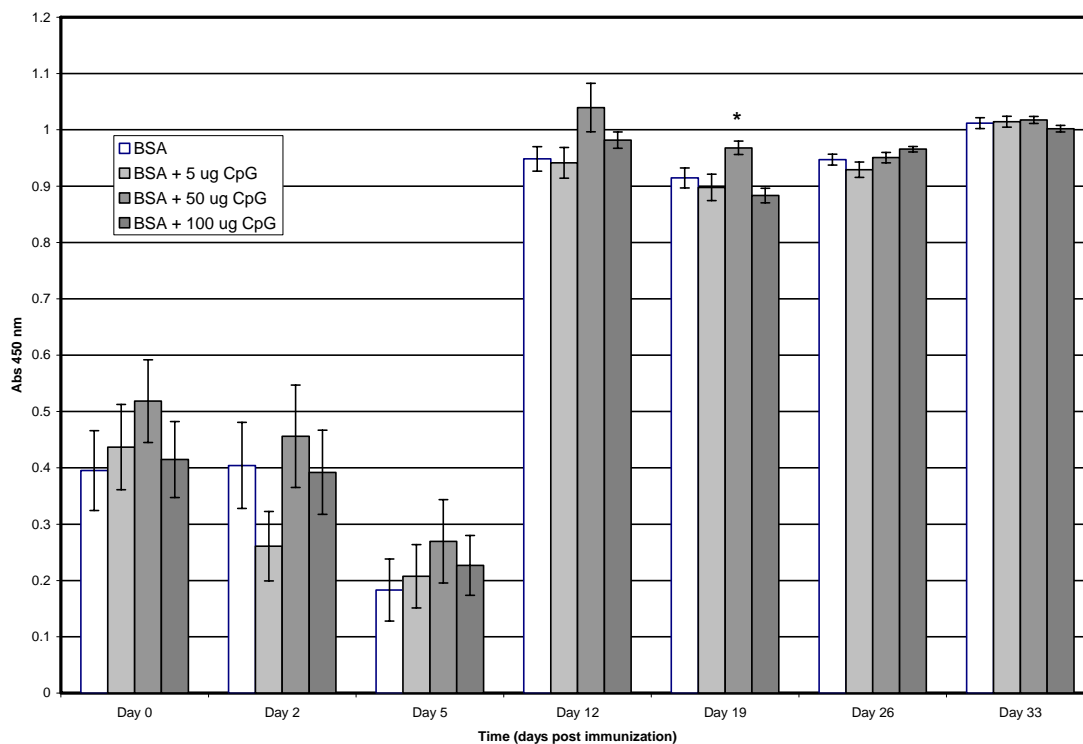
Figure 1: Specific serum IgG antibody responses after oral administration of BSA with increasing amounts of CpG-ODN. The groups were given 0, 5, 50, or 100 µg of CpG-ODN on day 0. All groups were also administered BSA in the drinking water at 1.4 mg/ml on days 0 through 6 as well as on day 26. Data represents mean ± S.E. of peak absorbance values determined by indirect ELISA performed on serum samples diluted 1:320. Each point is the mean of 20 birds. Standard errors were calculated per treatment at each time point. Means significantly different from the control group ($P<0.05$) were calculated by the Multivariate General Linear Model for Analysis of Covariance and are indicated by *.

Figure 2: Specific serum IgM antibody responses after oral administration of BSA with increasing amounts of CpG-ODN. The groups were given 0, 5, 50, or 100 μg of CpG-ODN on day 0. All groups were also administered BSA in the drinking water at 1.4 mg/ml on days 0 through 6 as well as on day 26. Data represents mean ± S.E. of peak absorbance values determined by indirect ELISA performed on serum samples diluted 1:320. Each point is the mean of 20 birds. Standard errors were calculated per treatment at each time point. Means significantly different from the control group (P<0.05) were calculated by the Multivariate General Linear Model for Analysis of Covariance and are indicated by *.
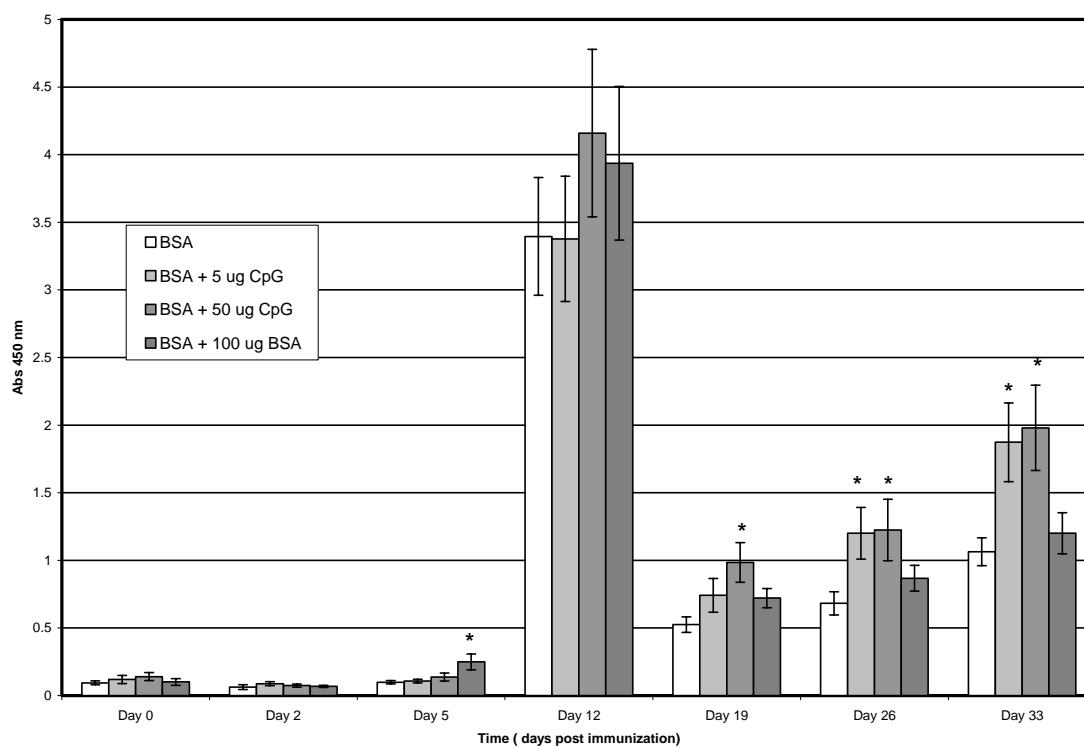
Figure 3: Specific serum IgA antibody responses after oral administration of BSA with increasing amounts of CpG-ODN. The groups were given 0, 5, 50, or 100 μg of CpG-ODN on day 0. All groups were also administered BSA in the drinking water at 1.4 mg/ml on days 0 through 6 as well as on day 26. Data represents mean ± S.E. of peak absorbance values determined by indirect ELISA performed on serum samples diluted 1:320. Each point is the mean of 20 birds. Standard errors were calculated per treatment at each time point. Means significantly different from the control group (P<0.05) were calculated by the Multivariate General Linear Model for Analysis of Covariance and are indicated by *.

In the distal ceca (Table 6) a significant difference (P<0.05) was observed in IgG levels between the negative control and the CpG treated group by 12 dpi. At 19 and 33 dpi IgG levels differed (P<0.05 and P<0.001 respectively) in all treated groups as compared to the negative control. Significantly higher (P<0.05) IgM levels were observed for the CpG treated group as compared to all others by 19 dpi. By 33 dpi the IgM levels in all treated groups differed (P<0.05) from the negative controls, but not each other. This trend was also repeated for IgA levels in the ceca, except that at 19 dpi, a significantly higher level (P>0.05) for the CpG group was observed as compared to both the negative control and non-CpG groups. Overall results for the non-CpG ODN in experiment one were lower than for the CpG treated group and most often similar to the BSA only group. They were never significantly different from the BSA only group.

DISCUSSION

The use of CpG-ODNs as adjuvants has been studied in a variety of animal models including mice, sheep, primates, and chickens (Hartmann et al., 2000; McCluskie et al., 2000; Rankin et al., 2001; Vleugels et al., 2002). Studies in chickens have, until the present investigation, been limited to intramuscular (IM) or subcutaneous (SQ) routes of administration. Using these routes of delivery, CpG-ODNs have been demonstrated to increase protection against *E. coli* infection and increase primary IgG and IgM responses to BSA (McCluskie et al., 2000; Vleugels et al., 2002). An optimal motif for chickens has not been proposed to date. The above experiments used motifs

TABLE 4: Antibody levels in the distal ileum of chickens orally immunized with BSA with and without CpG-ODN or a non-CpG-ODN control

Isotype	Experimental Group	Day 2	Day 5	Day 12	Day 19	Day 33
IgG	Control	0.00 ± 0.004	0.00 ± 0.001	0.00 ± 0.001^b	0.00 ± 0.001^b	0.00 ± 0.002^b
	BSA	0.03 ± 0.014	0.00 ± 0.001	0.04 ± 0.008^{ab}	0.16 ± 0.037^a	0.34 ± 0.055^a
	Non-CpG	0.01 ± 0.003	0.00 ± 0.001	0.03 ± 0.009^{ab}	0.14 ± 0.023^a	0.27 ± 0.057^a
	CpG	0.01 ± 0.004	0.04 ± 0.025	0.09 ± 0.046^a	0.26 ± 0.062^a	0.33 ± 0.103^a
IgM	Control	0.00 ± 0.000	0.00 ± 0.001	0.00 ± 0.002	0.00 ± 0.001^c	0.00 ± 0.001^b
	BSA	0.00 ± 0.000	0.00 ± 0.002	0.00 ± 0.001	0.01 ± 0.002^b	0.01 ± 0.003^{ab}
	Non-CpG	0.00 ± 0.000	0.00 ± 0.002	0.00 ± 0.001	0.01 ± 0.003^{bc}	0.02 ± 0.007^a
	CpG	0.00 ± 0.001	0.01 ± 0.004	0.00 ± 0.002	0.02 ± 0.007^a	0.02 ± 0.007^a
IgA	Control	0.00 ± 0.001	0.00 ± 0.008	0.00 ± 0.004	0.00 ± 0.003^C	0.00 ± 0.001^b
	BSA	0.00 ± 0.001	0.00 ± 0.015	0.01 ± 0.005	0.05 ± 0.014^B	0.05 ± 0.009^a
	Non-CpG	0.00 ± 0.001	0.00 ± 0.004	0.01 ± 0.003	0.07 ± 0.010^{AB}	0.05 ± 0.011^a
	CpG	0.00 ± 0.002	0.01 ± 0.008	0.00 ± 0.008	0.09 ± 0.013^A	0.08 ± 0.022^a

Data represent mean +/- S.E. of peak absorbance values determined by indirect ELISA. Serum samples were diluted 1:640, and media from intestinal culture were diluted 1:10. Means $\pm$ S.E. with no common superscript differ significantly at the following levels of significance: [a]P<0.05 or [A]P<0.001.

TABLE 5: Antibody levels in the cecal tonsils of chickens orally immunized with BSA with and without CpG-ODN or a non-CpG-ODN control

Isotype	Experimental Group	Day 2	Day 5	Day 12	Day 19	Day 33
IgG	Control	0.00 ± 0.001	-0.01 ± 0.012	0.01 ± 0.003	0.00 ± 0.000^b	0.00 ± 0.001^b
	BSA	0.00 ± 0.002	0.00 ± 0.003	0.05 ± 0.028	0.15 ± 0.036^a	0.17 ± 0.050^a
	Non-CpG	0.00 ± 0.003	0.02 ± 0.013	0.01 ± 0.015	0.21 ± 0.070^a	0.20 ± 0.047^a
	CpG	0.01 ± 0.013	0.01 ± 0.006	0.02 ± 0.013	0.23 ± 0.048^a	0.23 ± 0.035^a
IgM	Control	-0.01 ± 0.003	0.00 ± 0.001	0.00 ± 0.001	0.00 ± 0.000^b	0.00 ± 0.004^b
	BSA	0.00 ± 0.002	0.00 ± 0.000	0.00 ± 0.001	0.02 ± 0.001^a	0.01 ± 0.004^a
	Non-CpG	0.00 ± 0.009	0.00 ± 0.001	0.00 ± 0.003	0.01 ± 0.001^a	0.02 ± 0.006^a
	CpG	0.00 ± 0.004	0.00 ± 0.001	0.00 ± 0.002	0.02 ± 0.007^a	0.02 ± 0.004^a
IgA	Control	-0.01 ± 0.006	0.00 ± 0.001	0.00 ± 0.000	0.00 ± 0.004^b	0.00 ± 0.005^b
	BSA	0.00 ± 0.004	0.00 ± 0.000	0.00 ± 0.000	0.03 ± 0.003^a	0.05 ± 0.013^a
	Non-CpG	0.01 ± 0.009	0.00 ± 0.006	0.00 ± 0.000	0.02 ± 0.002^a	0.05 ± 0.015^a
	CpG	0.00 ± 0.002	0.00 ± 0.001	0.00 ± 0.001	0.04 ± 0.012^a	0.05 ± 0.028^a

Data represent mean +/- S.E. of peak absorbance values determined by indirect ELISA. Serum samples were diluted 1:640, and media from intestinal culture were diluted 1:10. Means ± S.E. with no common superscript differ significantly at the following levels of significance: [a]$P<0.05$ or [A]$P<0.001$.

TABLE 6: Antibody levels in the ceca of chickens orally immunized with BSA with and without CpG-ODN or a non-CpG-ODN control

Isotype	Experimental Group	Day 2	Day 5	Day 12	Day 19	Day 33
IgG	Control	0.00 ± 0.001	0.00 ± 0.001	0.00 ± 0.001^b	0.00 ± 0.001^b	0.00 ± 0.001^B
	BSA	0.01 ± 0.002	0.01 ± 0.010	0.13 ± 0.050^{ab}	0.20 ± 0.081^a	0.39 ± 0.082^A
	Non-CpG	0.02 ± 0.010	0.01 ± 0.002	0.04 ± 0.011^b	0.28 ± 0.101^a	0.35 ± 0.054^A
	CpG	0.01 ± 0.015	0.03 ± 0.021	0.31 ± 0.146^a	0.33 ± 0.074^a	0.49 ± 0.062^A
IgM	Control	0.00 ± 0.005	0.00 ± 0.003	0.00 ± 0.003	0.00 ± 0.001^b	0.00 ± 0.001^b
	BSA	0.00 ± 0.003	0.00 ± 0.001	0.01 ± 0.003	0.01 ± 0.002^b	0.02 ± 0.004^a
	Non-CpG	0.00 ± 0.001	0.00 ± 0.001	0.00 ± 0.001	0.01 ± 0.003^b	0.02 ± 0.007^a
	CpG	0.00 ± 0.003	0.01 ± 0.007	0.01 ± 0.007	0.03 ± 0.010^a	0.03 ± 0.006^a
IgA	Control	0.01 ± 0.003	0.00 ± 0.006	0.01 ± 0.002	0.00 ± 0.002^b	0.00 ± 0.004^B
	BSA	0.02 ± 0.005	0.00 ± 0.009	0.01 ± 0.006	0.04 ± 0.012^{ab}	0.13 ± 0.018^A
	Non-CpG	0.02 ± 0.005	0.00 ± 0.006	0.00 ± 0.004	0.02 ± 0.005^b	0.09 ± 0.007^A
	CpG	0.00 ± 0.006	0.00 ± 0.006	0.02 ± 0.008	0.07 ± 0.019^a	0.08 ± 0.024^A

Data represent mean +/- S.E. of peak absorbance values determined by indirect ELISA. Serum samples were diluted 1:640, and media from intestinal culture were diluted 1:10. Means $\pm$ S.E. with no common superscript differ significantly at the following levels of significance: [a]P<0.05 or [A]P<0.001.

optimal for humans and mice, respectively. The fact that both motifs demonstrated

efficacy, and that a wide range of motifs have been shown to induce cytokine and NO

production (He et al., 2003) as well as lymphocyte proliferation (Rankin et al., 2001) in

chickens, suggests that different motifs may have dissimilar properties.

In this study, we focused on the use of a CpG-ODN as a potential mucosal

adjuvant. For these experiments, we used a model of oral immunization previously

demonstrated to be effective by our laboratory and others (Klipper et al., 2000).

Specifically, we immunized chickens with BSA by adding it to the drinking water for six

consecutive days and then boosted with antigen in the same manner at day 26. When the

serum antibody responses of CpG treated birds were compared to those given BSA

alone, it was generally found that the addition of CpG-ODNs increased the production of

anti-BSA specific antibodies. IgG was significantly increased on day 19 only, using 50

and 75 µg of CpG-ODN, but not with 5 or 100 µg. IgM was increased on day 19 with

50, 75 or 100 µg , on day 26 with 5, 50, and 100 µg, and on day 33 with 50, 75, and 100

µg. Serum IgA levels were increased on day 19 for 50 and 75 µg and days 26 and 33 for

5 and 50 µg. These results, over two experiments, suggest that the oral administration of

CpG-ODNs increases antibody production in the serum for all isotypes at specific time

points. Further, it appears to prolong this increase for IgM and IgA dependent upon the

dose administered. This is in direct contrast to results obtained by injection of CpGs with

FIA and BSA. That study reported an increase in IgG both pre- and post-boost, an early

increase in IgM, and no difference in IgA production in the serum (Vleugels et al.,

2002). This distinction demonstrates that the route of the adjuvant administration

influences, to some extent, its effects on antibody levels. Since IgA is thought to help protect against pathogens that both colonize mucosal surfaces and also those that invade via these routes (Lamm, 1997), oral administration could be very important for protection against such pathogens.

Intestinal responses were also altered by dual administration of both CpG-ODNs and BSA, but not to the same extent as in the serum. In the distal ileum, significant increases were only observed for IgM and IgA on day 19 as compared to the BSA alone group. These increases did not persist beyond the booster immunization in either case. Cecal tonsil antibodies showed no difference between CpG treated birds and non-treated birds for any isotype. In the cecum the only increase in antibody level was observed for IgM on day 19.

In conclusion, these experiments suggest that CpG-ODNs can significantly enhance antigen-specific humoral responses in the chicken following a single oral dose administered early in neonatal life. This route of administration specifically enhances both the IgM and IgA responses in the serum, distal ileum and cecum, suggesting this route of CpG-ODN administration may be very attractive for generating protection against enteric pathogens in commercial poultry. As oral administration can be extended relatively easily to large scale operations, this may be an additional advantage to further development of this strategy for mass vaccination of commercial poultry flocks.

CHAPTER V

INVESTIGATION OF POTENTIAL ADJUVANT OR PROPHYLACTIC

EFFICACY OF CPG-ODNS DURING IMMUNIZATION OR CLINICAL

CHALLENGE WITH *EIMERIA*

INTRODUCTION

The etiological agents of chicken coccidiosis are a number of *Eimeria* species including, but not limited to, *E. acervulina*, *E. tenella* and *E. maxima*. Each species has characteristic prevalence, site of infection and pathogenicity. All species, however, parasitize the epithelial cells of the intestinal lining and cause pathological changes ranging from local destruction of the mucosa to systemic effects such as blood loss, shock, and death (Vermeulen et al., 2001). These changes lead to economic losses resulting primarily from decreased performance, such as feed conversion and weight gain. In a recent review (Allen and Fetterer, 2002), coccidial infections in poultry were estimated to cost the U.S. poultry producing industry in excess of $450 million annually. When worldwide production was taken into account, this increased to over $800 million per year. While these losses include the cost of prophylactic medication and losses due to morbidity, mortality and poor feed conversion, it was further estimated that less than 20% of these losses were due to prophylaxis, with the other 80% due to decreased weight gain and poor feed conversion (Vermeulen et al., 2001).

In addition to the current costs due to medications and performance losses, drug-resistant strains of coccidia are emerging worldwide. Such strains have forced producers

to implement elaborate schemes where various coccidiostats (medications) are alternated between flocks in an effort to minimize the emergence of drug resistant strains between flocks. Even with these precautions being taken, drug-resistant strains continue to emerge across the U.S. and the world forcing considerable interest in the development of alternative methods of control (Williams, 2002).

Live oocysts vaccines are currently the only viable alternative to the use of anti-coccidial drugs (Chapman et al., 2002). The basis for their use is that after an infection, the host is immune to subsequent infections by the same species of *Eimeria* (Yun et al., 2000b). This immunity is species specific, therefore vaccines are formulated with multiple species. To simulate the manner in which the bird might be exposed to shed oocysts following vaccination, researchers have shown that low numbers of parasite administered multiple times (trickle immunization) also results in species specific protective immunity (Vermeulen et al., 2001). Live vaccines have been used by the poultry industry for over 50 years, primarily for broiler breeders and replacement layer stock (Chapman et al., 2002). The reluctance to using live vaccines on a broader basis in broilers stems from decreased weight gain and feed conversion due to vaccination, when compared to performance in medicated birds. The potential for introducing new species into the rearing house has also been a detractor (Allen and Fetterer, 2002). While improved application methods and attenuated vaccines have led to an increased use in broilers (Chapman et al., 2002), an adjuvant capable of offsetting the deleterious effects would be advantageous to the poultry industry.

Immunostimulatory DNA sequences (ISS) represent a potential new class of mucosal adjuvants. ISS are DNA sequences containing unmethylated CpG (cytosine-phosphate-guanosine) dinucleotides in a specific base context, or motif. They are most commonly given in the form of synthetic oligodeoxynucleotides (ODN) made with a nuclease resistant phosphorothioate backbone (McCluskie et al., 2000). Several characteristics of CpG-ODNs highlight its potential usefulness as a mucosal adjuvant. CpG-ODNs have been reported to stimulate B cells to proliferate, secrete immunoglobulins, and secrete IL-6 and IL-12, as well as protect them from undergoing apoptosis (Krieg, 2001)**.** Additionally, CpG-ODNs have been shown to enhance expression of class II MHC and B7 costimulatory molecules on antigen presenting cells (APCs) leading to enhanced antigen presentation to lymphocytes. Activation of monocytes, macrophages, and dendritic cells to secrete select cytokines and chemokines that increase T-helper functions has also been reported (Krieg, 2002).

Research into the effectiveness of CpG-ODNs in chickens can be divided into two major types of investigations. The first type evaluated antibody response to a protein antigen. This includes administration of BSA, incomplete Freunds adjuvant, and CpG-ODNs subcutaneously (SQ) and subsequent measurement of the antibody response over thirty-five days (Vleugels et al., 2002). Work performed in our laboratory, using oral administration of BSA with and without CpG-ODNs, also falls into this category (please see Chapter IV of this manuscript). The second type of investigation has evaluated protection from a pathogen associated with CpG-ODN administration. Gomis (Gomis et al., 2003) used CpG-ODNs as an immunostimulant to protect from *Escherichia coli*

infections. Wang et. al. (Wang et al., 2003) evaluated the usefulness of CpG-ODNs as an adjuvant to IM DNA vaccination against infectious bursal disease virus (IBDV). Recently CpG-ODNs were also used to modulate infection with *Eimeria acervulina* (Dalloul et al., 2004). While all three studies observed a decrease in pathogenesis when CpG-ODNs were administered I.V., Dalloul et. al. (Dalloul et al., 2004) observed no protection related to body weight gain when CpG-ODNs were given orally prior to an infectious challenge. As the basis for all currently available vaccines is the repeated exposure of low levels of parasite over time (trickle immunization), we evaluated this immunostimulatory agent as a potential mucosal adjuvant in this context. Thus, the objectives of this study were to investigate the effects of three models of CpG-ODN administration on the response to challenge or immunization by two *Eimeria* species. The first objective was to determine whether a single dose of CpG-ODNs given with low doses of live parasite could influence body weight gain, lesion score, and feed conversion both before and after a clinical *Eimeria acervulina* challenge. The second objective was to determine whether a single dose of CpG-ODNs could influence body weight and lesion scores when given simultaneously with a clinical challenge dose of *E. acervulina* or *E. tenella.* The third objective was to determine whether a single dose of CpG-ODNs could influence body weight gain and lesion scores when given one day before administration of a clinical challenge dose of *E. acervulina* or *E. tenella*.

MATERIALS AND METHODS

Experimental Animals

In all experiments male single comb white Leghorn (SCWL) chicks were

obtained from a local commercial hatchery (Hyline International, Bryan, TX) on day of

hatch. All chicks were randomized and placed in Petersime brooder batteries. Chicks

were maintained at age appropriate temperatures and given *ad libitum* access to water

and a complete chick starter ration formulated to meet or exceed current NRC

recommendations for poultry (National Research Council, 1994).

Synthetic CpG-ODN

Both CpG-ODNs (Gomis et al., 2003) (5'-TCGT<u>CG</u>TTGT<u>CG</u>TTTTGT<u>CG</u>TT-

3') and non-CpG-ODNs (5'-CTGGTCTTTCTGGTTTTTTTCTGG-3') were obtained

from Integrated DNA Technologies, Inc (Coralville, IA). The ODNs were prepared with

a phosphorothioate backbone and reconstituted in pyrogen free PBS immediately before

administration.

Parasites

Strain #12 of *Eimeria acervulina* and Strain WLR-1 of *Eimeria tenella* were

obtained from Mr. Gary Wilkins and Dr. Mark Jenkins of the Animal Parasite Diseases

Laboratory (APDL), USDA-ARS, Beltsville MD.

Experimental Design

Trial 1: Trickle Immunization. In all replicates of both experiments, day of

hatch chicks were divided into six groups; non-immunized / non-challenged control,

non-immunized / challenged control, immunized / non-challenged, immunized /

challenged, CpG-ODN immunized / challenged, and non-CpG-ODN immunized / challenged. Beginning on day of hatch, for all replicate trials, all immunized birds were given either 500 sporulated oocysts (Experiment 1) or 50 sporulated oocysts (Experiment 2) for five consecutive days. CpG-ODN and non-CpG-ODN groups received 50 μg of the respective ODN on day of hatch only, with the first administration of oocysts. On day 19, all challenged birds were given a single clinical challenge dose of 1.25×10^5 sporulated oocysts of *Eimeria acervulina* #12.

Trial 2: Simultaneous Administration. In all replicates of all experiments, day of hatch chicks were divided into four groups; non-challenged control, challenged control, challenged with 50 μg CpG-ODNs or challenged with 50 μg non-CpG-ODNs administered simultaneous with challenge. Chickens in Experiment 1 were administered ODNs on day 15 along with 1.25×10^5 sporulated oocysts of *Eimeria acervulina*. Chickens in Experiment 2 were given ODNs on day 15 along with 1×10^5 sporulated oocysts of *E. tenella*.

Trial 3: Pre-administration. In all replicates of all experiments, day of hatch chicks were divided into four groups; non-challenged control, challenged control, challenged with 50 μg CpG-ODNs or challenged with 50 μg non-CpG-ODNs administered one day prior to challenge. Chickens in Experiment 1 were given ODNs on day 14 of life and administered 2×10^5 sporulated oocysts of *Eimeria acervulina* on day 15. Chickens in Experiment 2 were given ODNs on day 14 of life and administered 1×10^5 sporulated oocysts of *E. tenella* on day 15.

Sampling

Trial 1: Trickle Immunization. In order to calculate body weight gain and feed conversion, all birds were weighed individually on 0, 19, and 25 days post hatch. Body weight gain was calculated for each bird on days 19 and 25 post hatch. Feed conversion was calculated for each group on days 19 and 25 post hatch, and individual birds were lesion scored on day 25 post hatch.

Trials 2 and 3: Simultaneous and Pre-administration. In order to calculate body weight gain all birds were weighed individually on 0, 15, and 21 days post hatch. Body weight gain was calculated and lesions were scored for each bird on day 21 post hatch.

Statistical Analysis

Individual body weight gains and lesion scores were analyzed using the Univariate General Linear Model procedure for analysis of variance (SPSS, v. 11.0). Statistically different means ($P < 0.05$) were further separated using Duncan's Multiple Range Test (SPSS, v. 11.0). Due to the experimental design feed conversion was not statistically analyzed for trickle immunization.

RESULTS

To determine whether CpG-ODNs could influence body weight gain and feed conversion during trickle immunization, or body weight gain and lesion scores after a clinical challenge, a dose of 50 μg was chosen. This dose was selected based upon antibody responses as determined in previous experiments (please see Chapter IV of this manuscript). This dose is also towards the high end of the stimulatory range reported in previous *in vivo* experiments (McCluskie and Davis, 2000) which might alleviate any

presumptive loss of efficacy associated with oral administration due to proteolytic degradation within the GI tract. During the first set of experiments two *Eimeria* immunization doses were chosen with Experiment 1 evaluating 500 oocysts for five days and Experiment 2 evaluating 50 oocysts for five days. These were chosen in order to determine whether the immunizing dose would affect the efficacy of CpG-ODNs. In all three sets of experiments the clinical challenge dose was determined by performing a dose titration and selecting a dose which resulted in an average lesion score of 2 in challenged chickens.

Trial 1: Trickle Immunization

Pre-challenge body weight gain. Body weight gain during and subsequent to the immunization period for those groups given either CpG-ODNs or non-CpG-ODNs was significantly ($P<0.001$) greater as compared to chickens given the immunizing dose of parasite only or those in the non-immunized control group in both replicates of Experiment 1 and in one replicate of Experiment 2 (Table 7). In one of two replicates in each experiment, the immunized group had significantly ($P>0.001$) greater weight gain than the non-immunized group (Table 7).

Pre-challenge feed conversion. Feed conversion ratios followed a contrasting pattern in both replicates of both experiments. While we could not test for significance in calculated feed conversion ratios, we observed that feed conversion values in ODN administered groups were consistently in between the calculated values of non-immunized controls and those immunized with parasite alone (Table 7).

Post-challenge Body Weight Gain. In Experiment 1 post-challenge body weight gain in those birds given CpG-ODNs with the first immunizing dose of parasite was significantly greater (P<0.001) than the immunized / non-challenged, non-immunized / challenged, and immunized / challenged groups in both replicates (Table 8). However, the CpG-ODN administered groups did not differ from the non-CpG-ODN groups in either replicate and only differed from the non-immunized / non-challenged group in one of two replicates. In Experiment 2 post-challenge body weight gain in those birds given CpG-ODNs was significantly greater (P<0.05) than the non-immunized / challenged and immunized / challenged groups in both replicates (Table 9). However the CpG-ODN administered groups did not differ from the immunized / non-challenged or non-CpG-ODN groups in either replicate and was greater than (P<0.05) the non-immunized / non-challenged group in only one of the two replicates (Table 8).

Lesion Scores. Lesion scores in Experiment 1 for those birds immunized with parasite and CpG-ODNs or non-CpG-ODNs were significantly lower (P<0.001) than either the parasite only immunized / challenged and non-immunized / challenged groups in both replicates (Table 8). In addition, lesion scores for the ODN immunized birds were significantly greater (P<0.001) than both non-challenged groups in only one of two replicates. In contrast, no lesions were observed in Experiment 2 for any of the groups except the non-immunized / challenged group. While this group did differ significantly in replicates 1 and 2 (P<0.001 and P<0.05 respectively), there were no other differences among the remaining experimental groups (Table 9).

Post-challenge Feed Conversion. In direct contrast to pre-challenge feed conversion, there were no consistent trends observed for post-challenge feed conversion (Table9).

Trial 2: Simultaneous Administration

In Experiment 1, CpG-ODNs were given simultaneously with 1.25 X 10^5 oocysts of *E. acervulina*. No differences were seen between any of the groups with respect to weight gain in any of the three replicates (Table 10). There was no decrease in body weight gain due to challenge but lesions were observed only in the challenged groups, although there were no differences in their severity (Table 10). In Experiment 2 CpG-ODNs were given with 1.0 X 10^5 *E. tenella* oocysts. In replicates one and two, weight gain for the non-challenged and challenged only groups differed significantly ($P<0.05$ and $P<0.001$ respectively) from each other, but both the CpG-ODN and non-CpG-ODN groups did not differ from the challenge only in either replicate (Table 11). Lesion scores differed between the non-challenged and challenged groups, but there were no differences related to CpG or non-CpG-ODN administration (Table 11).

Trial 3: Pre-administration

In Experiment 1, although clinical challenge with 2.0 X 10^5 *E. acervulina* oocysts did result in decreased body weight gain and increased lesion score as compared to the non-challenged control, no differences were observed in a single experiment due to the administration of CpG-ODNs or non-CpG-ODNs one day prior to challenge (Table 12). In Experiment 2, when CpG-ODNs were given twenty-four hours prior to challenge with 1.0 X 10^5 *E. tenella* oocysts weight gain also did not differ between any

TABLE 7: Pre-challenge body weight gain in Experiments 1 and 2 of chickens immunized with 500 or 50 *Eimeria acervulina* oocysts from days 1-5 of life with and without ODNs on day 1

| | Experiment 1 (500 oocysts) | | | | | | Experiment 2 (50 oocysts) | | | | | |
| | Replicate 1 | | | Replicate 2 | | | Replicate 1 | | | Replicate 2 | | |
	N=	BWG	Feed Conversion	N=	BWG	Feed Conversion	N=	BWG	Feed Conversion	N=	BWG	Feed Conversion
Non-Immunized	117	86.6 ± 1.62^C	2.62	50	95.0 ± 2.29^B	2.42	30	101.2 ± 2.70^C	3.22	30	91.1 ± 1.70^A	3.06
Immunized	114	98.3 ± 1.78^B	2.97	49	99.4 ± 2.28^B	2.64	29	112.8 ± 2.42^B	3.83	29	92.8 ± 1.16^A	3.83
ODN Immunized	116	113.8 ± 2.02^A	2.93	50	116.5 ± 2.62^A	2.55	28	127.2 ± 2.9^A	3.52	30	95.7 ± 2.03^A	3.23

Data represent mean +/- S.E. of body weight gain for days 1-19 of life. Means ± S.E. within columns with no common superscript differ significantly at the following levels of significance: [A]P<0.001.

TABLE 8: Post-challenge body weight gain, lesion score and feed conversion in Experiment 1 in chickens immunized with 500 oocysts on days 1-5 of life with and without CpG-ODN or a non-CpG-ODN control on the first day

Group	N=	BWG	LS	Feed Conversion	N=	BWG	LS	Feed Conversion
		Replicate 1				Replicate 2		
Non-Immunized / Non-Challenged	60	73.4 ± 2.5^{BC}	0 ± 0^{D}	1.59	25	64.7 ± 1.5^{ABC}	0 ± 0^{C}	3.1
Immunized / Non-Challenged	57	71.5 ± 1.85^{C}	0 ± 0^{D}	1.65	24	61.1 ± 2.28^{BC}	0 ± 0^{C}	3.08
Non-Immunized / Challenged	57	69.3 ± 1.9^{C}	1.88 ± 0.11^{A}	2.32	25	59.7 ± 3.42^{C}	1.44 ± 0.014^{A}	2.56
Immunized / Challenged	57	70.1 ± 1.62^{C}	0.56 ± 0.07^{B}	2.16	25	58.3 ± 2.35^{C}	0.24 ± 0.09^{B}	2.56
CpG Immunized / Challenged	58	81.7 ± 2.68^{A}	0.22 ± 0.06^{C}	2.00	25	69.9 ± 4.01^{A}	0 ± 0^{C}	3.00
Non-CpG Immunized / Challenged	58	77.9 ± 2.05^{AB}	0.29 ± 0.06^{C}	1.99	25	69.4 ± 2.62^{AB}	0 ± 0^{C}	3.61

Data represent mean +/- S.E. of body weight gain for days 19 -25 of life or lesion scores on day 25. Means ± S.E. within columns with no common superscript differ significantly at the following levels of significance: [A]P<0.001.

TABLE 9: Post-challenge body weight gain, lesion score and feed conversion in Experiment 2 in chickens immunized with 50 oocysts of *Eimeria acervulina* on days 1-5 of life with and without CpG-ODN or a non-CpG-ODN control on the first day

Group	N=	BWG (Rep 1)	LS (Rep 1)	Feed Conversion (Rep 1)	N=	BWG (Rep 2)	LS (Rep 2)	Feed Conversion (Rep 2)
Non-Immunized / Non-Challenged	15	67.9 ± 1.9^b	0 ± 0^B	2.60	15	83.1 ± 2.48^A	0 ± 0^B	3.51
Immunized / Non-Challenged	15	70.9 ± 1.02^{ab}	0 ± 0^B	3.13	15	73.7 ± 1.90^{BC}	0 ± 0^B	3.24
Non-Immunized / Challenged	15	64.6 ± 2.53^b	2.07 ± 0.18^A	4.23	15	66.2 ± 4.30^C	1.93 ± 0.21^A	3.81
Immunized / Challenged	14	67.3 ± 2.35^b	0 ± 0^B	3.85	14	68.0 ± 2.80^C	0 ± 0^B	3.10
CpG Immunized / Challenged	14	75.8 ± 2.24^a	0 ± 0^B	3.44	15	80.9 ± 2.06^{AB}	0 ± 0^B	2.98
Non-CpG Immunized / Challenged	14	70.2 ± 2.98^{ab}	0 ± 0^B	3.10	15	74.3 ± 3.75^{ABC}	0 ± 0^B	2.45

Data represent mean +/- S.E. of body weight gain for days 19 -25 of life or lesion scores on day 25. Means ± S.E. within columns with no common superscript differ significantly at the following levels of significance: [a]$P<0.05$, [A]$P<0.001$

of the challenged groups in either replicate (Table 12). However, a significant (P<0.001) improvement of lesion scores was observed in the CpG-ODN administered group as compared to the non-CpG-ODN and challenged only groups in both replicates (Table 12).

DISCUSSION

The use of CpG-ODNs as adjuvants has been studied in a variety of vertebrate models including mice, sheep, primates, salmon, and chickens (Bridle et al., 2003; Hartmann et al., 2000; McCluskie et al., 2000; Rankin et al., 2001; Vleugels et al., 2002). Studies in chickens include both *in vitro* stimulation assays and *in vivo* protection assays. *In vitro* application of CpG-ODNs has been shown to stimulate lymphocyte proliferation, induce nitric oxide (NO) production, and IL-1β mRNA synthesis in the HD11 macrophage cell line, induce IFN-γ mRNA expression and nitric oxide production in peripheral blood mononuclear cells (PBMCs), and induce degranulation and generation of reactive oxygen species (ROS) in heterophils (He et al., 2003; He et al., 2005; Rankin et al., 2001; Xie et al., 2003). *In vivo* studies have investigated CpG-ODNs adjuvant effects through intramuscular (i.m.), subcutaneous (s.c.), and oral administration, and its protection in infectious challenge studies by subcutaneous (s.c.), i.m., i.p., intravenous (i.v.), *in ovo*, and oral administration. Increases in antigen specific antibodies were observed when CpG-ODN and BSA were injected subcutaneously both with and without incomplete Freund's adjuvant (IFA) (Vleugels et al., 2002). A modest increase was also observed in antigen specific antibodies when both BSA and CpG-ODNs were given orally (please see Chapter IV of this manuscript). When CpG-ODNs

were injected i.m. with a DNA vaccine against infectious bursal disease (IBD), an increase in antibody titer, and a decrease in mortalility, morbitity, and lesions were observed as compared to vaccine alone animals (Wang et al., 2003). Protective effects of CpG-ODNs against bacterial challenges were investigated with *Salmonella enteritidis* when given i.p. and *Escherichia coli* when given s.q., i.m., and *in ovo*. In both cases all routes provided protection from invasion and mortality (Gomis et al., 2004; Gomis et al., 2003; He et al., 2005). Protection against protozoan infection has also been investigated. Dalloul et. al. (2004) observed that i.v. and s.c., but not oral, administration of CpG-ODNs could increase body weight gain and decrease oocyst shedding due to subsequent infection with *Eimeria acervulina*.

The above experiments used motifs optimal for either humans or mice since an optimal motif for chickens has yet to be determined. The fact that a wide range of motifs have demonstrated efficacy as measured by the ability to induce cytokine and NO production, as well as lymphocyte proliferation in chickens, suggests that different motifs may have dissimilar properties (He et al., 2003; Rankin et al., 2001). Therefore, a study examining the efficacy of different motifs and their influence on immunity to *Eimeria* may be advantageous.

In the first study, we focused on the use of CpG-ODNs as mucosal adjuvants delivered orally with low doses of the protozoan parasite *Eimeria acervulina*. Specifically, we immunized chicks with a subclinical dose (50 or 500 oocysts) for the first five days of life both with and without CpG-ODNs or non-CpG-ODNs which was

administered with oocysts on the first day of immunization. This was followed with a

clinical challenge at 19 days of age. Protection was determined by body weight

gain and feed conversion on days 19 and 25, and lesion development in the intestines on

day 25. CpG-ODN and non-CpG-ODN immunized chickens had higher body weight

gains as compared to the immunized alone group in three of four replicate experiments.

Additionally, feed conversion was consistently in between that of immunized and non-

immunized groups. After clinical challenge, body weight gain was higher in the CpG-

ODN administered groups than the immunized alone groups in both replicates of both

experiments. However, the CpG-ODN group did not differ from the non-CpG-ODN

groups in any of the experiments and there were no consistent trends for post-challenge

feed conversion.

In the second and third trials in this investigation, we focused on the potential

protective effects of CpG-ODNs when given either simultaneously with or one day prior

to a clinically infectious dose of *E. acervulina* or *E. tenella*. When CpG-ODNs were

given simultaneously with an infectious dose of *E. acervulina* no decrease in body

weight gain was observed due to challenge. Lesions were observed only in the

challenged groups although there were no differences in their severity. When CpG-

ODNs were given simultaneously with *E. tenella* we did observe a decrease in weight

gain due to challenge as well as lesions in the challenged groups. However, there were

no differences due to either CpG-ODN or non-CpG-ODN administration.

TABLE 10: Post challenge weight gain and lesion scores in Experiment 1 after co-administration of CpG-ODNs with 125,000 *Eimeria acervulina* oocysts

Group	N=	Replicate 1 Weight Gain	Replicate 1 Lesion Score	N=	Replicate 2 Weight Gain	Replicate 2 Lesion Score	N=	Replicate 3 Weight Gain	Replicate 3 Lesion Score
Non-Challenged	15	57.2 ± 2.16	0 ± 0^B	15	57.1 ± 1.49	0 ± 0^B	15	59.9 ± 1.69	0 ± 0^B
Challenged	15	63.0 ± 2.62	1.9 ± 0.17^A	15	58.3 ± 2.30	1.8 ± 0.17^A	15	60.2 ± 2.14	1.8 ± 0.17^A
Challenged & CpG-ODN	15	61.2 ± 1.96	1.9 ± 0.17^A	15	60.6 ± 2.56	1.8 ± 0.17^A	15	61.3 ± 1.79	1.6 ± 0.16^A
Challenged & non-CpG-ODN	15	57.8 ± 2.27	1.6 ± 0.16^A	15	57.4 ± 2.67	1.4 ± 0.16^A	15	61.8 ± 2.43	1.6 ± 0.16^A

Data represent mean +/- S.E. of body weight gain for days 15 - 21 of life or lesion scores on day 21. Means ± S.E. within columns with no common superscript differ significantly at the following levels of significance: [a]$P<0.05$, [A]$P<0.001$

TABLE 11: Post-challenge weight gain and lesions scores in Experiment 2 after co-administration of CpG or non-CpG-ODNs with 100,000 *Eimeria tenella* oocysts

Group	N=	Replicate 1 Weight Gain	Replicate 1 Lesion Score	N=	Replicate 2 Weight Gain	Replicate 2 Lesion Score
Non-Challenged	12	72.5 ± 2.84^a	0 ± 0^B	12	66.7 ± 2.11^a	0 ± 0^B
Challenged	12	59.3 ± 3.89^b	2.00 ± 0.21^A	11	55.7 ± 3.67^b	2.00 ± 0.30^A
Challenged & CpG-ODN	12	64.1 ± 3.14^{ab}	2.17 ± 0.24^A	13	63.1 ± 1.97^{ab}	1.77 ± 0.23^A
Challenged & non-CpG-ODN	11	63.0 ± 3.42^{ab}	1.91 ± 0.21^A	13	57.6 ± 3.53^b	2.31 ± 0.21^A

Data represent mean +/- S.E. of body weight gain for days 15 - 21 of life or lesion scores on day 21. Means ± S.E. within columns with no common superscript differ significantly at the following levels of significance: [a]$P<0.05$, [A]$P<0.001$

TABLE 12: Post challenge weight gain and lesion scores in Experiment 3 after pre-administration of CpG-ODNs one day before clinical challenge with 200,000 oocysts of *Eimeria acervulina* or 100,000 *Eimeria tenella* oocysts

		Eimeria acervulina			*Eimeria tenella* Replicate 1			*Eimeria tenella* Replicate 2	
Group	N=	Weight Gain	Lesion Score	N=	Weight Gain	Lesion Score	N=	Weight Gain	Lesion Score
Non-Challenged	12	91.08 ± 3.45^A	0 ± 0^B	12	86.43 ± 4.00^a	0 ± 0^C	15	70.6 ± 2.14^a	0 ± 0^C
Challenged	12	74.26 ± 1.71^B	2.17 ± 0.21^A	12	63.78 ± 4.93^b	2.08 ± 0.23^A	15	57.6 ± 1.94^b	2.27 ± 0.21^A
Challenged & CpG-ODN	12	71.01 ± 2.53^B	2.25 ± 0.18^A	12	64.18 ± 5.19^b	1.50 ± 0.15^B	15	64.4 ± 3.18^{ab}	$1.13 \pm .19^B$
Challenged & non-CpG-ODN	12	66.61 ± 2.75^B	2.42 ± 0.19^A	12	73.01 ± 4.52^b	2.33 ± 0.19^A	13	63.4 ± 3.37^{ab}	$1.85 \pm .22^A$

Data represent mean +/- S.E. of body weight gain for days 15 - 21 of life or lesion scores on day 21. Means ± S.E. within columns with no common superscript differ significantly at the following levels of significance: $^aP<0.05$, $^AP<0.001$

When CpG-ODNs were given to chickens one day prior to infectious challenge with *E. acervulina* we did observe a decrease in body weight gain and an increase in lesions compared to the non-challenged control group. However, there were no differences respective to CpG-ODN or non-CpG-ODN administration. When ODNs were given twenty-four hours prior to challenge with *E. tenella,* we also observed a decrease in body weight gain and an increase in lesions due to challenge. While there were no differences in weight gain due to CpG-ODN or non-CpG-ODN administration, we did observe a reduction in lesions scores in the CpG-ODN administered group as compared to the non-CpG-ODN and challenged only groups.

These results of the first trial in this study suggest that administration of CpG-ODNs with an immunizing dose of *Eimeria acervulina* may improve weight gain and feed conversion both before and after subsequent clinical *Eimeria* challenge. While differences between the two immunizing doses were not observed, it is worth noting that both doses were well below a clinical challenge dose and also differed from immunizing doses used in other experiments (Galmes et al., 1991). Although a previous investigation did not report protection from *Eimeria acervulina* with oral administration of CpG-ODNs (Dalloul et al., 2004), the first trial used a lower immunizing dose followed by a clinical *E. acervulina* challenge. It is possible that giving a large dose of parasite through the same route could abrogate the immunostimulatory effects of the CpG-ODNs, while low consistent exposure to the parasite may not. The rational behind this speculation is that a low dose of parasite would be less likely to overwhelm the gut mucosa and give the CpG-ODNs time to stimulate mucosal immunity.

The results of the second and third trials are in agreement the findings of Dalloul et. al. (2004) in that the oral pre-administration of CpG-ODNs does not protect from an infectious dose of *E. acervulina* given twenty-four hours later. However, it was also observed that pre-administration of CpG-ODNs can reduce lesion scores, but not improve body weight gain, in chickens given a clinical dose of *E. tenella* twenty-four hours later. As a decrease in body weight gain between the challenged only and non-challenged groups was observed in both experiments the dissimilarity in the results is likely due to the species of *Eimeria* and not the dose given.

Although the precise immunostimulatory affects of CpG-ODNs in chickens have not been fully defined, i*n vitro* studies have demonstrated that CpG-ODNs can stimulate lymphocyte proliferation, induce nitric oxide (NO) production and IL-1β mRNA synthesis in the HD11 macrophage cell line, induce IFN-γ mRNA expression and nitric oxide production in peripheral blood mononuclear cells (PBMCs), and induce degranulation and generation of reactive oxygen species (ROS) in heterophils (He et al., 2003; He et al., 2005; Rankin et al., 2001; Xie et al., 2003). In addition, differential cytokine responses in the duodenum and ceca of chickens infected with *E. acervulina* or *E. tenella,* suggest that responses to and protection from infection differ between the two species (Choi et al., 1999; Laurent et al., 2001).

In conclusion, while administration of CpG-ODNs with an immunizing dose of *Eimeria acervulina* may be able to improve weight gain and feed conversion both before and after subsequent clinical challenge, neither co-administration nor pre-administration of CpG-ODNs with a clinical challenge dose of *E. acervulina* or *E. tenella* seems to

influence body weight gain. However, while no differences were observed for lesion development related to *E. acervulina* challenge, a significant decrease in lesion score due to pre-administration of CpG-ODNs was observed with *E. tenella* challenge. Taken together the above data suggests that orally administered CpG-ODNs may modulate the chickens immune response thereby improving performance during vaccination and providing either protection from or reduction of immunopathology resulting from *E. tenella* infection. Whether the differences discussed above would be seen in chickens capable of more rapid growth is unknown at this time, however given the oral route of administration, the use of CpG-ODNS in mass vaccination may be feasible.

CHAPTER VI

CONCLUSIONS

In Chapter III of this manuscript, antigen specific antibody production to the protein antigen BSA was evaluated seven days after administration by one of three routes. These were i.p. injection with an alum adjuvant at 12 and 17 days of age, oral gavage on 12 through 18 days of age, and drinking water administration on 12 through 18 days of age. In Experiment 1, serum and bile were assayed for IgG and IgA respectively. In Experiment 2, using an *ex vivo* culture method, the ileum, cecal tonsils, and ceca were assayed for both IgG and IgA secretion in addition to measuring serum IgG and biliary IgA. In both experiments, drinking water administration and i.p. injection resulted in higher serum IgG than oral gavage or the control groups (Tables 1 and 2). Biliary IgA, however, was only significantly increased in the group administered BSA in the drinking water, as compared to gavage, i.p. injection, or the controls (Tables 1 and 2). When intestinal IgG was measured, both the i.p. injected group and the drinking water group had higher levels than oral gavage or the controls (Table 2), however there were no differences between the groups with regards to intestinal IgA. These observations were interpreted to indicate that purified protein antigen given in the drinking water can elicit a primary antigen specific antibody response in the serum, bile, and intestine.

Previous research has established that oral administration of protein in solution is immunogenic and not tolerogenic in domestic chickens. (Klipper et al., 2000; Miller and Cook, 1994). This has been demonstrated by feeding discrete amounts of solubilized

antigen to chickens of an immunocompetent age for six to 14 consecutive days (Klipper et al., 2000; Miller and Cook, 1994). Following these regimes, specific antibody production has been reported in both the serum and the bile. Alternative approaches of antigen administration, such as once a week feedings, do not induce an humoral response in the absence of adjuvant (Hoshi et al., 1998; Hoshi et al., 1999).

The present experiments confirm that oral administration of solubilized BSA induces both serum and bile antibody responses and also establishes that a local intestinal antibody response is measurable seven days following the last time of immunization, indicating specific antibodies were produced by lymphocytes in the distal ileum, cecal tonsils, and distal cecum in response to oral BSA immunization. Although measured responses consisted primarily of IgG secretion, it bears mentioning that only one time point during this evaluation of intestinal antibody secretion was evaluated. The observation that sIgA levels did not differ at seven days after the last immunization, where IgG secretions did in fact differ, is not unique. Other studies have reported similar findings in response to challenge with *Eimeria* species or a purified recombinant antigen from *E. tenella* (Girard et al., 1997; Girard et al., 1999). It is indeed likely that if measured at a later day, IgA production may have been elevated in the intestinal sections tested, as seen in the studies previously mentioned.

As stated above, *ad libitum*, oral administration of solubilized protein antigen elicits an immune response in commercial lines of SCWL chickens. While this study was limited to SCWL chickens, other studies have reported a similar responsiveness to oral BSA administration in broiler chickens (Klipper et al., 2000). When compared to

negative controls and crop-gavaged chickens, animals receiving BSA by drinking water administration produced higher levels of antigen specific antibodies. It is worthwhile to note that IgG levels in those chickens were not significantly different than levels in chickens given two i.p. injections of BSA with alum as an adjuvant.

Observations of drinking water administration being more efficacious in eliciting humoral immune responsiveness were also seen by Klipper and co-workers (Klipper et al., 2001). One possible explanation may involve crop distention associated with the single bolus *per os* administration. Crop distention, in this scenario, may likely result in the release of proteolytic pepsinogen and HCl by the proventriculus, which might result in the degradation of the protein before adequate amounts could reach lymphoid tissue at distal sites in the intestine. The findings of Klipper *et al*. (2000), indicating that feeding powdered forms of BSA to mature chicks induced neither an immune response or tolerance, and Hoshi *et al*. (1998) by demonstrating that gavage administration did not induce immunity and may induce tolerance, adds support to this speculation.

Regardless of the mechanisms involved in the observed responsiveness to oral antigen in the chicken, the present investigation clearly confirms that solubilized oral protein administration induces both systemic and local humoral immune responses. These findings suggest that subunit protein vaccines, administered by a drinking water route of delivery, may be a feasible option for commercial productions or smaller breeder flocks. While additional investigation into such a route of mass vaccination is clearly needed to support such a claim, the ease of administration to large numbers of animals by this approach represents a distinct advantage, even more so than what exists

for many live and attenuated vaccines for enteric pathogens, which are presently given orally. Additional research should also include investigation into variability in immunogenicity of antigens isolated from specific pathogens, as well as the level of protection induced by these antigens. The duration of immunity elicited by oral protein antigen administration with and without adjuvant also needs to be studied.

In Chapter IV of this manuscript the model developed in Chapter III, oral administration of BSA, was used to investigate whether the recently described mucosal adjuvant CpG-ODN could influence antibody production to an orally administered protein antigen in chickens. In Experiment 1, chickens were divided into four groups. These were a negative control, a BSA only group, and groups administered 75 μg of either CpG-ODNs or non-CpG-ODNs on the first day of BSA administration only. IgG, IgM, and IgA were assayed in the serum and, using the *ex vivo* culture method mentioned above, antibody secretion was measured from the distal ileum, cecal tonsils, and cecum. Samples were taken on days 0, 2, 5, 12, 19, and 33 dpi. In Experiment 2 a dose effect was investigated and chickens received 0, 5, 50, or 100 μg of CpG-ODN administered identically to Experiment 1. Serum was assayed for IgG, IgA and IgM. Samples were taken on 0, 2, 5, 12, 19, 26, and 33 dpi. In both experiments a booster administration of BSA was given on day 26 dpi.

Serum levels of BSA specific IgG were greater than the BSA only controls in the groups given 50 and 75 μg CpG-ODNs before the boost, but there were no post-boost differences (Table 3 and Figure 1). Serum IgM, however, was increased over the BSA only control at all levels of CpG-ODNs before the booster administration, and at all

levels but 100 μg after the booster (Table3 and Figure 2). Serum IgA was increased over the BSA only control at 5 and 50 μg both before and after the boost, but only before the boost at 75 μg. In intestinal sections no increase in IgG was observed in any of the sections tested, an increase was seen for IgM in both the ileum and ceca before the booster, and for IgA in the ileum only, also before the booster. These results suggest that the oral administration of CpG ODNs both increases antibody production in the serum for all isotypes at specific time points and prolongs this increase for IgM and IgA dependent upon the dose.

Given the present experiments, CpG-ODNs can significantly enhance antigen-specific humoral responses in the chicken after a single oral dose administered early in neonatal life. In addition, this route of administration specifically enhances both the IgM and IgA responses in the serum, distal ileum and cecum, suggesting this route of CpG-ODN administration may be very attractive for generating protection against enteric pathogens in commercial poultry. As oral administration can be extended relatively easily to large scale operations, this may be an additional advantage to further development of this strategy for mass vaccination of commercial poultry flocks.

The use of CpG-ODNs as adjuvants have been studied in a variety of vertebrate models including mice, sheep, primates, salmon, and chickens (Bridle et al., 2003; Hartmann et al., 2000; McCluskie et al., 2000; Rankin et al., 2001; Vleugels et al., 2002). Studies in chickens include both *in vitro* stimulation assays and *in vivo* protection assays. *In vitro* application of CpG-ODNs has been observed to stimulate lymphocyte proliferation, induce nitric oxide (NO) production and IL-1β mRNA synthesis in the

HD11 macrophage cell line, induce IFN-γ mRNA expression and nitric oxide production in peripheral blood monocytes (PBMCs), and induce degranulation and generation of reactive oxygen species (ROS) in heterophils (He et al., 2003; He et al., 2005; Rankin et al., 2001; Xie et al., 2003). *In vivo* studies have investigated CpG-ODNs adjuvant effects through both intramuscular (i.m.) and subcutaneous (s.c.) injections and its protective effects by s.c., i.m., i.p., intravenous (i.v.), *in ovo*, and oral administration. Increases in antigen specific antibodies were observed when CpG-ODN and BSA were injected subcutaneously both with and without incomplete Freund's adjuvant (IFA) (Vleugels et al., 2002). When CpG-ODNs were injected IM with a DNA vaccines against infectious bursal disease (IBD) an increase in antibody titer, and a decrease in mortalility, morbitity, and lesions were observed as compared to the vaccine only (Wang et al., 2003). The protective effects of CpG-ODNs against bacteria were investigated with *Salmonella enteritidis* when given i.p. and *Escherichia coli* when given s.q., i.m., and in ovo. In both cases all routes provided protection from invasion and mortality (Gomis et al., 2004; Gomis et al., 2003; He et al., 2005). Protection against protozoan infection has also been investigated. Dalloul et. al. (2000) observed that i.v. and s.q., but not oral, administration of CpG-ODNs could increase body weight gain and decrease oocyst shedding due to subsequent infection with *Eimeria acervulina*.

The above experiments used motifs optimal for either humans or mice, since an optimal motif for chickens has not been determined yet. The fact that a wide range of motifs have demonstrated efficacy as measured by the ability to induce cytokine and NO

production (He et al., 2003) as well as lymphocyte proliferation (Rankin et al., 2001) in chickens, suggests that different motifs may have dissimilar properties.

In Chapter V, the dose information from Chapter IV was used to investigate the adjuvant and protective properties of CpG-ODNs related to *Eimeria* protection or immunity. In order to do this, three administration models were used, all utilizing the same dose of CpG-ODNs. The first was to give a single dose of CpG-ODNs with low levels of the parasite *Eimeria acervulina* given over five days (trickle immunization). The second was to give the CpG-ODNs simultaneously with a clinically infectious dose of either *E. acervulina* or *E. tenella*. The third was to give the CpG-ODNs one day before a clinically infectious dose of either *E. acervulina* or *E. tenella*.

In the first study, an immunizing dose of either 50 or 500 oocysts was given for the first five days of life both with and without CpG-ODN or non-CpG-ODN administration on the first day of immunization. This was followed with a clinical challenge at 19 days of age. Protection was determined by body weight gain and feed conversion on days 19 and 25, and lesion scores on day 25. CpG-ODN and non-CpG-ODN immunized chickens had higher body weight gain as compared to the immunized alone group in three of four trials (Table 7). Additionally, feed conversion was consistently in between that of immunized and non-immunized groups (Table 7). After clinical challenge, body weight gain was higher in the CpG-ODN administered groups than the immunized alone groups in both replicates of both experiments (Tables 8 and 9). However, the CpG-ODN group did not differ from the non-CpG-ODN groups in any

of the experiments and there were no consistent trends for post-challenge feed conversion (Tables 8 and 9).

In the second study CpG-ODNs were given either simultaneously with a clinical infectious dose of *E. acervulina* or *E. tenella*. When CpG-ODNs were given simultaneously with an infectious dose of *E. acervulina* no decrease in body weight gain was observed due to challenge (Table 10). Lesions were observed only in the challenged groups, although there were no differences in their severity (Table 10). When CpG-ODNs were given simultaneously with *E. tenella* we did observe a decrease in weight gain due to challenge as well as lesions in the challenged groups. However, there were no differences in either due to CpG-ODN or non-CpG-ODN administration (Table 11).

When CpG-ODNs were given one day prior to an infectious dose of *E. acervulina* we did observe a decrease in body weight gain and an increase in lesions as compared to the non-challenged control (Table 12). However, there were no differences with regards to CpG-ODN or non-CpG-ODN administration. When ODNs were given twenty-four hours prior to challenge with *E. tenella,* we also observed a decrease in body weight gain and an increase in lesions due to challenge. While there were no differences in weight gain due to CpG-ODN or non-CpG-ODN administration, we did observe a reduction in lesions scores in the CpG-ODN administered group as compared to the non-CpG-ODN and challenged only groups (Table 12).

These results of the first study suggest that co-administration of CpG-ODNs with an immunizing dose of *Eimeria acervulina* may improve weight gain and feed conversion both before and after subsequent clinical challenge. While differences

between the two immunizing doses were not observed, it is worth noting that both doses were well below a clinical challenge dose and immunizing doses used in other experiments (Galmes et al., 1991). Although a previous investigation did not report protection from *Eimeria acervulina* with oral administration of CpG-ODNs (Dalloul et al., 2004), the first study used a lower immunizing dose followed by a clinical *E. acervulina* challenge. It is possible that giving a large dose of parasite through the same route could abrogate the immunostimulatory effects of the CpG-ODNs, while low consistent exposure to the parasite may not. The rational behind this speculation is that a low dose of parasite would be less likely to overwhelm the gut mucosa and give the CpG-ODNs time to stimulate mucosal immunity.

The results of the second and third sets of experiments are in agreement with Dalloul et. al. (2004) in that pre-administration of CpG-ODNs orally does not protect from an infectious dose of *E. acervulina* given twenty-four hours later. However, it was also observed that pre-administration of CpG-ODNs can reduce lesion scores, but not improve body weight gain, in chickens given a clinical dose of *E. tenella* twenty-four hours later. As a decrease in body weight gain between the challenged only and non-challenged groups was observed in both experiments the dissimilarity in the results is likely due to the species and not the dose given.

Although the precise immunostimulatory affects of CpG-ODNs in chickens have not been fully defined, *in vitro* studies have demonstrated that CpG-ODNs can stimulate lymphocyte proliferation, induce nitric oxide (NO) production and IL-1β mRNA synthesis in the HD11 macrophage cell line, induce IFN-γ mRNA expression and nitric

oxide production in peripheral blood mononuclear cells (PBMCs), and induce degranulation and generation of reactive oxygen species (ROS) in heterophils (He et al., 2003; He et al., 2005; Rankin et al., 2001; Xie et al., 2003). In addition, differential cytokine responses of the duodenum and ceca of chickens infected with *E. acervulina* or *E. tenella* suggest that responses to and protection from infection differ between the two species (Choi et al., 1999; Laurent et al., 2001).

In summary, while administration of CpG-ODNs with an immunizing dose of *Eimeria acervulina* may be able to improve weight gain and feed conversion both before and after subsequent clinical challenge, neither co-administration nor pre-administration of CpG-ODNs with clinical doses of *E. acervulina* or *E. tenella* seems to influence body weight gain. However, while no differences were observed for lesions scores in *E. acervulina* challenges, a significant decrease in lesion scores due to pre-administration of CpG-ODNs was observed with *E. tenella* challenge. Taken together the above data suggest that orally administered CpG-ODNs may modulate the chicken's immune response, thereby improving performance during vaccination and providing either protection from or reduction of immunopathology resulting from *E. tenella*. Whether the differences discussed above would be seen in birds capable of more rapid growth is unknown at this time, however given the oral route of administration, the use of CpG-ODNS in mass vaccination may be feasible

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