ENHANCING DNA VACCINE-INDUCED AVIAN INFLUENZA HEMAGGLUTININ SUBTYPE-SPECIFIC REFERENCE ANTISERA PRODUCTION

by

JENNIFER PFEIFFER

(Under the Direction of David L. Suarez)

ABSTRACT

When avian influenza virus is isolated, it must be subtyped by determining which of the 16 hemagglutinin (HA) and nine neuraminidase (NA) proteins it has. Hemagglutination inhibition (HI) test used for identifying HA subtype, uses a panel of subtype-specific antibodies against each of the HAs. We previously demonstrated that DNA vaccines against different HA subtypes worked as efficiently as the traditional method using whole, inactivated virus, but multiple vaccinations are needed for an equal response. We tested various adjuvants including CpG motifs, different transfection reagents, and chicken cytokines. We also compared different routes of vaccine delivery. Chickens were vaccinated, bled at two-week intervals, and boostered at monthly intervals. HIs were used to assess antibody titers. Interleukin-2 and interferon-1 improved the antibody response against the less antigenic HA tested. Intradermal delivery route also showed potential for use at producing these diagnostic tools.

INDEX WORDS: Avian influenza, Adjuvant, DNA vaccines, Hemagglutinin, HI test

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DEDICATION

To Mom and Dad for your continuous and ongoing support,

Beepa, Grandma Faye, and Grandma Lucille for always feeding me positive comments,

Troy Boy for your simple yet such profound advice: Don't Think. The best New Year's resolution I ever made! This short phrase helped me survive the past year, while retaining a small bit of my sanity!

Andrea: Happy Birthday! "Check it out!" – an official, published Thesis Paper for us!!

Chicken, We're almost half way there…Three more years of our weekly sessions….but for now, hang loose!

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CHAPTER 1

INTRODUCTION

Background of the study

Avian influenza virus (AIV) in poultry presents itself in the form of either a low pathogenic (LPAI) or highly pathogenic (HPAI) disease. Both have negative effects on the poultry industry, ranging from decreased egg production to sudden deaths of entire flocks, which ultimately result in major economic losses. Determining the subtype(s) of the virus affecting a particular flock is necessary for deciding the most appropriate measures to be implemented in eradicating the disease. One method for subtyping the virus is by the hemagglutination inhibition (HI) test. However, because 16 defined HA subtypes have been described for AIV, at least 16 different reference antibodies (Abs) against each of the 16 hemagglutinins (HA) are necessary diagnostic reagents to allow for subtyping. The typical method for the production of the HA subtype-specific reference antibodies is by injection of chickens with killed whole virus vaccines. This method of antibody production can have several potential drawbacks. First, because whole virus is used, antibodies will be produced to other viral proteins such as the neuraminidase protein. Neuraminidase antibodies can interfere with the HI test results by steric hindrance.

Currently, commercially available vaccines against AIV are either whole, inactivated virus or recombinant fowlpox virus vectored vaccines. Because they both involve live viruses at some point in their production, biosafety level 2 laboratories are

needed. This is important because the virus must be prevented from leaving the laboratory and infecting birds in its close vicinity. Because this disease can be zoonotic, those working with the virus are at potential risk as well. In addition, the fowlpox vaccines are only available for the H5, and they do not produce high antibody titers. Economically speaking, high titers of reference antibodies should ideally be produced.

DNA vaccines involve injection of plasmid DNA encoding for antigenic proteins. For AIV, they can be designed to express HA subtype-specific proteins. Since a non-infectious plasmid DNA is used to express the HA protein, only a biosafety level 1 laboratory is needed for their production. In addition, the Abs induced by DNA vaccination are specific for the hemagglutinin being used. This is also useful diagnostically as well as for trade reasons because they could be used as DIVA vaccines, since the vaccinated birds would only have antibodies against the HA protein and not the internal proteins.

Objective

In a previous study conducted at our laboratory (46), DNA vaccines were effectively used to produce reference antisera against 15 HA subtypes. The use of this type of vaccine was advantageous because polyclonal HA-specific Abs were produced, but Abs to other influenza proteins were not produced. Although the previous study successfully produced antibodies for all 15 HA subtypes, the process required multiple vaccinations and resulted in inconsistent production of Abs from the birds. Additionally, some HA subtypes produced a poor antibody response even after three vaccinations. The goal of this project was to enhance HA subtype-specific Ab production to produce higher levels of antibody, preferably with smaller doses of DNA and fewer booster shots,

induced by the DNA vaccine. Several strategies for improving performance were evaluated, including various adjuvants such as CpG oligodeoxynucleotides, DNA plasmids encoding for various cytokines, comparing different cationic lipids, and various routes of vaccine delivery.

DNA vaccines, though they have numerous advantages over the whole, killed or recombinant vaccines, are currently more expensive, require more booster vaccinations than the presently available vaccines for AI, and a variability of the immune responses occurs from bird to bird. Another goal of this project was to improve both the efficacy and consistency of the DNA vaccines at inducing Ab production, which would allow for progress toward the use of DNA vaccines in the field.

CHAPTER 2

LITERATURE REVIEW

Overview

AIV is a member of the family *Orthomyxoviridae* and of the genus Influenza virus type A (32,41,76). Three other genera within this family include Influenza virus types B, C, and Thogotovirus (76,41). Influenza types B and C infect primarily humans, while type A influenza infects not only humans, but birds, swine, and horses as well (82).

Avian influenza (AI) is a disease in poultry which presents in either a low pathogenic form (LP) or a highly pathogenic (HP) phenotype and is caused by *avian influenza* virus (AIV) (76). The symptoms associated with LPAI range from asymptomatic infection, respiratory disease, or drops in egg production (9,76). LPAI is usually associated with low mortality, but in cases of concurrent bacterial or viral infection or poor environmental conditions, a mortality rate of 30% or higher has been observed (76). Highly pathogenic AI (HPAI) can cause a mucosal as well as a systemic disease with up to 100% mortality in chickens (9, 76). AI is of great economic importance to the poultry industry in many aspects (20, 76). The most severe economic losses are a result of HPAI outbreaks on commercial farms with intensive poultry production (76). Direct losses of such outbreaks include costs of depopulation and disposal, losses due to high morbidity and mortality, quarantine and surveillance costs, indemnities to compensate for the elimination of marketing birds, and loss of export markets for trade (20, 76). LPAI outbreaks have also resulted in great economic losses

for chicken, turkey, and duck producers, particularly when opportunistic secondary bacterial and viral pathogens are present (2, 20, 82). However, these losses are less significant than those resulting from HPAI because of lower mortality rates and fewer interruptions to national or international trade (76). Avian influenza was initially associated exclusively with chickens but it also affects turkeys, ducks, quail, and various other domestically raised avian species (20, 69, 76).

History

HPAI was likely first described in 1878, when Perroncito reported 'fowl plague' in chickens in Italy (20, 32,76). Rivolta and Delprato further described that 'fowl plague' was different from fowl cholera in 1880, which they called *typhus exudatious gallinarum* (69). Centanni and Savonuzzi, in 1901, next determined that the cause of 'fowl plague' was a filterable agent (69, 76) isolated from an infected chicken, suggesting that its etiology was viral (2, 32). Hirst then characterized the hemagglutination activity of influenza virus in 1941, and Schafer further characterized 'fowl plague' as a member of the influenza A virus group in 1955 (32).

HPAI outbreaks have been known to spread widely since being first described in Italy. For example, during 1894, a severe outbreak of HPAI in Northern Italy spread to chickens in various parts of Europe (32, 69, 76) where it remained endemic until the mid-1930s (76). HPAI was first discovered in the United States, in 1924-25, and then again in 1929 (36). In both instances, it was fully eradicated (36) By the middle of the twentieth century, AI had been diagnosed in most of Europe, Russia, Netherlands, Hungary, Great Britain, Egypt, China, Japan, Brazil, and Argentina (76).

By the mid 1900s, the first isolate of LPAI, an H10, was obtained from chickens in Germany (76). During the first half of the twentieth century, it had been assumed that all H5 and H7 hemagglutinin subtypes of AI were HP (76). The false notion that all H5 and H7 subtypes of AIV were HP was reversed after 1971, when many LP-causing H5 and H7 AIVs were isolated (32, 76).

The first isolation of a HPAI H5N3 influenza virus from wild birds was in 1961, found in common terns in South Africa (2, 17). The next discovery in the history of AI was made in the 1970s during surveillance for exotic Newcastle disease in California, when AIV was isolated from migratory waterfowl (32, 76). The data gained from these surveys helped to determine that AIV causes asymptomatic infection in wild birds (32, 76). Since then, healthy wild birds, particularly Anseriformes (shorebirds) and Charadniiformes (waterfowl) have been found to be asymptomatic reservoirs of AIV (2, 32, 76). AI is most commonly introduced into domestic poultry by wild migratory waterfowl (36, 82). Fortunately, most AI from these wild birds is LP (32, 76). However, a presumption has been made that HPAI emerges only after the virus has crossed to poultry from wild birds (2).

Until recently, the presence of HPAI was rare in domestic poultry and is considered to be a foreign animal disease or exotic disease in the U.S (9, 45). A mere 17 episodes were reported worldwide from 1959-1998 (9). The last major outbreak of HPAI in the U.S. occurred in 1983, when an H5N2 outbreak occurred in Pennsylvania, costing nearly 60 million dollars just for the control effort and resulted in the death or destruction of nearly 17 million birds (76). The Pennsylvania outbreak was the first documented case of LPAI virus mutating to the highly pathogenic form of the virus (45).

The source of the LPAI virus associated with the outbreak was not determined, but one speculation is that the LPAI had been endemic in live bird markets (LBM) in this region (32, 45). The role of LBMs in avian influenza epidemiology was not recognized until 1986 when LP H5N2 that was related to the HPAI outbreak AI was first isolated, and the H5N2 Pennsylvania lineage of virus was not completely eradicated until 1989 (73). Since 1997, eight more episodes of HPAI have occurred over a seven year period (9), including an unusual H5N2 outbreak in Texas in 2004 (9, 43). Of interest, an increase in LPAI outbreaks, particularly of the H5 and H7 subtypes, has been reported in various other countries (9). These incidences have occurred in Pakistan, Mexico, Central America, Australia, Italy, Ireland, parts of Europe, North America, and in Southeast Asia (9), causing it to be an international disease of economic concern (76).

For a long time, interspecies transmission of AIV from bird to human was considered to be highly unlikely and seldom occurred (32, 69). However, in Hong Kong in 1997, 18 people became infected with a HPAI strain of H5N1 containing a genome entirely of avian origin (32, 41). Of these 18 infected humans, six people died (32). New human cases of lethal H5N1 AI are currently being reported in Thailand and Viet Nam nearly every week (WHO). This phenomenon has raised fears that the H5 strain could cause the next global influenza pandemic among humans, should it become better adapted for direct human-to-human transmission (32).

Etiology

Classification

Avian influenza is a member of the family *Orthomyxoviridae* (40). "Ortho" means "other" and "myxo" means "mucous" in Greek (40). This family was so named because of its ability to bind to mucous (hemagglutination) and also as a means of differentiating it from other negative sense RNA viruses, such as members of the *Paramyxoviridae* family(40). The orthomyxovirus family is comprised of influenza A, B, and C, and thogotovirus, of which influenza A is known to infect birds (40). The name influenza is an Italian word derived from the Latin word "influentia" meaning "epidemic", so chosen because disease epidemics were thought to occur as a result of occult influences (40).

Orthomyxoviridae viruses are enveloped, segmented, single-stranded, negative sense RNA viruses (40). The term negative sense refers to the fact that the viral mRNA lacks a 7-methyl guanosine cap and can not be directly translated into viral proteins (40). Thus, it must be transcribed into positive sense RNA from their eight viral RNA segments (40).

Influenza viral RNA serves two purposes: 1) mRNA synthesis; proteins comprising the virus particles are manufactured from these and 2) template or cRNA production; the negative sense RNA serves as a template for producing more genomic negative-sense RNA (40).

Unique characteristics of influenza viruses that separate them from other RNA viruses are that both transcription and replication occur in the nucleus of the infected cell

(40). For initiation of mRNA synthesis, an influenza virus-encoded cap-dependent endonuclease snatches the 7-methyl guanosine-caps of cellular mRNA to use as primers for mRNA synthesis (40). Influenza also takes advantage of the cellular splicing machinery (40). In doing so, it also utilizes its compact genome to great capacity; its replication involves some spliced mRNAs with overlapping reading frames, bicistronic mRNAs with overlapping reading frames, and finally, coupled translation of tandem repeats (40).

Differentiating the various types of influenza viruses (A, B, or C) is based on antigenic differences between the nucleocapsid (NP) and matrix (M) proteins (Fields). Type A influenza viruses naturally infect various avian species, humans, and other mammalian species (*i.e.* swine and horses) (40). Further subtyping of type A influenza is carried out based on the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) (40, 76). These two glycoproteins show greater variability in their amino acid sequences compared to those of the other types of influenza (40).

Virion morphology

The typical shape of virions is spherical to pleomorphic but may also be filamentous (40, 76). The size ranges from 80-120 nm in diameter (40, 76). Each particle is surrounded by a lipid envelope derived from the plasma membrane of the infected host cell from whence the viral particle budded (40, 76). Protruding from the envelope are two different types of spikes (fields, Diseases). The hemagglutinins (HA) are rod-shaped trimers and are approximately four to five times more abundant than the

mushroom-shaped neuraminidase tetramer spikes (40, 76). Nestled within the lipid envelope layer is a third integral membrane protein, the ion channel (M_2) (40).

Just beneath the envelope lies the matrix (M_1) protein (40). Associated with the matrix are the various-sized ribonucleoprotein (RNP) complexes (40). Each RNP complex is comprised of nucleocapsid proteins (NP), which are helical-shaped and are intertwined closely with one of the eight segments of ss viral RNA (40). In addition to the NP and viral RNA, the RNA-dependent RNA polymerase complex composed of PB1, PB2, and PA also takes part in forming the RNP complexes (40). One RNP complex exists per virus RNA segment (40). The eight segments of genomic viral RNA encode for at least 10 proteins (10); nine comprise the virus particle itself (HA, NA, NP, PB2, PB1, PA, M_1, M_2, and NS_2), and two are found in the cytoplasm of infected cells (NS_1 and NS_2) (40).

Virus genome and proteins

As previously mentioned, AI has eight RNA segments which are single-stranded and negative sense. Each segment has been designated a number according to its size in nucleotides (40). In order from largest to smallest, the segments are as follows: 1 is PB2, 2 is PB1, 3 is PA, 4 is HA, 5 is NP, 6 is NA, 7 is M_1 and M_2, and 8 is NS_1 and NS_2 (40). The size of the entire genome is approximately 13, 588 nucleotides, but it varies according to each particular virus strain (40).

Polymerase Proteins (PB2, PB1, and PA)

The polymerase proteins are encoded by the three largest viral RNA segments (40). Their molecular weights range from 85-96 kDa (40). They were named according to their behavior on isoelectric focusing gels; PB1 and PB2 have a basic net charge of +28 at pH 6.5 while PA has an acidic charge of –13.5 at pH 6.5 (40).

RNA segments 1 and 2 are each 2,341 nucleotides and encode for proteins of 759 and 757 amino acids (aa), respectively (40). The third segment is 2,233 nucleotides long and encodes for a protein of 716 aa (40).

Together, these three polymerase proteins form a complex in the cytoplasm and nucleus of the host cell (40). The complex sedimentation values on sucrose gradients are 11S-22S (40). These proteins are synthesized in the cytoplasm and due to their intrinsic karyophilic properties, are transported to the nucleus; each of the proteins contains a nuclear localization signal (40).

The PB1 protein is involved in the recognition and snatching of the 7-methyl-guanosine cap of the 5' end of host cell mRNA (22, 39). As mentioned previously, this converts (-) to (+) sense mRNA. It is also part of the transcriptase complex (39, 40). PB2 has endonuclease activity and catalyzes nucleotide addition (40, 76). It also is part of the transcriptase and replication complex (40, 76). Lastly, the PA protein is involved in viral RNA replication and has proteolytic activity (22, 40, 76).

Nucleocapsid Protein (NP)

The NP is the primary structural protein that interacts with each viral RNA segment to form the RNPs (40). It is a type-specific antigen in that the different types (A,

B, or C) of influenza virus can be distinguished based on this protein (40). Viral RNA segment five, which is 1,565 nucleotides long, encodes for NP, which is 498 aa in length (40). The protein has a molecular weight of approximately 56 kDa (40). NP is rich in arginine residues and has a basic charge of +14 at pH 6.5 (40). It has no particular clusters of basic residues, which is an implication that multiple regions of NP may participate in its binding to the genomic RNA (40). Like the polymerase proteins, NP is synthesized in the cytoplasm and then transported to the nucleus, due to its two nuclear localization signals (40). During late infection, a cellular protease involved in apoptosis, named caspase, cleaves NP (40). This cleavage may have a function in virus – host interaction or may merely be a marker for apoptosis; this is currently unclear (40). NP molecules carry out various functions in infected cells (76). They are involved in transporting vRNPs from the cytoplasm to the nucleus of an infected cell and are necessary for the synthesis of full-length vRNA (40). NP molecules are the major targets for cytotoxic T lymphocytes (40, 80). DNA vaccine studies involving NP DNA have shown to induce both $CD4^+$ and $CD8^+$ T cells and elicit limited heterosubtypic protection against influenza challenge (80).

Hemagglutinin (HA)

Hemagglutinin (HA) is by far the most important AI protein. Without HA, the virus particle would be unable to carry out its main purpose: to produce more virus particles. It was so named because of its ability to agglutinate erythrocytes (40). The HA has three main roles during the replication cycle (40). First, it provides the virus attachment to host receptors containing sialic acid residues, which are found lining the

upper respiratory and intestinal tracts (20, 34, 66, 76). This is important in that if the virus is not able to attach to the cell, it is unable to infect the host, and can not replicate. Next, HA mediates fusion between the virus and endosomal membranes, which ultimately results in the release of viral RNA into the cytoplasm (40). Finally, HA is the major antigenic protein against which neutralizing antibodies are produced (40, 76). It is against this protein that vaccines are targeted. As previously mentioned, variations in its structure resulting from antigenic drift or shift promote epidemics to occur (40, 82).

Epidemic patterns of influenza viruses occur in humans (82) as a result of two types of processes (22, 82). Antigenic drift occurs in both the HA and neuraminidase (NA) proteins when point mutations arise (22, 82). These mutations enable the virus to evade host immunity against previously circulating viruses in a single person or an entire population (22, 82). The resulting new variants of influenza are the cause of yearly epidemics (82).

Antigenic shift occurs only in segmented viruses (22, 82). This phenomenon arises when HA and NA not previously circulating in the population spontaneously emerge (82). It can occur by direct transmission from hosts of other species or during coinfection by two distinct subtypes of influenza A (22). During coinfection, the viruses exchange segments, resulting in the emergence of new reassortants (22). Antigenic shift has been the notorious cause of pandemics associated with worldwide morbidity and mortality (22, 82).

Viral RNA segment four encodes for HA (40). It is synthesized on membrane-bound ribosomes and then translocated to the lumen of the endoplasmic reticulum (ER) as a precursor polypeptide, HA_0 (40). Its N-terminal signal sequence containing 16

amino acid residues is cleaved by signal peptidase in the ER (39). Being a type I membrane glycoprotein, its amino-terminus (N-terminus) is in the ectodomain while its carboxy-terminus (C-terminus) is found in the proximal transmembrane region (40). It is cotranslationally modified in that its ectodomain acquires up to seven oligosaccharide chains (of which one is needed for proper folding in the ER) and proximal cysteines at its C-terminus acquire three thioether-linked palmitate residues (40, 52, 53).

The HA protein exists as the HA_0 precursor with a molecular weight of 76 kDa or as a cleaved protein made up of HA_1 and HA_2 subunits (47 kDa and 29 kDa, respectively) linked together by disulfide chains (40, 66). Cleavage of HA_0 to HA_1 and HA_2 is important (40, 66) because it is a necessity for virus infectivity and thus determines pathogenicity and spread of infection (40, 68). The newly exposed N-terminus of the HA_2 formed upon cleavage of the molecule, referred to as the fusion peptide, is more conserved among different strains of influenza and is required for HA endosomal fusion activity (40, 68).

In intracellular environments with low pHs, such as the endosome, cleaved HAs undergo irreversible conformational changes which move the fusion peptide toward the endosomal membrane, ultimately resulting in fusion of the viral and endosomal membranes (22, 40). Cleaved HAs at a neutral pH are considered to be in a metastable form (40). The low pH enables them to take on a more stable form, which renders the HAs more susceptible to digestion by proteases found within the host (40). At this time, some antigenic sites are lost while others are created (40).

The HA gene on RNA segment four was the first influenza gene to be fully sequenced and it encodes for 16 different known subtypes (23, 40). The 16th subtype

was just recently described (23, 40). The HA protein is approximately 1,750 nucleotides in length and encodes a protein of about 564 amino acids (40s). In the mature virus, each HA is a homotrimer composed of three monomers (40). Following cleavage, the fusion peptide is buried in the interior of the trimer (40). The trimeric structure is divided into two regions: 1) the stalk and 2) the globular head (40). The stalk contains the HA_2 part and is proximal to the membrane and the membrane-distal head contains the HA_1 part (40).

A receptor-binding pocket, inaccessible to antibodies, is located in the distal head of each monomer (40). The amino acid sequences forming this pocket are conserved among various subtypes, resulting in different binding specificities to host cells (28, 40). In human trachea, the sialic acid receptor is linked to galactose via α2-6 linkages, while the intestines of avian species contain α2-3 linkages and swine trachea contain both types (28, 40). The differences in receptor specificity are thought to contribute to host specificity, but it is not the only determinant. (28). Most neutralizing antibodies attach to epitopes near the receptor binding sites, that are characterized into five antigenic binding sites located on the globular heads. (40).

Once the virus attaches to sialic acid and is internalized into the cell inside the endosome, the environment in the endosme changes to a lower pH (40). This pH change causes the cleaved HA to undergo irreversible conformational changes, which move the fusion peptide toward the endosomal membrane, ultimately resulting in fusion of the viral and endosomal membranes (22, 40).

There is experimental data supporting the correlation between the number of aa at cleavage sites and virus virulence (9). Cleavage sites containing two basic aa, at

positions -1 and -3 or -4, for H7 and H5 subtypes, respectively, are viruses of low virulence (9, 40, 66). These cleavage sites, which contain only one arginine residue, require exogenous proteases such as trypsin, which are restricted to areas along the respiratory and intestinal tracts (40). Conversely, all HPAI viruses possess multiple basic aa (arginine and lysine) adjacent to the cleavage site (9). These multiple basic aa were apparently a result of insertion or substitution (9). The presence of multiple basic aa result in motifs that are targets for ubiquitous intracellular proteases such as furin (9, 40, 66). This concept explains why LPAI is localized to mucosal regions while HPAI is mucosal and systemic.

Another factor thought to be involved in determining virulence is the presence or absence of a sugar chain near a cleavage site containing multiple basic amino acids (40). The idea has been proposed that this sugar would block furin from accessing the cleavage site and disease of low virulence would result (40, 32). However, when no sugar is in the way of the cleavage site, furin has access to the cleavage site and disease of the highly pathogenic form may result (40).

Neuraminidase (NA)

The neuraminidase (NA) is another integral membrane protein found on the influenza virus particle that is involved in subtyping the virus (40). This protein is a homotetramer of 220 kDa (40). The molecule contains a distal head region with enzymatic activity and a stalk region that is proximal to the virus particle membrane (40). NA have two important purposes: 1) they remove sialic acid from glycoproteins (*i.e.* HA, NA, and cell surfaces), preventing influenza viruses from attaching to each other or

remaining bound to cells, which would effectively reduce the level of further infection and 2) they are antigenic determinants and undergo antigenic variation (40). These proteins may also allow the virus to move through the respiratory tract's mucin layer and find the target epithelial cells (28, 40). Finally, some AI NAs have receptor-binding sites which cause hemagglutination (40).

Segment six of the genomic RNA encodes for NA and nine protein subtypes have been identified (40). The gene is approximately 1,400 nucleotides in length and the protein contains about 450 aa (40). The NA protein contains one hydrophobic region, which is located in the N-terminus and spans the lipid bilayer (40). This region serves as both an uncleaved signal and an anchor domain by targeting NA to the ER membrane and allowing for stable attachment in the membrane, respectively (40). The NA's amino-terminus is in the cytoplasm, which puts them in the type II class of membrane glycoproteins (40). There are five possible N-linked carbohydrate sites for most neuraminidase proteins (40, 63).

The head of NA is box-shaped, with large pockets on the surfaces of each monomer for ligand-binding (40). Hemagglutinating activity associated with NA occurs at a sialic acid binding site separate from the enzyme active site (40). On each NA molecule there are four main antibody-binding domains which cluster in regions distal to the membrane but are not neutralizing (40). As a result, antibodies against NA prevent viral spread from infected cells but do not prevent infection (40).

Matrix (M_1) and ion channel (M_2)

The matrix (M_1) protein is the most abundant protein in the virus particle and lies just beneath the virus envelope, providing structural support (40). The ion channel (M_2) protein is not as abundant in the virion but it serves an important function; it allows the influx of hydrogen ions (H^+) into the virus particle while the virus is in the endosome (40). This ultimately results in a drop in pH inside the virion that results in a conformational change in the HA (40).

Viral RNA segment seven encodes for both of these two proteins (40). The gene segment is typically 1027 nucleotides long (40). A colinear transcript mRNA codes for M_1 and a spliced mRNA encodes for M_2 protein, which overlaps M_1 by 14 residues (40).

The M_1 protein is typically 252 amino acids that is approximately 28 kDa (40). It, like NP, is a type-specific antigen and its aa sequence is highly conserved among influenza type A subtypes (40). M_1 is a peripheral membrane protein which interacts with the cytoplasmic tails of the three integral membrane proteins (40). When M_1 remains intact with RNPs, during uncoating the RNPs are not transported to the nucleus (40). Later on in the replication process of infection, M_1 must enter the nucleus in order for newly synthesized RNPs to exit the nucleus (40).

M_2 is abundant within the plasma membrane of the virus-infected cells, but only about 40 of these proteins are actually incorporated into the virus particles (40). It spans the membrane only once and because it has an N-terminal ectodomain but no cleavable signal sequence, it is considered a type III integral membrane protein (40). Instead of a signal sequence, it depends on signal recognition particles in order to be cotranslationally inserted into the ER membrane (40). This protein is posttranslationally modified but

these modifications are not mandatory for its proper functioning (40). It is a homotetramer that forms a pore in the membrane ion channel (40). The influenza-specific anti-viral drug amantadine targets M_2 and blocks its ion channel activity which prevents the virus from uncoating (40).

M_2 has two important roles for the virus: 1) it permits the influx of hydrogen ions (H^+) into the virion during uncoating and 2) it regulates the pH of the Golgi apparatus (40). This ion channel is activated at low pH levels within endosomes and the trans golgi network (40). An important characteristic pertinent to these functions is that M_2 is highly specific for H+ ions (40).

Nonstructural Proteins (NS_1 and NS_2)

The smallest segment of viral RNA, segment eight, encodes for two nonstructural proteins, NS_1 and NS_2 (40). The NS_2 mRNA was the first proof that splicing occurs with an RNA virus that lacks any DNA intermediates during replication (40).

Viral RNA segment eight is typically 890 nucleotides in length (40). The NS_1 protein for which it encodes has a molecular weight of approximately 26 kDa and is made up of 230 aa while the NS_2 protein is about 14 kDa and is typically 121 aa in length (40). The NS_1 is directly encoded by mRNA and the NS_2 mRNA is spliced (40). They both share a 56-nucleotide leader sequence which contains the codon involved in initiation of protein synthesis (40).

The NS_1 protein exists abundantly in the nuclei of infected cells (40, 78). However, none have been detected in virions (41). In the nuclei, they interact with polysomes (40). They contain two nuclear localization signals (NLS) and one nuclear

export signal (NES) which regulate the export of proteins from the nucleus (40). The NS_1 protein is also important in endowing the virus with resistance to host cell interferon (40). Double stranded (ds) RNA usually triggers the Protein Kinase R (PKR) pathway, either directly or via triggering the production of IFN (29, 40, 48). The NS_1 protein binds to ds viral RNA, which blocks the PKR pathway (40, 70). Hence, the PKR inhibition of protein synthesis is abrogated (40).

The NS_2 proteins does exist in virions and is associated with the M_1 protein (40). They are located in the nucleus and cytoplasm and each protein contains an NES, which aids in transporting viral mRNAs to the cytoplasm for translation (22, 40).

Viral Replication and Assembly

The replication process of influenza virus begins with the attachment of the virus via HA to sialic acid residues found on cell surface glycoproteins and glycolipids (40). AIV specifically binds to mucoproteins with sialic acid α2,3 linkages (13, 32, 40, 66). Individual cleaved, native HAs have a low affinity for sialic acid but when multiple HAs are present, high avidity occurs, allowing for initiation of infection (13, 40).

AIV enters the cell by receptor-mediated endocytosis (22, 40). When H^+ ions are pumped into the endosome, causing a drop in pH to 5-6 (40), this triggers the HA to undergo a conformational change (40). The hydrophobic fusion peptide at the N-terminus of HA_2 becomes exposed and inserts into the endosomal membrane, bringing both endosomal and viral membranes into close proximity (13, 40, 66). A highly bent stalk structure forms, ultimately resulting in fusion pore formation (13, 40). Meanwhile, H^+ ions flow from the endosome into the virion via M_2 (40). This drop in pH within the

virus particle causes disruption of protein-protein interactions between M_1 and RNPs (40). M_1 dissociates from RNPs (40). The internal contents of the virus mix with the cytoplasm of the endosome by passing through multiple pores (13, 40). At this point, viral RNPs are able to be directed into the nucleus (13). Nuclear localization signals (NLS) found on RNP proteins enable the RNP to be actively transported through nuclear pores (40). Both old and freshly synthesized M_1 enter the nucleus via passive diffusion (40). Synthesis of viral mRNA (vmRNA) is catalyzed by PB2, involved in the cap binding, and PB1, involved in elongation (40). These proteins are functional only in the presence of vRNA (40). When the 5' end of vRNA binds to PB1, PB2's cellular mRNA cap binding acitivity is triggered (40). Next, the 3' end of vRNA binds to PB1 and activates PB1 to cut off the cell mRNA's cap and subsequently elongate the vmRNA (40).

The next step to occur is virion RNA replication which involves two parts: 1) template (+ sense) RNA synthesis, followed by 2) vRNA (- sense) synthesis (40). The transition from vmRNA production to vRNA replication requires solitary NP molecules not associated with nucleocapsids (40). These NP molecules are important for cessation of cap snatching and antitermination at poly A sites (40). One theory is that NPs binds to the common 5' ends of nascent transcripts, followed by the addition of more NPs to the elongating chain of RNA (40). In this set up, the vmRNA is prevented from slipping backward along the vRNA template and the reiterative As are blocked (40). According to this theory, the number of NP molecules in infected cells regulates the amounts of vmRNA vs. genomic RNA produced because full-length RNAs are synthesized only when NP molecules are present (40).

The second part involving the copying of the vRNA template to produce (–) sense vRNA also requires NP to add on to elongating vRNA (40). However, this time, it binds to full-length, (+) sense RNA (40). The result is new vRNA in nucleocapsid form that is ready for packaging into progeny virions (40). In addition to NPs, vRNA templates also require replication polymerase (PA), which is present only in infected cells and not in virus particles (40). On the other hand, PB2 is not needed at this stage of virus replication (40). The requirements for (+) and (–) sense vRNA but not vmRNA for NP help to explain why they occur encapsidated with NP subunits and do not involve cap-primed synthesis (40).

Once vRNPs are assembled, each forms a complex with M_1 and NS_2 (40). The NES found on NS_2 overrides the NLSs found on the polymerase and NPs (40, 54). Hence, RNPs are exported from the nucleus (40, 54).

Infection with AI is divided into two phases, based on the quantities of the individual RNA segments transcribed: 1) Early phase and 2) Late phase (40). During the early phase, synthesis of particular vRNAs, vmRNAs, and viral proteins are all coupled (40). Following primary transcription, equimolar amounts of (+) sense template RNA is made at the start of this phase (40). With the exception of particular vRNAs, there occurs a sharp decline in their synthesis (40). Both NP and NS_1 are made early because they are involved in the initiation and synthesis of all template and genomic vRNA (40). M_1 vmRNA and protein synthesis are both delayed (40). Upon entry into the late phase, the synthesis of all vRNAs reaches a peak, but the rate drops drastically soon thereafter (40). Protein as well as genomic vRNA synthesis continue to proceed through the end of this stage (40). M_1 and HA proteins are produced in particularly large amounts in the late

phase (40). This feature makes sense due to the fact that M_1 stops transcription of vRNA into vmRNA and is also involved in the transport of RNPs to the cytoplasm (40, 54).

As previously mentioned, posttranscriptional processing of NS_1 and M_1 vmRNAs occurs with the assistance of cellular machinery in the nucleus (40). Two smaller mRNAs encoding for NS_2 and M_2, respectively, are produced (40). Only about 10% of such vmRNA produced is spliced and the amount of splicing is determined by the rates of splicing and the rates of nuclear export of the vmRNA (40). All spliced and unspliced vmRNA are exported to the cytoplasm for translation (40).

During infection with influenza, the virus takes control over host cell translation by two main aspects (40). First, the NS_1 protein prevents type I IFN induction, which reduces the interferon cellular response of blocking protein synthesis, which ensures efficient translation of virus-specific proteins in infected cells (40). Additionally, translation of cellular mRNAs is inhibited and viral mRNA is preferentially translated (40).

Virus Assembly and Release

The HA, NA, and M_2 proteins are all synthesized on membrane-bound ribosomes and are translocated across the ER membrane in a signal recognition particle (SRP)-dependent manner (40). The HA's N-terminal signal sequence is cleaved in the ER by signal peptidase (40). The other two surface proteins do not contain such signal sequences (40). N-linked carbohydrate chains are transferred to HA and NA (40). Following folding and assembly of these three proteins, they are transported from the ER to the Golgi apparatus for further processing of the oligosaccharide chains (40).

Interesting to note is that both HA and NA lack terminal sialic acids on their complex carbohydrate chains, most likely due to NA action (40). HA, NA, and M_2 proteins are expressed at the plasma membrane with the HA dispersed over the surface while M_2 and NA occur in clusters (40).

Bud formation and release of virus particles are the final steps in viral replication and production of new infectious virions (40, 54). The precursor to the envelope of the budding virion is a patch of cell membrane containing envelope proteins (40, 54). Typical budding sites are regions among the cell membrane where lipid rafts occur (40, 54). vRNA-M_1-NS_1 complexes exiting the nucleus are transported to these rafts containing HA and NA (22). Virus assembly and budding requires the interactions between the viral envelope, M_1, and vRNP, with M_1 serving as a bridge between the two (54). The M_1 may also serve as a bridge between the envelope proteins and the vRNP (54). Interactions between M_1 and M_1 assist in concentrating the viral components and in excluding cellular components (40, 54). Outward curvature of the cell plasma membrane at virus assembly sites leads to bud formation (54). Virus particles bud from the apical plasma membranes of polarized epithelial cells (40, 54). Fusion of the opposing membranes results in bud closure and the separation of the virus particle from the host plasma membrane (54). The virus particle is released into the extracellular environment to infect other cells (54). The complete release of virus particles requires NA activity to release them from the sialic acid on the cellular proteins which allows for spread from cell to cell (40, 54).

Clinical Signs

An important determinant in the clinical symptoms of AI is the pathotype – either HPAI or LPAI (76). The symptoms are variable, depending on the host species, sex, age, the simultaneous presence of any other infections, acquired immunity, and environmental conditions (76).

With rare exception, wild birds are only infected with LPAI with no clinical signs of disease (76). In domestic poultry such as chickens and turkeys, the most common signs of clinical disease with LPAI include mild to severe respiratory signs such as coughing, sneezing, rales, rattles, and excessive lacrimation (76). Increased broodiness and decreased egg production occurs in breeder and layer hens (76). In addition, huddling, ruffled feathers, depression, decreased activity, decreased intake of food and water, and some diarrhea may occur (76). A slight increase in daily mortality may also be observed (76). Many flocks infected with LPAI may seroconvert without evidence of clinical disease (76). In experimental inoculations of specific pathogen free chickens with LPAI, clinical disease is seldom observed (71).

In domestic chickens, turkeys, and other galliformes infected HPAI, clinical manifestations tend to be more systemic and vary, depending on the affected organs (71, 76). HPAI can replicate and cause damage directly to a number of visceral organs as well as the cardiovascular and nervous systems (76). Often, chickens and turkeys die before any clinical signs occur (76). If the birds survive for a few days, they may begin to exhibit nervous disorders including tremors of the head and neck, inability to stand, torticolis, and other uncommon positions of the head and appendages (76). Depression

and decreased intake of food and water are also displayed (76). A drastic decrease or possibly complete cessation of egg laying within six days may occur in breeders and layers (76). Respiratory signs similar to those seen in LPAI may also occur (76). Hemmorrhage or necrosis of the combs and wattles, legs, and serosal surfaces of internal organs may also be observed (76).

Morbidity and mortality rates in chickens and turkeys are as variable as the clinical signs (76). With LPAI, there is usually high morbidity, however, mortality can vary widely depending primarily on if secondary infections are present (76). Generally both morbidity and mortality rates are high with HPAI in gallinaceous birds, sometimes reaching up to 100% mortality in flocks (76). For ducks and geese, mortality is usually much lower for HPAI, but recent H5N1 isolates from Asia may be an exception to this rule (43).

Immunity

Active Immunity

Infection and vaccination with AIV elicits both systemic and mucosal Ab responses (71, 76.). The intensities and levels of the antibody responses vary from species to species, with a general trend of highest to lowest levels beginning with chickens > pheasants > turkeys > quail > ducks (71, 76). As previously mentioned, Abs against HA can be both neutralizing and protective while Abs against NA do not prevent infection, but can reduce clinical disease and viral shedding (40, 71, 76). The degree of protection against mucosal challenge and shedding of the virus depends on the sequence similarity between the vaccine and challenge virus antigens such as HA and NA (71, 76).

The duration of protection against clinical signs and death has been shown to last up to 30 weeks in the field (76). Birds which have recovered after field exposure are normally protected against homologous HA and NA subtypes (76). Immune responses against the internal virus proteins have demonstrated no protection from clinical signs or death but may curtail the replication and shedding period, likely through cell mediated immunity (76).

Serology

The most commonly used diagnostic test in the U.S. for type A influenza viruses is a double immunodiffusion test, also known as the agar gel immunodiffusion (AGID) or agar gel precipitation (AGP) test (55, 76, 82). Concentrated virus and antiserum against the NP or M_1 proteins are used because these proteins are highly conserved among the type A viruses (76, 82). This test is relatively simple to perform and requires a minimum of equipment. An Enzyme-linked Immunosorbant Assay (ELISA) against the influenza type A-specific antigens is also available (76, 82). Several commercial ELISA tests are available, and are commonly used as a screening test with confirmation with the AGID test. Further serologic analysis for subtype of the virus is carried out using the hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests (55, 76, 82). Both tests rely on a panel of virus all 16 HA and nine NA subtypes (55, 82). The drawback to the HI test is that non-specific inhibitors found in the serum sometimes have to be removed before running the test (55, 82). Within the past couple of years, real time-RT PCR reactions have become the standard procedure used for diagnosis (55, 67).

Assessing Pathogenicity

Once an isolate of avian influenza has been identified and subtyped, its pathogenicity is next evaluated (55). One method for evaluating pathogenicity is by the Intravenous Pathogenicity Index (IVPI) (55). Eight four- to eight-week old susceptible chickens are inoculated with 0.2 ml of a 1/10 dilution of infective allantoic fluid (55). If six or more of these birds die within 10 days, it is considered HPAI (55). In addition, if the virus is not of the H5 or H7 subtype but kills one to five chickens, the virus is grown in cell culture, such as Madin Darby Canine Kidney cells (55). If the virus causes cytopathic effects or plaque formation occurs in the absence of trypsin, it is considered HPAI (55). Finally, LP H5 and H7 viruses grown in cell culture without trypsin that have sequence similarity with other HPAI isolates, particularly multiple basic amino acids at the cleavage site, are considered to be HPAI (55). To date, all HPAIs identified have been of the H5 or H7 subtypes (40).

Vaccines

A variety of different types of vaccines have been developed and when tested experimentally, have shown to protect against AI (71). Most of these vaccines have been evaluated for protection from HPAI (71). Greater resistance would be seen as a consequence of antibody formation (71). Vaccines can be divided into two categories: 1) conventional killed whole virus and 2) subunit vaccines which contain only a part of the entire virus (71). Some of the various types of subunit vaccines that have been tested include retrovirus-expressed HA (35), baculovirus-derived HA (15), and recombinant

fowlpox vaccines containing HA or NA gene inserts (59, 77). The vaccines currently used are the whole virus in oil emulsion adjuvant and a fowlpox recombinant with an H5 insert (18, 71, 75).

Another type of subunit vaccine that has been tested and is still being optimized is that of plasmid DNA containing an HA gene insert (38, 61). DNA inoculation of plasmid DNA with a eukaryotic promoter encoding an antigenic protein can induce an immune response against that protein (31). Delivery routes for DNA vaccines are either intradermal via gene guns or intramuscular via needle (31). Plasmid DNA vectors contain characteristic components to allow for the most efficient functioning possible (31). One example is the pCI eukaryotic expression vector, which contains the cytomegalovirus promoter. This plasmid was shown to produce better expression when compared to other plasmids that contained different promoters (72) in plasmids tested in chickens. Downstream of the promoter region is the actual gene of interest, which in turn is followed by a simian virus (SV40) polyA signal which ensures correct termination of mRNA and allows little read through transcription (31).

Hypothetically speaking, during intramuscular injection, the DNA is delivered to the muscle and some antigen presenting cells (61). Some of the transfected cells then leave the injection site and circulate to the spleen where antigen presentation likely takes place (61). This can result in stimulation of both the cellular and humoral immune responses (38, 61).

DNA vaccines have several advantages over conventional or other subunit vaccines (24). The DNA plasmids are relatively easy to manipulate with regards to switching of genes and vectors (24). Only the antigen of interest required for protective

immunization is involved, but without the hassle of protein purification (24). Protein synthesis *in vivo* allows for processing, modification, and presentation of antigen to the host immune system similar to what would take place during a natural infection, evoking both MHC I and MHC II responses (5, 24). Long-lived immunity by B cells is also induced (38). There is no handling of the infectious organism which means that only a Biosafety Level 1 laboratory is required (38, 79). No disease pathology occurring from either concomitant infection by another pathogen, or from any injection site reaction results (30). Because DNA plasmids are stable at high ambient temperatures, the need to maintain cold chain is reduced (30).

Despite advantages of DNA vaccines, there are some drawbacks. Immune responses to these vaccines are not always consistently seen in the vaccinates (46). Compared to killed adjuvanted vaccines, the antibody response is delayed (46). Finally, large amounts of immunizing DNA as well as multiple boosts are often required to achieve Ab responses equivalent to those from conventional vaccines (46).

As previously mentioned, one way to subtype an AIV isolate is by using Abs against each of the 16 HA subtypes in an HI test (55, 82). These Abs are typically produced by vaccinating chickens with whole, inactivated vaccines or with live virus. Unfortunately, this classic method of production has several potential drawbacks. First, incorrect subtyping may occur if any interfering viral proteins (such as NA) interfere with RBC binding (46). To test the efficacy of DNA vaccines in producing HA subtype-specific Abs for use in HIs, chickens were vaccinated against each of the 15 HAs using such vaccines (46). However, with the DNA vaccines, it is sometimes difficult to get consistently high (if any) Ab responses (46). An area of current research interest is in

improving the Ab responses to DNA vaccines when using the Abs for diagnostic purposes. One approach involves the use of various adjuvants including CpG motifs, liposomes, and cytokines (6, 30, 60, 83,).

An adjuvant is an agent that increases the antigenic response of an Ag (4). It acts to accelerate, extend, or enhance the magnitude of a specific immune response to the vaccine Ag (4). In addition, an adjuvant is used to reduce the amount or number of immunizations required for protective immunity (4). Antigens are categorized into groups which include 1) bacteria-derived adjuvants, 2) liposomes, 3) cytokines, and various others (57).

Bacteria-derived adjuvants enhance the immune response against coadministered Ags despite the fact that by themselves, they are not very immunogenic (4). This type of adjuvant activity is mediated by activating toll-like receptors (TLR), found on such cells as macrophages and leukocytes, which trigger danger signals that activate the immune system (60). In particular, CpG motifs, derived from bacteria, were discovered when a correlation was made between bacterial DNA containing many CpG motifs and adjuvant activity by the bacteria (39). CpG motifs are dinucleotides which occur in the order of cytosine-guanosine and are surrounded by two purines upstream and two pyrimidines downstream (39). These dinucleotide sequences are naturally found in bacteria in unmethylated form, in contrast to vertebrate CpGs, which are methylated (39). When a vertebrate host's innate immune system detects this unmethylated DNA, an immune response is triggered, resulting in increased B cell proliferation and antibody production (39). When synthetic CpG deoxynucleotides have been coadministered with antigenic

proteins, an enhanced immune response, including antibody production, has occurred (81).

A second group of adjuvants are liposomes, which are synthetic spheres comprised of lipid layers which encapsidate Ags and act as vaccine delivery vehicles and as adjuvants (57). Enhancing immune responses to DNA vaccines may depend on increasing the transfection of cells using different cationic liposomes such as Lipofectin or Lipfectamine 2000. Cationic liposomes are known to enhance the uptake of plasmids by the cells by promoting the release of plasmids from endosomes to the cytoplasm, and by protecting the DNA from degradation by DNases found in the interstitial fluid (61). The adjuvantal efficacy of liposomes depends on lipid layers, the electric charge, the composition, and the method of preparation (57). Liposomes enhance the humoral immunity to protein Ags by increasing the half-life of Ags in the blood, which ensures an increased exposure to antigen presenting cells following vaccination (3, 27).

Finally, cytokines are naturally derived, small molecular weight proteins that play a crucial role in controlling the immune system and are produced in response to infection (4). Their potent activities work in an autocrine, paracrine, or endocrine manner to activate or inhibit immune responses (1). The use of recombinant cytokines has been shown to be effective vaccine adjuvants when coadministered with the vaccine (49, 64). Cytokines have been coadministered both in protein form and in plasmid DNA (12,47, 49, 50, 64, 83). They play a major role in the stimulation of primary immune responses by providing signals between the different types of cells (83). One study involving the coadministration of GM-CSF-encoding plasmid DNA with antigen-encoding plasmids have suggested that the efficacy of such vaccines could be improved (83). Recently,

various chicken cytokine genes have been cloned and characterized, including IFN-1, IL-10, and IL-2 (62, 65, 74). When incorporated into DNA plasmid vectors, these cytokines were able to elicit adjuvant activities when coadministered with proteins encoded for within DNA plasmids (49, 64, 83).

Type I interferon (IFN-1) is a group of cytokines, interferon-α (IFNα) and interferon-β (IFNβ) most noted for their antiviral activity (1) and their ability to enhance primary antibody responses (58, 64). These cytokines are constitutively expressed at low levels in primary lymphoid organs but following viral infection, are induced to high levels (7, 42). Previous studies have shown that exogenous IFN-I had strong adjuvant activity by enhancing an Ab response against a poorly immunogenic soluble protein antigen, chicken gamma globulin (42). It also augmented the production of various subclasses of immunoglobulin G (IgG) during the primary antibody response and induced long-term Ab production and immunological memory after one injection of soluble protein antigen (42). In addition, it has been shown to educe higher titers of IgG2a, promoting a Th1 response when coadministered with vaccines (58). Mo et al. (2001) demonstrated experimentally that recombinant chicken IFN α ameliorated Infectious Bursal Disease Virus (IBDV) and Newcastle Disease Virus (NDV) infections when the cytokine was administered following infection. This supported the supposition that IFN-1 is necessary for the resolution of viral infections that are controlled by antibodies (7).

Interleukin 10 (IL-10), like IFN-1, is also a multifunctional cytokine (51). It was originally called cytokine synthesis inhibitory factor (CSIF) (25) because of its ability to inhibit activation and effector functions of T cells, monocytes, and macrophages (51). It also limits and ultimately terminates inflammatory responses induced by Th1-related

cytokines (1, 51). It is now known to enhance humoral immunity (11, 21, 25, 30) and regulates the growth and differentiation of B cells (21, 30). Choi et al. demonstrated that the long term culture of B cells stimulated by anti-CD40 or activated T cells or follicular dendritic cells (FDCs) and IL-10 resulted in the differentiation of B cells into plasma cells (instead of memory cells). A separate study done by Go et al. demonstrated that B cells from unstimulated mice had increased expression in MHC II Ags and increased viability when cultured with recombinant IL-10.

Interleukin-2 (IL-2) is a cytokine produced by activated CD4+ T cells as well as DCs which acts as a growth factor and a stimulus for Ab synthesis in B cells (1, 26). It also acts on T and natural killer (NK) cells and is important in adaptive immune responses and induces Th-1 responses (1,4). When a plasmid encoding IL-2 was coadministered with a plasmid encoding an IBDV Ag, a significant increase in IBDV-specific neutralizing Abs was seen when compared to the response to the vaccine alone (47). Coadministration of IL-2 plasmid also induced Ab production up to two weeks earlier than the vaccine alone (47). Chow (et al.) also found that when the IL-2 gene was coexpressed with Hepatitis B surface Ag, humoral immunity was enhanced. An increase in Th-1 cells was also detected in splenocytes from mice vaccinated with the IL-2-coexpressing plasmids (12).

Another approach to improving DNA vaccine efficiency involves the delivery systems or routes taken to administer them to vaccinates (5). Intramuscular (im) injection is the most common route of administration used (33). It is based on transfection of a limited number of muscle fibers, resulting in the expression of the transgenic DNA for prolonged time periods due to the lack of turnover of muscle fibers (56). The drawback

is that large quantities of purified plasmid are required and there is a risk of autoimmunity (31). On the other hand, intradermal inoculation, typically with a gene gun, has been shown to elicit an immune response equal or superior to that educed by im vaccination, with as much as 100- to 1000-fold less DNA (61). More APCs, primarily dendritic cells (Langerhans cells), are present in the skin than in muscle (61). These dendritic cells (DCs) are important for initiating the immune response via their ability to efficiently present Ag (61). One possible means to enhance the immune response to DNA vaccines may be by intradermal delivery by other means. Various “needle free” devices to deliver antigen intradermally (id), subcutaneously (subQ), or im are being tested. These may have commercial applications to id DNA vaccination. This would allow for a more economical, yet efficient approach to vaccination by targeting the DCs in the skin.

Though the above approaches have been tested to some degree, no research thus far has been tried to assess the efficacy of DNA vaccines in enhancing the Ab response in chickens using AI HA as a model Ag. By using DNA plasmid containing an HA gene for the antigenic protein, we were able to find a way to enhance the antibody response.

References

1. Abbas A. K. and A. H. Lichtman. 2003. Cellular and Molecular Immunology. Independence Square West. Philadelphia, PA.

2. Alexander D. J. 2000. A review of avian influenza in different bird species. Veterinary Microbiology. 74:3-13.

3. Allison A. C., and G. Gregoriadis. 1974. Liposomes as immunological adjuvants. Nature. 252: 252-258.

4. Asif M., K. A. Jenkins, L. S. Hilton, W. G. Kimpton, A. G. G. Bean, and J. W. Lowenthal. 2004. Cytokines as adjuvants for avian vaccines. Immunology and Cell Biology. 82:638-643.

5. Babiuk L. A., R. Pontarollo, S. Babiuk, B. Loehr, S. van Drunen Little-van den Hurk. 2003. Induction of immune responses by DNA vaccines in large animals. Vaccine. 21:649-658.

6. Bracci L., I. Canini, M. Venditti, M. Spada, S. Puzelli, I. Donatelli, F. Belardelli, and E. Proietti. 2005. Type I IFN as a vaccine adjuvant for both systemic and mucosal vaccination against influenza virus. Vaccine. In Press.

7. Braun D., I. Caramalho, and J. Demengeot. 2002. IFN-α/β enhances BCR-dependent B cell responses. International Immunology. 14:411-419.

8. Capua I. and S. Marangon. 2003. The use of vaccination as an option for the control of avian influenza. OIE. 71st General Session. p. 1-10.

9. Capua, I. and D. Alexander. 2004. Avian Influenza: recent developments. Avian Pathology. 3:393-404.

10. Chen W., P. A. Calvo, D. Malid, J. Gibbs, U. Schubert, I. Bacik, S. Basta, R. O'Neill, J. Schickli, P. Palese, P. Henklein, J. R. Bennink, and J. W. Yewdell. 2001. A novel influenza A virus mitochondrial protein that induces cell death. Nature Medicine. 7: 1306-1312.

11. Choi. J., and Y. S. Choi. 1998. IL-10 interrupts memory B cell expansion in the germinal center by inducing differentiation into plasma cells. Eur. J. Immunol. 28:508-515.

12. Chow Y., W. Huang, W. Chi, Y. chu, and M. Tao. 1997. Improvement of hepatitis B virus DNA vaccines by plasmids coexpressing hepatitis B surface antigen and interleukin-2. J. Virology. 71:169-178.

13. Cross K. J., L. M. Burleigh, and D. A. Steinhauer. 2001. Mechanisms of cell entry by influenza virus. *In* Expert reviews in molecular medicine. Cambridge University Press, 1-18.

14. Crawford J. M. , M. Garcia, H. Stone, D. Swayne, R. Slemons, and M. L. Perdue. 1998. Molecular characterization of the hemagglutinin gene and oral immunization with a waterfowl-origin avian influenza virus. Avian Diseases. 42:486-496.

15. Crawford J., B. Wilkinson, A. Vosnesensky, G. Smith, M. Garcia, H. Stone, and M. Perdue. 1999. Baculovirus-derived hemagglutinin vaccines protect against lethal influenza infections by avian H5 and H7 subtypes. Vaccine. 17:2265-2274.

16. Davis A.R., T.J. Bos, and D.P. Nayak. 1983. Active influenza virus neuraminidase is expressed in monkey cells from cDNA clones in simian virus 40 vectors. Proc. Natl. Acad. Sci.80:3976-3980.

17. Easterday B. C.1975. p. 449-480. *In* E. D. Kilbourne (ed.), The Influenza Viruses and Influenza. Academic Press, New York, NY.

18. FAO. 2004. FAO Recommendations on the Prevention, Control and eradication of highly pathogenic avian influenza (HPAI) in Asia. FAO Position Paper.

19. Feltquate D. M., S. Heaney, R. G. Webster, and H. L. Robinson. 1997. Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. J. Immunol. 158:2278-2284.

20. Fenner, F., P. A. Bachman, E. P. J. Gibbs, F. A. Murphy, M. J. Studdert, and D. O. White. 1975. Orthomyxoviridae. p. 473-484. *In* Veterinary Virology. Academic Press, New York, NY.

21. Fickenscher H., S. Hor, H. Kupers, A. Knappe, S. Wittmann, and H. Sticht. 2002. The interlukin-10 family of cytokines. Trends in immunology. 23:89-96.

22. Flint S. J., L. W. Enquist, R. M. Krug, V. R. Racaniello, A. M. Skalka. 2000. Principles of virology: molecular biology, pathogenesis, and control. ASM Press. Washington D.C.

23. Fouchier R. A. M. , V. Munster, A. Wallensten, T. M. Bestebroer, S. Herfst, D. Smith, G. F. Rimmelzwaan, B. Olsen, and A. D. M. E. Osterhaus. 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J. Virol. 79:2814-2822.

24. Fynam E. F., R. G. Webster, D. H. Fuller, J. R. Haynes, J. C. Sanoro, and H. L. Robinson. 1995. DNAvaccines: a novel approach to immunization. Int. J. Immunopharmac. 17:79-83.

25. Go, N.F., B. E. Castle, R. Barrett, R. Kastelein, W. Dang, T. R. Mosmann, D. W. Moore, and M. Howard. 1990. Interleukin 10, a novel B cell stimulatory factor: Unresponsiveness of X chromosome-linked immunodeficiency B cells. J. Exp. Med. 172:1625-1631.

26. Granucci F., D. Andrews, M. A. Degli-Esposti, and P. Ricciardi-Castagnoli. IL-2 mediated adjuvant effect of dendritic cells. Trends in Immunology. 23:169-171.

27. Gursel, M., S. Tunca, M. Ozkan, G. Ozcengiz, and G. Alaeddinoglu. 1999. Immunoadjuvant action of plasmid DNA in liposomes. Vaccine. 17: 1376-1383.

28. Harvey R., A. C. R. Martin, M. Zambon, and W. S. Barclay. 2004. Restrictions to the Adaptation of Influenza A Virus H5 Hemagglutinin to the Human Host. J. Virol. 78:502-507.

29. Hatada E. and R. Fukuda. 1992. Binding of influenza A virus NS1 protein to ds RNA in vitro. J. Gen. Virol. 73: 3325-3329.

30. Haygreen, L., F. Davison, P. Kaiser. 2005. DNA Vaccines for poultry: the jump from theory to practice. Expert Rev. Vaccines. 4:51-62.

31. Henke A. 2002. DNA immunization- a new chance in vaccine research? Med. Microbiol. Immunol. 191:187-190.

32. Horimoto, T., and Y. Kawaoka. 2001. Pandemic threat posed by avian influenza A viruses. Clinical Microbiology Reviews. 14: 129-149.

33. Hulse D. J., and C. H. Romero. 2004. Partial protection against infectious bursal disease virus through DNA-mediated vaccination with the VP2 capsid protein and chicken IL-2 genes. Vaccine. 22:1249-1259.

34. Humiston S. G., D. A. Pappano. 2004. Influenza and influenza vaccination. Emergency medicine reports. 25:321-332.

35. Hunt L. A., D. W. Brown, H. L. Robinson, C. W. Naeve, and R. G. Webster. 1988. Retrovirus-expressed hemaglutinin protects against lethal influenza virus infection. J. Virol. 62:3014-3019.

36. Jacob, J.P., G. D. Butchner, F. B. Mather, and R. D. Miles. 1998. Avian influenza in poultry. p. 1-4. *In* University of Florida Extension Service, Institue of Food and Agriculatural Sciences, University of Florida.

37. Kaiser P. 2002. Turkey and chidken IL-1β share high sequence identidty, but have different poly A sites in their 3' UTR. Dev. Comp. Immunol. 26:681-7.

38. Kodihalli S., D. L. Kobasa, and R. G. Webster. 2000. Strategies for inducing protection against avian influenza A virus subtypes with DNA vaccines. Vaccine. 18:2592-2599.

39. Krieg A., A.K. Yi, J. Schorr, and H.L. Davis. 1998. The role of CpG dinucleotides in DNA vaccines. Trends Mivrobiol. 6:23-7.

40. Lamb, R. A., and R. M. Krug. 1998. Orthomyxoviridae: the viruses and their replication. P. 1353-1395. *In* D. M. Knipe, P. M. Howley (ed.), Fields Virology, third ed. Lippincott Williams & Wilkins, Philadelphia, Penn.

41. Lamb, R. A., and R. M. Krug. 2001. Orthomyxoviridae: the viruses and their replication. p. 1487-1532. *In* D. M. Knipe, P. M. Howley (ed.), Fields Virology, fourth ed. Lippincott Williams & Wilkins, Philadelphia, Penn.

42. Le Bon A., G. Schiaboni, G. D'Agostino, I. Gresser, F. Belardelli, and D. F. Tough. 2001. Type I interferons potently enhance humoral immunity and can promote isotype switching by stimulating dendritic cells *in vivo*. Immunity. 14:461-470.

43. Lee C. W., D. E. Swayne, J. A. Linares, D. A. Senne, and D. L. Suarez. 2005. H5N2 avian influenza outbreak in Texas in 2004: the first highly pathogenic strain in the United States in 20 years? In press.

44. Lee C.W., D.A. Senne, and D.L. Suarez. 2004. Generation of reassortant influenza vaccines by reverse genetics that allows utilization of a DIVA (Differentiating Infected from Vaccinated Animals) strategy for the control of avian influenza. Vaccine. 22: 3175-3181.

45. Lee C.W., D. A. Senne, J. A. Linares, P. R. Woolcock, D. E. Stallknecht, E. Spackman, D. E. Swayne, and D. L. Suarez. 2004. Characterization of recent H5 subype avian influenza viruses from US poultry. Avian Pathology. 33:288-297.

46. Lee C.W., D.A. Senne, and D.L. Suarez. 2003. Development of hemagglutinin subtype-specific reference antisera by DNA vaccination of chickens. Avian Diseases. 47:1051-1056.

47. Li J., X. Liang, Y. Huang, S. meng, R. Xie, R. Deng, and L. Yu. 2004. Enhancement of the immunogenicity of DNA vaccine against infectious bursal disease virus by co-delivery with plasmid encoding chicken interleukin-2. Virology. 329:89-100.

48. Lu Y., M. Wambach, M. G. Katze, and R. M. Krug. 1995. Binding of the influenza virus NS1 protein to double-stranded RNA inhibits the activation of the protein kinase that phospyorylated the ilf-2 translation initiation factor. Virology. 214: 222-228.

49. Min W., H. S. Lillehoj, J. Burnside, D. C. Weining, P. Staeheli, and J. Zhu. 2002. Adjuvant effects of IL-1β, IL-2, IL-8, IL-15, IFN-α, IFN-γ, TGF-β4, and lymphotactin on DNA vaccination against *Eimeria acervulina.* Vaccine. 20:267-274.

50. Mo C. W., Y. C. Cao, and B. L. Lim. 2001. The in vivo and in vitro effects of chicken interferon α on infectious bursal disease virus and Newcastle disease virus infection. Avian Diseases. 45:389-399.

51. Moore K. W., R. de Waal Malefyt, R. L. Coffman, and A. O'Garra. 2001. Interleukin-10 and the interleukin-10 Receptor. Annu. Rev. Immunol. 19:683-763.

52. Naeve C. W., D. Williams. 1990. Fatty acids on the A/Japan/305/57 influenza virus hemagglutinin have a role in membrane fusion. *EMBO J.* 9: 3857-3866.

53. Naim H.Y. and M.G. 1993. Roth. Basis for selective incorporation of glycoproteins into the influenza virus envelope. J. Virol. 67:4831-4841.

54. Nayak D. P., E. K. Hui, and S. Barman. 2004. Assembly and budding of influenza virus. Virus Research. 106:147-165.

55. OIE. 2004. Highly pathogenic avian influenza. *In* Manual of diagnostic tests and vaccines for terrestrial animals, 5th edition.

56. Pertmer T. M., T. R. Roberts, and J. R. Haynes. 1996. Influenza virus nucleoprotein-specific immunoglobulin G subclass and cytokine responses elicited by DNA vaccination are dependent on the route of vector DNA delivery. J. Virology. 70:6119-6125.

57. Petrovsky N., and J. C. Aguilar. 2004. Vaccine adjuvants: current state and future trends. Immunl and Cell Biol. 82:488-496.

58. Proietti E., L. Bracci, S. Puzelli, T. Di Pucchio, P. Sestili, E. De Vincenzi, M. Venditti, I. Capone, I. Seif, E. De Maeyer, D. Tough, I. Donatelli, and F. Belardelli. 2002. Type I IFN as a natural adjuvant for a protective immune response: Lessons from the influenza vaccine model. J. of Immunol. 169:375-383.

59. Qiao C., K Yu, Y Jiang, Y Jia, G Tian, M. Liu, G. Deng, X. Wang, Q. Meng, and X. Tang. 2003. Protection of chickens against highly lethal H5N1 and H7N1 avian influenza viruses with a recombinant fowlpox virus co-expressing H5 haemagglutinin and N1 neuraminidase genes. Avian Pathology. 32:25-31.

60. Rami A. D., H. S. Lillehoj, M. Okamura, H. Xie, W. Min, X. Ding, and R. A. Heckert. 2004. *In vivo* effects of CpG oligodeoxynucleotide on *Eimeria* infection in chickens. Avian Diseases. 48: 783-790.

61. Robinson H. L., and C. A. T. Torres. 1997. DNA vaccines. seminars in immunology. 9:271-283.

62. Rothwell L., J. R. Young, R. Zoorob, C. A. Whittaker, P. Hesketh, A. Archer, A. L. Smithe, and P. Kaiser. 2004. Cloning and characterization of chicken IL-10 and its role in the immune response to *Eimeria maxima*. The Journal of Immunology. 173:2675-2681.

63. Saito T. and I. Yamaguchi. 2000. Effect of glycosylation and glucose trimming inhibitors on the influenza A virus glycoproteins. J. Vet. Med. Sci. 62:575-581.

64. Schijns V. E. C. J., K. C. Weining, P. Nuijten, E. O. Rijke, and P. Staeheli. 2000. Immunoadjuvant activities of *E. coli*- and plasmid-expressed recombinant chicken IFN-α/β, IFN-γ, and IL-1β in 1-day- and 3-week-old chickens. Vaccine. 18:2147-2154.

65. Sekellick M. J., A. F. Ferrandino, D. A. Hopkins, and P. I. Marcus. 1994. Chicken interferon gene: cloning, expression, and analysis. Journal of Interferon Research. 14:71-79.

66. Skehel J. J., and D. C. Wiley. 2000. Receptor binding and membrane fusion in virus entry: The influenza hemagglutinin. Annu Rev. Biochem. 69:531-569.

67. Spackman E., D.A. Senne, T.J. Myers, L.L. Bulage, L.P. Garber, M.L. Perdue, K. Lohman, L.T. Daum, and D.L. Suarez. 2002. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J. Clin. Microbiol. 40:3256-3260.

68. Steinhauer D. A. 1999. Role of hemagglutinin cleavage for the pathogenicity of influenza virus. Virology. 258:1-20.

69. Stubbs E. L.1948. Fowl Pest. p. 603-614. *In* H. E. Biester, L.H. Schwarte (ed.), Diseases of Poultry. The Iowa State University Press, Ames, Ia.

70. Suarez D.L. and M.L. Perdue. 1998. Multiple alignment comparison of the non-structural genes of influenza A viruses. Virus Research. 54: 59-69.

71. Suarez D. L. and S. Schultz-Cherry. 2000a. Immunology of avian influenza virus: a review. Developmental and Comparative Immunology. 24: 269-283.

72. Suarez D. L. and S. Schultz-Cherry. 2000b. The effect of eukaryotic expression vectors and adjuvants on DNA vaccines in chickens using an avian influenza model. Avian Diseases. 44:861-868.

73. Suarez, D. L. and D. E. Senne. 2000c. Sequence analysis of related low-pathogenic and highly pathogenic H5N2 avian influenza isolates from United States live bird markets and poultry farms from 1983-1989. Avian Diseases. 44:356-364.

74. Sundick R. S., and C. Gill-Dixon. 1997. A cloned chicken lymphokine homologous to both mammalian IL-2 and IL-15. J Immunol. 159:720-725.

75. Swayne D. E. 2003a. Vaccines for List A Poultry Diseases: Emphasis on avian influenza. Dev. Biol. Basel. 114:201-212.

76. Swayne, D. E., and D. A. Halvorson. 2003b. Influenza. p. 135-160. *In* Y. M. Said, H. J. Barnes, J. R. Glisson, A. M. Fadly, L. R. McDougald, D. E. Swayne (ed.), Diseases of Poultry. Iowa State University Press, Ames, Ia.

77. Swayne D. E., M. Garcia, J. R. Beck, N. Kinney, and D. L. Suarez. 2000. Protection against diverse highly pathogenic H5 avian influenza viruses in chickens immunized with a recombinant fowlpox vaccine containing an H5 avian influenza hemagglutinin gene insert. Vaccine. 18:1088-1095.

78. Tumpey, T.M., R. Alvarez, D.E. Swayne, and D.L. Suarez. 2005. Diagnostic approach for differentiating infected from vaccinated poultry on the basis of antibodies to NS1, the nonstructural protein of influenza A virus. J. Clin. Microbiol. 43: 676-683.

79. Ulmer, J. B., J. J. Donnelly, S. E. Parker, G. H. Rhodes, P. L. Felgner, V. J. Dwarki, S. H. Gromkowski, R. R. Deck, C. M. DeWitt, A. Friedman, L. A. Hawe, K. R. Leander, D. Martinez, H. C. Perry, J. W. Shiver, D. L. Montgomery, and M. A. Liu. 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science. 259:1745-1749.

80. Ulmer, J. B., T. M. Fu, R. R. Deck, A. Friedman, L. Guan, C. DeWitt, X Liu, S. Wang, M. A. Liu, J. J. Donnelly, and M. J. Caulfield. 1998. Protective $CD4^+$ and $CD8^+$ T cells against influenza virus induced by vaccination with nucleoprotein DNA. J. Virol. 72:5648-5653.

81. Vleugels B, C. Ververken, and B.M. Goddeeris. 2002. Stimulatory effect of CpG sequences on humoral responses in chickens. Poultry Science. 81:1317-1321.

82. WHO. 2002. Surveillance for Influenza. WHO Manual on Animal Influenza Diagnosis and Surveillance.

83. Xiang Z. and H. C. J. Erti. 1995. Manipulation of the immune response to a plasmid-encoded viral antigen by coinculation with plasmids expressing cytokines. Immunity. 2:129-135.

84. Yankauckas M.A., J.E. Morrow, S.E. Parker, A. Abai, G.H. Rhodes, V.J. Dwarki, and S.H. Gromkowski. 1993. Long-term anti-nucleoprotein cellular and humoral immunity is induced by intramuscular injection of plasmmid DNA containing NP gene. CAN Cell Biol. 9:771-776.

CHAPTER 3

ENHANCING DNA VACCINE-INDUCED AVIAN INFLUENZA HEMAGGLUTININ SUBTYPE-SPECIFIC REFERENCE ANTISERA PRODUCTION[1]

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ABSTRACT. Various methods of enhancing DNA vaccine were tested. The genes of two different HA subtypes, H5 or H7, were encoded separately in a eukaryotic expression vector (EEV) and were coadministered with one of various adjuvants including CpG oligodeoxynucleotides, cationic lipids, or various chicken cytokines. The genes for chicken IFN-1 (IFN), interleukin-10 (IL-10), and interleukin-2 (IL-2) were cloned individually into EEVs and were coadministered with HA plasmids. Specific pathogen free (SPF) chickens between the ages of two and a half and four weeks were vaccinated intramuscularly with 100 μg HA plasmid, and boosted up to two times at monthly intervals. The hemagglutination inhibition (HI) test was used to evaluate Ab titers. In general, most adjuvants improved the H7-specific Ab response, particularly the IFN and IL-2, as determined by the HI test. However, none of the approaches had a significant effect on H5-specific antibody responses. Various routes of inoculation of the H5 DNA vaccine, including intramuscular (im), intradermal (id), and subcutaneous (subQ), were also evaluated. The im route provided the best results, but the id route of delivery also induced a good response that was dose dependant. Compared to the conventional subcutaneous route using whole inactivated virus vaccines, DNA vaccines are a feasible alternative means of producing HA subtype-specific diagnostic reference antisera. We were able to enhance this production by several different approaches, making it a more viable option.

INTRODUCTION

Avian influenza (AI) virus is a type A influenza virus in the family *Orthomyxoviridae* (Fields). Type A influenza viruses are further subtyped based on expression of surface glycoproteins, hemagglutinin (HA), and neuraminidase (NA), of which there are sixteen and nine subtypes, respectively (7, 29). The natural reservoirs of type A influenza are wild waterfowl and shorebirds, which are typically asymptomatic when infected and AI only replicates in the intestinal tract (29). Poultry are considered to be aberrant hosts (27). AI has two phenotypes in domestic poultry (*i.e*. chickens); clinical signs of low pathogenic (LP) AI may not be apparent but include respiratory disease and decreased egg production(29). Conversely, highly pathogenic (HP) AI disease causes a serious disease that may result in acute death of birds as early as 24 hours after exposure (29). HPAI disease is a reportable List A disease according to the Office International des Epizooties (OIE) (17). Only the H5 and H7 subtypes have been associated with the HPAI phenotype in chickens, however not all H5 or H7 subtypes result in the HP form of disease (29). If LPAI enters a flock, it has the potential to mutate and become more virulent (26) which could have severe economic effects on the poultry industry (29). Hence, the identification of HPAI is necessary to ensure that poultry being exported out of the country are free of infection (26). In addition, HPAI H5N1 strain has caused disease in humans (3).

Because both LPAI and HPAI can have devastating effects on chickens and may also infect humans, identification is essential. One means of subtyping AI isolates is by the hemagglutination inhibition (HI) test using a panel of reference antisera against all 16 HA subtypes. Typically, polyclonal hemagglutinin-specific antibodies (Ab) are produced

by the vaccinating chickens with whole, inactivated virus. This method produces antibody not only to the hemagglutinin protein, but also to other influenza proteins including the neuraminidase protein. We have previously demonstrated the use of DNA vaccines for producing HA subtype-specific reference antisera (11). This method provides two advantages for producing reference sera. First, DNA vaccines induce antivbodies only to the HA and none against other viral proteins. Second, since DNA vaccines use plasmid DNA, you do not need to work with live influenza viruses. However, not all HA subtypes elicit the same level of antibody. Also, there is still room for improving the effectiveness of DNA vaccines as well as decreasing the cost and large amounts of DNA required to elicit an immune response. In this study, we tested the efficacy of different adjuvants and vaccine delivery routes in order to increase Ab production induced by DNA vaccines for diagnostic purposes. Potential adjuvants as well as delivery routes will be discussed.

MATERIALS & METHODS

Chickens. Specific-pathogen-free (SPF) white leghorn chickens between the ages of 2 ½ and 4 weeks were used. The animals received food and water *ad libitum* and were maintained in a biosafety level 2 facility at the Southeast Poultry Research Laboratory, Athens, GA.

DNA preparation.

Plasmid DNA containing antigen-encoding sequences. The H7 gene was subcloned from the pAMP vector (Life Technologies, Gaithersburg, MD) by digestion with restriction

enzymes Mlu I and Sal I into the pCI eukaryotic expression vector (EEV) (Promega, Madison, WI), containing the cytomegalovirus (CMV) immediate early promoter. The coding sequence of the H5 gene, already inserted into pCI (kind gift from David Suarez), and H7 were confirmed by DNA sequencing. In addition, each EEV was tested in cell culture to confirm expression of the HA protein (76). Briefly, 90% confluent 293-T cells were transfected with 1 μg of each EEV using Lipofectamine 2000 transfection reagent (Invitrogen). Cells were incubated 24 hrs at 37°C before fixing them with 2% paraformaldehyde (Sigma, St. Louis, MO) and reacted with either H5 or H7 subtype-specific antisera obtained from vaccinated chickens. After fluorescein isothiocyanate-conjugated anti-chicken IgG (Sigma) was added, the cells were observed for fluoresecence. EEVs used for vaccination were prepared either by using Qiagen Mega Prep plasmid purification kit (Qiagen Inc., Valenci, CA) or by a commercial company (Aldevron, Fargo, ND).

Plasmid DNA containing CpG motif-flanked antigen coding sequences. Primers specific for H5 or H7 flanked by a CpG motif (*e.g.* 5'AACGTT 3') (8) at the 5' and 3' ends were designed. The 5' end of both HA primers contained the Mlu I restriction site and the 3' end of reverse HA primers contained the Sal I restriction site. The CpG-H5 primer set is as follows: (CpG-H5 +primer) 5'AGCTACGCGTAACGTTATGGAAAGAATAGTGATT 3' and (CpG-H5 -primer) 5'AGCTGTCGACAACGTTCTAGATGCAAATTCTGCATT 3'. The CpG-H7 + and – primers are 5'AGCTACGCGTAACGTTATGAACACTCAAATTCTGGCA 3' and 5'AGCTGTCGACAACGTTTTTTCTCAAACTATATACA 3', respectively. PCR was

used to amplify the H5 or H7 sequences contained in the above mentioned pCI EEVs. Their sequences were confirmed as above, by DNA sequencing and indirect immunofluorescent antibody (I-IFA) staining.

Cloning of chicken cytokines.

IFN. Chicken embryo fibroblast (CEF) cells were produced using 10-day-old embryonated eggs to seed 6-well plates. After 4 days of growth, DNA was extracted using Trizol LS reagent following the manufacturer's instructions (Life Technologies Inc., Grand Island, NY). Primers containing the Mlu I site or the Xba I site in the 5' or 3' primer, respectively, were designed according to the IFN-1 coding sequence determined by Sekellick et al.(1994) to amplify IFN-1 cDNA. The + IFN-1 and – IFN-1 primers used were: 5' AGCTACGCGTATGGCTGTGCCTGCAAGCCCA 3' and 5' AGCTTCTAGACTAAGTGCGCGTGTTGCCTG 3', respectively. A predicted 602 base pair (bp) fragment was amplified using 50 μl PCR reactions. The protocol consisted of 2 min. predenaturation at 94°C, followed by 30 cycles of 45s at 94°C for denaturation, 15s at 56°C for annealing, 1 min. at 72 °C for elongation, and finally, a single 5 min. elongation step at 72°C.

Restriction digestion of the PCR product by Mlu I and Xba I was performed and the resulting fragment was ligated into Mlu I / Xba I-digested pCI using T4 DNA ligase (New England Biolabs, Ipswich, MA). DNA sequencing was used to determine correct incorporation of IFN into pCI. Expression of mRNA was confirmed by transfecting 4 μg plasmid DNA into 6-well plates of CEFs, extracting the cellular mRNA using Qiagen Oligotex mRNA Kit, and performing RT-PCR for IFN. A 602 base pair product was

noted in transfected cells. Some control nontransfected cells displayed faint bands, most likely from RNA, already intrinsically present.

IL-10 and IL-2. Tissue samples of the thymus, cecal tonsils, and bursa of Fabricious were obtained from a 3-week-old SPF chicken and frozen on dry ice until transferred to a -70°C freezer. Each different tissue sample was pulverized and RNA was extracted. Briefly, tissue was removed from the freezer and immediately set into a sterile mortar containing liquid nitrogen and sand. It is necessary to keep the sample from thawing. The tissue was ground with the pestle until liquid nitrogen evaporated, whereupon it was scraped into a sterile 1.5 ml microfuge tube containing 750 µl Trizol LS reagent. The standard protocol was followed, according to the manufacturer's instructions.

Qiagen 1-step RT-PCR was performed on extracted RNA using 25 µl reactions. Primers specifically designed for either chicken IL-10 or IL-2 sequences (20, 23, respectively) were used. The forward and reverse sequences contained the Mlu I or Xba I site, respectively. The forward and reverse IL-10 primer sequences were as follows, respectively: 5' AGCTACGCGTATGCAGACCTGCTGCCAA 3' and 5'AGCTTCTAGATCACTTCCTCCTCCTCATCA 3'. The forward and reverse IL-2 primer sequences were: 5' AGCTACGCGTATGATGTGCAAAGTACTGAT 3' and 5' AGCGCTTCTAGATTATTTTTGCAGATATCTCA 3', respectively. The RT-PCR program consisted of reverse transcription at 50°C for 30 min., an initial PCR activation step at 95°C for 15 min., followed by PCR consisting of 32 cycles at 94°C for 45 s, 53°C for 30s, 72°C for 1:30 min., and an elongation step at 72°C for 5 min. Sequences of 528

and 432-bp amplified PCR products, corresponding to the IL-10 and IL-2 genes, respectively, were confirmed and restriction digestion was carried out as with IFN cDNA. Both the IL-10 and IL-2 gene sequences were ligated into the pCI EEV. These EEVs for both cytokines used in vaccination were prepared by Aldevron.

CpG oligodeoxynucleotides. Fifteen base pair oligonucleotides containing either CpG (experimental group) or GpC (negative CpG control) were obtained from Integrated DNA Technologies (Coralville, IA). The sequences of the CpG and GpC oligos used were: 5'GCTAGACGTTAGC*G*T 3' and 5'GCTAGAGCTTAGG*C*T 3', respectively (31). Asterisks denote phosphorothioate bonds between bases , which were added to increase the stability of the oligos in vivo.

Transfection reagents. For every 100 μg DNA injected into birds, 20 μl Lipofectin (Life Technologies, Gaithersburg, MD) transfection reagent was used (25). Briefly, Lipofectin was added to sterile phosphate-buffered saline (PBS) (100 μl PBS for every 20 μl Lipofectin used) and incubated for 35 min. at room temperature to allow for liposome formation. Plasmid DNA was diluted into PBS (enough to bring the final volume of each dose up to 0.4 ml), mixed with the liposome, and left standing at room temperature for 15 min. before vaccination. Volumes of Lipofectin were adjusted according to the amounts of DNA used. Lipofectamine 2000 was prepared in volumes equivalent to Lipofectin. However, upon addition of Lipofectamine 2000 to PBS, a five min. incubation time elapsed before mixing with plasmid DNA. The Lipofectamine 2000 and DNA mixture was left standing 20 min. before inoculation. GeneJammer reagent

(Stratagene , La Jolla, CA) was prepared by adding 30 µl for every 100 µg plasmid DNA used. This volume was consistent with the transfection reagent: DNA ratios of the other reagents used. GeneJammer was added to PBS and allowed to sit at room temperature for 10 min. Next, DNA was added to and mixed with the GeneJammer. A 10 min. incubation period elapsed before inoculation. All vaccines were maintained at room temperature until administered to birds.

Oil emulsion vaccine preparation. A/TK/WI/68 (H5N9) virus was grown in 10-day-old-embryonating chicken eggs, and allantoic fluid was pooled. The hemagglutination titer of 512 was determined prior to inactivation with 0.1% β-propiolactone (Sigma, St. Louis, MO) by using the hemagglutination test. Oil emulsion vaccine preparation was followed according to a previous protocol (24). Briefly, one part aqueous antigen (10 ml) was emulsified in four parts (40 ml) oil phase. The oil phase consisted of 36 ml Drakeol 6 VR mineral oil (Penreco, Burler, PA), 3 ml 7.5% sorbitan monooleate (Arlacel 80, ICI United States, Inc., Wilmington, DE), and 1 ml 2.5% polysorbate (Tween 80, ICI United States, Inc.).

Vaccination.

Experiment 1.

Adjuvant effects of CpG motif, Lipofectamine 2000, and IFN.

Seventy-seven four-week-old specific pathogen free (SPF) chickens were divided into two groups, receiving pCI-containing H5 or H7 genes (H5 or H7 plasmids), respectively. These H5 and H7 groups were further divided into five and six groups, respectively, with

seven birds in each group (Table 1). All of these vaccines were prepared using Lipofectin, unless otherwise specified. The H5 groups all received 100 μg plasmid DNA. In addition to the H5 plasmids, CpG oligos, GpC oligos, and IFN plasmids were coadministered to groups 2-4, respectively. Group 5 received H5 plasmids in Lipofectamine 2000. H7 groups 6-10 received H7, along with CpG oligos, GpC oligos, IFN plasmids, or in Lipofectamine 2000, respectively. Group 11 received 100 μg of the CpG motif-flanked H7 plasmids (CpG-enriched plasmids) in Lipofectin. Birds were inoculated intramuscularly with 0.2 ml in each breast muscle. Serum samples were taken beginning two weeks after initial vaccination. All birds were boostered once, one month following initial vaccination.

Experiment 2.

Comparing efficacy of various routes of vaccine delivery.

Eighty-one two and a half week old SPF chickens were divided into nine groups of nine birds per group. All DNA plasmids used for this experiment contained the H5 gene and were administered in Lipofectin in doses of 100 μg per bird, unless otherwise stated. Birds receiving oil emulsion vaccines were injected with 0.5 ml either in the nape of the neck or in the inguinal region. Each group was vaccinated as follows: group 1 received oil emulsion vaccine subcutaneously, group 2 received oil emulsion vaccine intradermally, group 3 received H5 plasmids in GeneJammer transfection reagent, intramuscularly, group 4 received the CpG-enhanced plasmids intramuscularly, groups 5 was vaccinated subcutaneously with H5 plasmids, group 6 received H5 plasmids intramuscularly, and groups 7-9 were injected intradermally, with 100 μg, 50 μg, and, 10

μg of H5 plasmids, respectively (Table 2). Serum samples were taken at two-week intervals beginning two weeks after initial vaccination. Booster vaccinations were administered twice; one- and two-months following initial vaccination. All booster vaccines were identical to the original vaccines.

Experiment 3.

Testing the adjuvant effects of plasmid DNA encoding for chicken cytokines.
Seventy-seven three-week-old birds were divided into 11 groups of seven birds per group. Groups one through five all received H5 plasmids in Lipofectin. In addition, group 2 received IL-10 plasmids, group 3 received IL-2 plasmids, group 4 received a cocktail of both IL-2 and IFN plasmids, and group 5 received IFN plasmids. Groups six through 11 all received H7 plasmids in Lipofectin, unless stated otherwise. Additionally, groups 7-10 received IL-10 plasmids, IL-2 plasmids, both IL-2 and IFN plasmids, and IFN plasmids, and group 11 received H7 plasmids in Lipofectamine 2000 (Table 3). All birds were injected intramuscularly with 0.2 ml into each breast muscle. Serum samples were taken beginning two weeks and until 12 weeks after initial vaccination. Booster vaccines were administered twice, at monthly intervals, beginning one month following initial vaccination, and contained identical doses of DNA as the initial vaccination.

HI test. Sera from vaccinated birds were tested for HA subtype-specific antibodies with the HI test. (25, 30). Briefly, inactivated avian influenza virus from A/TK/WI/68 or A/CK/NY/13142-5/94 was diluted in PBS. Twenty-five microliters of diluted antigen,

four hemagglutinating units, were added per well of a 96-well plate, where the test serum had been diluted two-fold. The plates were incubated for 15 min. at room temperature before adding washed chicken red blood cells (0.5%) to each well. Plates were mixed for 15 s, and incubated for 45 min. at room temperature. Results were interpreted as the reciprocal of the last well that had complete inhibition of hemagglutinating activity.

Statistical analysis. Student's *t*-test was performed to make statistical comparisons between the different groups.

RESULTS

DNA preparation.

Plasmid DNA containing antigenic coding sequences. When used in DNA vaccinations, the TK/WI/68 hemagglutinin is highly antigenic in chickens, whereas CK/NY/13142-5/94 has a consistently lower response (11). The HA genes of these two isolates were chosen for use in order to compare levels of enhancement induced by the various adjuvants.

The coding sequence of H7 was subcloned into pCI and sequencing verified proper orientation of both H5 and H7 into the pCI EEV. In addition, 293-T cells were transfected with these plasmids and iIFA confirmed the expression of both proteins (Fig.1). A CpG motif was incorporated both immediately upstream and immediately downstream of the coding regions of both HA genes in pCI (8) by using primers developed for this purpose. Sequence analysis confirmed proper orientation, and protein expression was also confirmed by iIFA. We hypothesized that close proximity of the CpG motif to HA antigens may act as an endogenous adjuvant.

Cloning chicken cytokine genes. The chicken cytokine genes of IFN-1, IL-2, and IL-10 were isolated by using primers designed based on their respective sequences (20, 21, 23, 28). The IFN gene was obtained from the CEF DNA. The expression of IFN was confirmed by obtaining mRNA from transfected cells and performing RT-PCR and PCR on it (Fig. 2). The IL-10 and IL-2 genes were cloned from specific tissues (20, 28). The tissue samples were kept frozen solid on dry ice, at -70°C, or in liquid nitrogen. The IL-10 gene was isolated from the cecal tonsils and bursa of Fabricious while the IL-2 was isolated from the thymus. By restriction digestion, we were able to clone each of the genes into pCI, according to sequence analysis. Currently, there are neither chicken IL-2 nor chicken IL-10 antibodies commercially available so we were not able to test for expression of either of these proteins by using iIFA.

Vaccination.

Experiment 1. *Adjuvant effects of CpG motif, Lipofectamine 2000, and IFN.* Chickens injected only with plasmid DNA containing the H5 or H7 genes with Lipofectin served as positive controls. In addition to these plasmids, experimental groups received one of various adjuvants including CpG motifs, plasmid DNA containing chicken IFN-1, or Lipofactamine 2000 cationic lipid as an alternative to Lipofectin (Table 1). The particular CpG sequence we chose was based on a previously reported motif that was shown shown to be efficient at stimulating B-cells in mice. It was tested in two-week old chickens by Vleugels et al (2002) and shown to enhance antibody production when administered in 20 μg doses. Because the phosphodiester CpGs oligos are prone to degradation by nucleases, particularly at the 3’ ends (Yu-phosphorothioate paper), two phosphorothioate

bonds were incorporated between the last three nucleotides at the 3' end of the oligos in order to decrease the likelihood of such degradation. Sequences containing GpC replacing CpG were used as CpG negative controls.

By the first month, the IFN plasmids, CpG oligos, and Lipofectamine 2000 increased the Ab responses in the H5 group compared to the H5 plasmid alone (Fig.3). The GpC group HI titers were significantly lower ($p < 0.05$) than the H5 positive control group (Fig. 3). After the booster, 10 weeks following the initial vaccination, only the CpG group maintained an average HI titer close to the H5 plasmid only control.

In the H7 groups, all of the adjuvants enhanced the antibody responses at different time points (Fig.4). For the first month, both the IFN and Lipofectamine 2000 caused a significant increase ($p < 0.05$) in HI titer. Two weeks after booster inoculation, all experimental groups, except the IFN group, caused a statistically significant increase (p, 0.05) in Ab titers. Two weeks following the booster, all groups, except the IFN group, showed significantly ($p < 0.05$) enhanced antibody titers compared to the control plasmid H7 group. One month after the booster injection, the GpC , Lipofectamine 2000, and the CpG-enhanced plasmid HI titers were significantly higher than ($p < 0.05$) the control H7 group.

Because DNA vaccination is similar to cell culture transfection, and transfection efficiency is an important determining factor in producing Ab responses, we chose to test various transfection reagents' capabilities to act as DNA vaccine adjuvants. In addition to increasing transfection efficiency, cationic liposomes also promote the release of DNA from endosome into cytoplasm and protect the plasmids from Dnases which may exist in the interstitial fluid (30). The Lipofectamine 2000 group maintained higher titers than

controls, beginning one month post inoculation. Though all of the adjuvants improved responses in the H7 groups, the Lipofectamine and IFN plasmids appeared to elicit an improved response the soonest, while significant increases seen in the other groups went into effect following the booster vaccination.

Experiment 2. *Effect of delivery routes on antibody responses.* We tested the efficacy of DNA vaccination by using different delivery routes. The traditional method of vaccination for antisera production, subcutaneous inoculation with an oil emulsion-adjuvanted whole killed vaccine, was used as a control to compare both vaccine type and delivery method to the traditional means of reference antiserum production. The traditional method induced the highest antibody responses at all time points, followed closely by the intradermally delivered oil emulsion vaccine. It should be noted that because the chicken skin is so thin, the intradermal vaccine was in reality a combination of intradermal and subcutaneous. The dose was also approximate, since some leakage of vaccine occurred in all cases. In regards to the DNA vaccines, the H5 group receiving intramuscular vaccination had the highest titers ($p < 0.05$), followed by the intradermally vaccinated group (Fig. 5). The subcutaneously vaccinated group had the lowest HI titers of the delivery route groups that received equal amounts of plasmid DNA. No improvement was made by using the CpG-enhanced H5 plasmids nor the other commercial reagent. A dose response could be seen when various amounts of plasmid were injected intradermally, with the best response being produced by the 100 µg injection ($p < 0.05$). (Fig. 6).

Experiment 3.*Testing the adjuvant effects of cytokine genes contained in plasmid DNA.*

We coadministered H5 or H7 with either one of the cytokines, or with a cocktail of cytokine-encoded plasmids (Table 3). All vaccines were administered in Lipofectin, intramuscularly, except one H7 group, which received the vaccination in Lipofectamine 2000. All birds were boostered twice at monthly intervals. There was no statistical difference ($p < 0.05$) between the H5 positive control group and any of the other groups coadministered with the cytokine DNA (Fig. 7). However, out of the H7 groups, the IFN and IL-2-encoding plasmids significantly enhanced the antibody responses (Fig. 8). The IFN began working by the first sampling date, two weeks post inoculation, and increased antibody titers were sustained throughout the duration of the experiment. The same scenario occurred in the IL-2 group beginning one month after initial inoculation. The cytokine plasmid cocktail did make a statistically significant difference in antibody response.

DISCUSSION.

When AI virus is isolated, part of the identification process involves subtyping of the HA and NA surface proteins. Typically, the HI test is used for determining the HA subtype. This test uses reference antisera against each of the 16 different HAs. The typical means of producing reference antisera is through the vaccination of chickens with whole, inactivated virus. The drawbacks to this method are that antibodies against other surface proteins such as NA may interfere with the HIs, resulting in misdiagnosis. In

addition, preparation of the vaccine used for the traditional method involves handling of live virus, which poses a potential risk to the handler.

It was previously shown that DNA vaccines could be used to produce HA subtype-specific reference antisera (11) with the advantage being that only antibodies against the HA were produced, eliminating the concern of steric hindrance with neuraminidase antibody. One drawback to this method was that the antisera production using the DNA vaccine was much lower than that induced by the inactivated, whole virus vaccine.

In this study, we set out to find an adjuvant which could enhance antibody responses to DNA vaccines when used as a diagnostic tool. We compared the response to CpG oligodeoxynucleotides, cationic lipids, and plasmid DNA into which different cytokine genes had been cloned. Naturally occurring unmethylated CpG dinucleotides found in bacterial DNA are known to trigger the innate immune system in vertebrates, resulting in B cell proliferation and increased antibody production (9). The CpG motifs, whether coadministered separately or engineered to flank the coding sequences of HA genes, both upstream and downstream, did not seem to have much of an impact on the antibody response. Optimal CpG motif sequences vary from species to species (31). The motif that we used had a good response in mice, but perhaps is not effective for chickens. Another possibility is that the dosage was not high enough or that they were degraded too rapidly despite the use of the 3’ phosphorothioate bonds. There was inconsistency with regards to the immune responses to GpC oligos, depending on the HA gene with which they had been inoculated. Similar results have occurred where a higher antibody response was seen in the CpG negative control than in the positive control group (6). As

for the CpG-enhanced plasmids, it is possible that the plasmid-encoded proteins were synthesized so soon after administration that the protein and not DNA sequences was detected by the immune system *ie* whatever protein is encoded for by the CpG motifs was made so the CpG sequence was not seen – it was upstream of the start codon.

While Lipofectamine 2000 moderately improved the antibody response, no improvement was seen with the other cationic lipid, GeneJammer. The Lipofectamine 2000 may have enabled the plasmid DNA to be transfected more efficiently or to linger in the tissues longer, safe from nucleases. The increased response of the Lipofectamine 2000 mirrors the improved response as seen in cell culture. Although some improvement was seen with the Lipofectamine 2000, the improved response likely does not overcome its much higher cost as an effective alternative to lipofectin.

Interferon-1 (IFN), interleukin-2 (IL-2), and interleukin-10 (IL-10) have all been found to have an impact on antibody production (2, 4, 5, 12, 15, 18, 22). We believed that by inserting these cytokine genes into plasmid DNA and coadministering them with DNA encoding an HA, an increased Ab response would result. The IFN is a component of the innate immune system, which may help to explain why such an increase could be seen in antibody titers in the H7 group beginning at the time of the first bleeding. The IL-2-encoding plasmids also helped to enhance the antibody responses to the H7 proteins, supporting its ability to increase antibody production. IL-10, on the other hand, did not seem to work as well as expected. IL-10 inhibits Th1 immune responses. Perhaps there was a "conflict" in responses initiated by the IL-10 (Th2) and the intramuscular vaccination (Th1), abrogating the dominance of one particular type of response over the other. Finally, the IL-2 + IFN plasmid cocktail coadministered worked the least

efficiently of the cytokine DNAs at improving the antibody response. This was surprising since IL-2 is thought to be involved in promoting the avian immune system to produce IFN-α (12), which is encoded for in this coadministered IFN gene. There also may be a limit to the capacity of DNA that transfection reagents are capable of delivering to cells, being that they were developed for *in vitro* use.

We also tested different routes of vaccine delivery, including subcutaneous, intramuscular, and intradermal. The typical method of DNA vaccination is via the intramuscular route. However, there are many more dendritic cells, known as Langerhans cells, present in the dermis, which are not present in large numbers in muscle tissue (8). We hypothesized that if we targeted this layer, there would be a greater uptake of the protein antigen, more antigen presentation, and ultimately, a greater antibody response. This method of vaccination, when using a needle, can be quite tedious and requires some skill. In our study, the intramuscular route was the most efficient means of DNA vaccination (Fig.5). However, if a method of more accurate delivery of the plasmid to the dermis can be identified, perhaps a better immune response can occur. Hence, if a standard technique such as needleless injection were employed, a greater uptake of DNA by the Langerhans cells may be achievable, making intradermal delivery more feasible. One study using intramuscular vaccination, either by a needle or through needleless injection, found that the needleless delivery resulted in an increased uptake of plasmid in the skin (14). Even though needleless delivery was directed toward the muscle, perhaps the mechanism behind this needleless delivery route is very efficient at directly transfecting cells in the skin. A dose response was seen, with 100 μg dosage eliciting the highest response of the three amounts administered (Fig.6). One alternative

measure of DNA vaccination to the dermis that could be considered is the Genegun technology (8). This system coats the plasmid onto gold beads which are then fired into the dermis. This system, although efficient at directly getting plasmid into the skin, is expensive per dose, and was not considered as a likely long-term solution (8.).

Overall, we found that coadministration of either IFN or IL-2 worked most efficiently at enhancing the antibody responses to the less immunogenic H7 protein, while none of the adjuvants or delivery routes seemed to positively alter the production of antibodies against H5. This leads to the idea that the antibody responses to each HA protein are based, in part, in response to the particular protein, and is not necessarily a universal response produced against all HA proteins by the host. It is possible that the H7 protein produced by DNA vaccination is less antigenic because it has some intrinsic characteristic which prevents IFN or IL-2 production by the host. Thus, by coadministration, this cytokine suppression is abrogated.

Further work is needed to evaluate the effects of the IFN or IL-2 plasmids on antibody production against other HA subtypes which are poorly antigenic. Another approach would be to study the levels of various cytokines produced in response to the different HAs.

CHAPTER 4

DISCUSSION

Avian influenza is an important disease of poultry, which is caused by *avian influenza* (AI) virus. This disease occurs in two forms, either low pathogenic (HPAI) or highly pathogenic (LPAI), both having potential to cause economic losses to the poultry industry. LPAI may cause no symptoms or decreased egg production while HPAI may cause 100% mortality to chicken flocks.

The rapid and accurate identification of the virus is necessary to characterize a virus. Initial characterization is done according to identifying its surface protein, hemagglutinin (HA), of which there are 16 different types. The hemagglutination inhibition (HI) test is the most common method used in this identification procedure and requires a panel of reference antisera against each HA. The current means of reference antisera production is by vaccinating chickens subcutaneously with whole, inactivated virus. One drawback to this method is that antibodies against all AI proteins are produced. We recently demonstrated that reference antisera could be produced successfully by DNA vaccination with the advantage that only antibodies against HA were produced (12).

One disadvantage to using DNA vaccination to produce reference antisera is that multiple vaccinations and large quantities of DNA are required to obtain amounts of antibodies equal to those induced by the traditional method. The purpose of this study was to enhance the efficacy of DNA vaccination so as to increase antibody production to

be used as a diagnostic tool. We tested CpG motifs, various transfection reagents, chicken cytokines, and different routes of delivery.

Unmethylated CpG motifs, found naturally in bacterial DNA, trigger the innate immune system of vertebrates. When administered as adjuvants, they have been shown to increase the antibody response. When we coadministered CpG oligodeoxynucleotides with DNA containing an H5 or H7 gene, an increased antibody response was seen, but it was only significant in the H7 group. Various factors could have been the cause of these results. The dose of oligos administered may not have been high enough. The amount we used on our four-week-old birds was the amount that had worked well in two-week old birds in the study we used as a reference. Additionally, there may be more optimal CpG motifs for activating the avian immune system than the one we chose to use. We also attempted to enhance the immune response to CpG motifs by flanking the coding sequence of the HA gene insert. This may not have worked as well as hypothesized as a result of the plasmids leaking out of the endosome, into the cytoplasm before the toll-like receptors (TLR) had a chance to detect the CpG motifs.

Since transfection reagents enhance uptake of plasmid DNA by cells *in vitro*, we tested them to see if they would work as well *in vivo*. The cationic lipid had previously been shown to be an effective adjuvant. Alternative adjuvants also worked reasonably well, with some significant increases seen in the H7 groups with Lipofectamine 2000, but the polyamine did not work as well. It may be the case that cationic lipids are more stable in living tissues than are polyamines, resulting in the inability of polyamines to protect DNA from degradation before they themselves are degraded.

We tested chicken cytokines IFN, IL-10, and IL-2. IFN, which is a component of the innate immune system and also is known to increase antibody responses, was one of the adjuvants showing optimal enhancement. IL-2 is a potent T-cell growth factor and also directly acts on B cells causing them to undergo proliferation. It also worked to significantly enhance the Ab response. IL-10 enhanced antibody production, though not as efficiently as the other two cytokines tested. IL-10 has been known to control innate immune responses and to inhibit dendritic cells. The cocktail of IL-2 and IFN was not as efficient as administration of the individual cytokine-encoding plasmids with the antigen-encoding DNA. The amount of DNA administered in this group was over twice as large as that administered to the control group.

More Langerhans cells are present in the dermis than in the muscle. We hypothesized that if plasmids were delivered to the dermis, ultimately, an increase in antigen presentation would occur, resulting in an enhanced antibody response. Out of subcutaneous, intramuscular, or intradermal delivery, the intramuscularly injected groups showed the highest antibody titers. It must be taken into account that intradermal delivery via needle is a difficult task to manage. During delivery, some of the vaccine was lost due to leakage or accidental delivery to the subcutaneous layer of skin. In addition, one study using needleless intramuscular delivery found that more plasmid was taken up in the skin compared to administration with the needle. Plasmids also had dispersed to distal sites when administered using needleless delivery. It is possible that similar to gene gun delivery, that the overall mechanism behind needleless delivery favors uptake by the skin. If a less tedious method of intradermal injection, such as by needleless delivery, could be employed, this route of delivery should be promising.

Thus far, the best adjuvants we found were the IFN and IL-2 cytokines with the intradermal delivery route showing great promise as well. Further studies which would lend some more light to the unresolved aspects of this study could be to test the effects of the coadministration of the IFN or IL-2 cytokines with DNA encoding other HA subtypes. From a different approach, the immune responses such as the cytokines produced in respose to vaccination of plasmid DNA encoding for other individual HA subtypes could be assessed. Finally, it would be interesting to assess the efficiency of intradermal delivery using needleless administration.

FIGURE LEGENDS

Fig. 1. Indirect immunofluorescent antibody assay. 293-T cells were transfected with H5 (a) or H7 pCIs (b), and were reacted with H5 or H7 antisera obtained from chickens. A non-transfected negative control (c) was also used and reacted with both types of antisera. Fluorescence within cell membranes was observed in both groups a and b of transfected cells. Magnification = 20X.

Fig. 2. Agar gel electrophoresis to test for expression of IFN pCI plasmids. Chicken embryo fibroblasts were transfected with IFN pCIs. Messenger RNA was isolated and both RT-PCR and PCR were performed to check for presence or absence of a band at the 602 bp location. Lane 1 = RT-PCR, Lane 2 = 100 base pair ladder, Lane 3 = PCR.

Fig. 3. Seven 4-week-old SPF chickens vaccinated with pCI EEV encoding the H5 gene alone, or along with CpG oligodeoxynucleotides, GpC oligodeoxynucleotides, IFN-1 gene-encoding pCI, or in Lipofectamine 2000 transfection reagent. Sera were collected for ten weeks, beginning two weeks post initial inoculation. HI test was used to determine hemagglutinin-specific antibody titers. Mean $\log_2$ HI titers of each group are indicated in the graph.

Fig. 4. Seven 4-week-old SPF chickens vaccinated with pCI EEV encoding for H7 gene sequence, either alone or coadministered with CpG oligodeoxynucleotides, IFN-1 gene-

encoding pCI plasmids, or in Lipofectamine 2000 transfection reagent. An additional group was administered CpG-enhanced pCI-H7 plasmids alone. Serum samples were collected from each bird for 10 weeks, beginning two weeks post initial vaccination. HI test was used to determine hemagglutinin-specific antibody titers. Mean $\log_2$ HI titers of each group are indicated in the graph.

Fig. 5. Groups of nine 2 ½ -week-old chickens vaccinated subcutaneously, intradermally, or intramuscularly with pCI EEV encoding for the TK/WI/68 H5 gene. Serum samples shown were taken from each bird beginning two weeks post initial vaccination, through 10 weeks post inoculation. HI test was used to evaluate hemagglutinin-specific antibody titers. Mean $\log_2$ HI titers and standard deviations of each group are indicated in the graph.

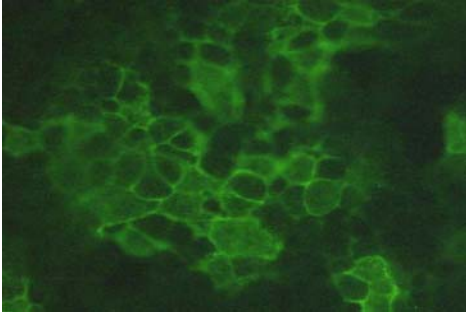
Fig. 6. Groups of nine 2 ½-week-old SPF chickens vaccinated with pCI EEV encoding for the TK/WI/68 H5 gene using doses of 100 μg, 50 μg, or 10 μg DNA per bird. Data shown are hemagglutinin-specific antibody titers which were assessed by using HI test on serum samples taken from each bird between two and 10 weeks post initial vaccination. Birds were boostered twice at monthly intervals beginning one month post inoculation. Standard deviations and mean $\log_2$ HI titers of each group are indicated in the graph.

Fig. 7. Seven 3-week-old SPF chickens vaccinated with pCI EEV containing an H5 gene TK/WI/68. In addition, some groups of birds received pCI encoding for IL-10, IL-2, IFN-1, or a combination of these. One group received the H5-encoding pCIs in

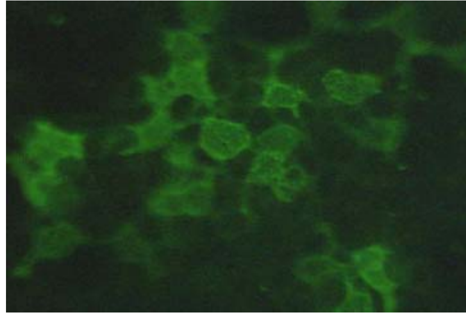
Lipofectamine 2000. Birds were bled beginning 2 weeks post initial vaccination and the hemagglutinin antibody-specific serum samples were tested using HI test. Booster vaccinations were administered twice at monthly intervals beginning one month following first vaccination. Mean $\log_2$ HI titers and standard deviations are indicated in the graph.

Fig. 8. Seven 3-week-old SPF chickens inoculated with pCI EEV containing an H7 gene insert or coadministered with other plasmids encoding various cytokines or administered in Lipofectamine 2000. Additional pCI EEVs encoding for IL-10, IL-2, or IFN-1 genes were coadministered in some of the groups. One other group received the H7-encoding pCIs in Lipofectamine 2000 transfection reagent unlike the rest, which were administered in Lipofectin. Birds were bled at biweekly intervals up to 10 weeks post initial vaccination and serum antibodies against the hemagglutinin were titrated by HI test. Mean $\log_2$ HI titers and standard deviations are indicated in the graph.

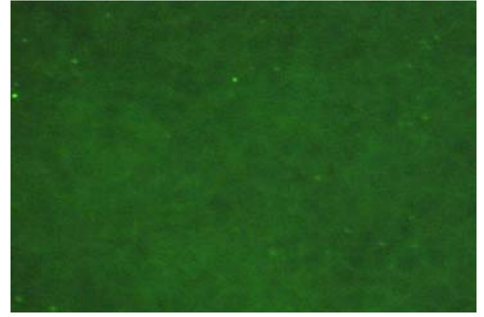
Figure 1.



pCI-H5
(a)



pCI-H7
(b)



pCI
(c)

Figure 2.

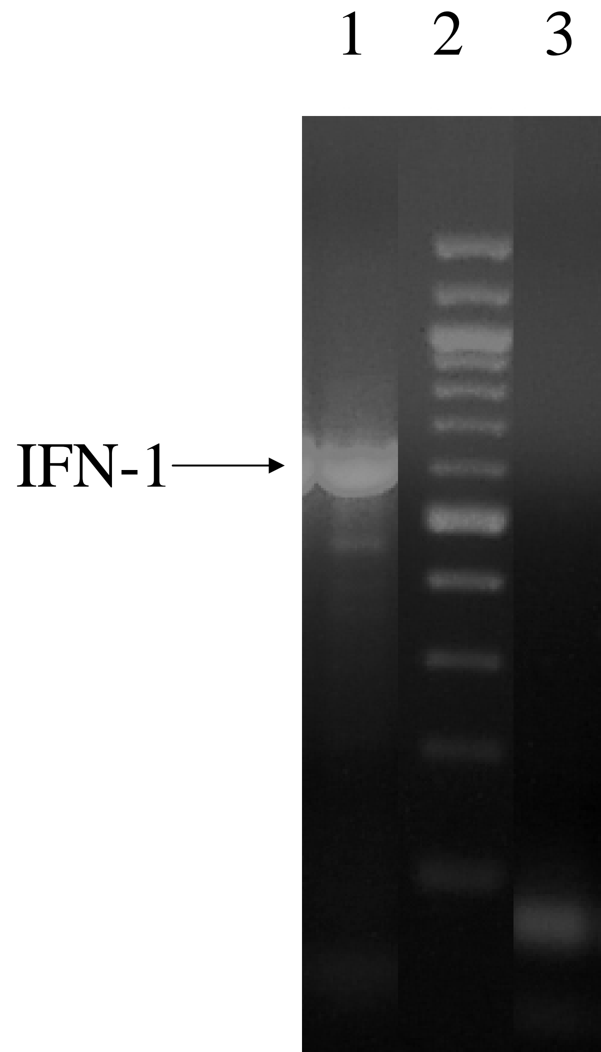


Figure 3.

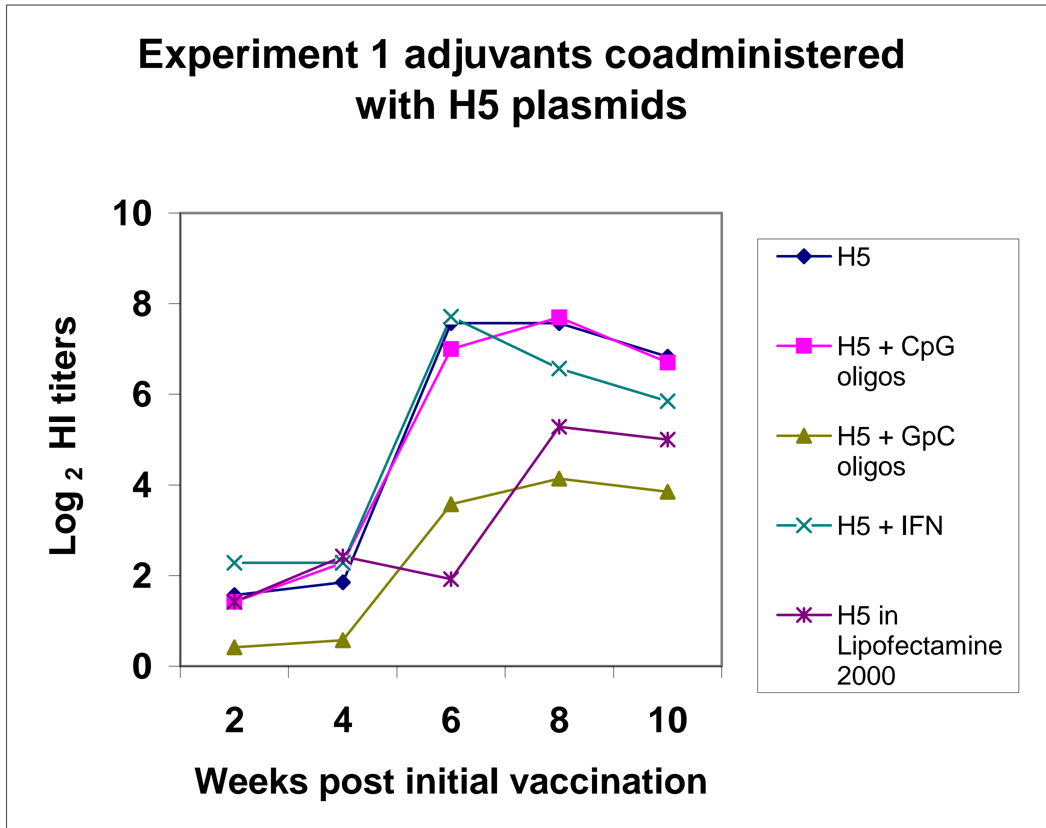


Figure 4.

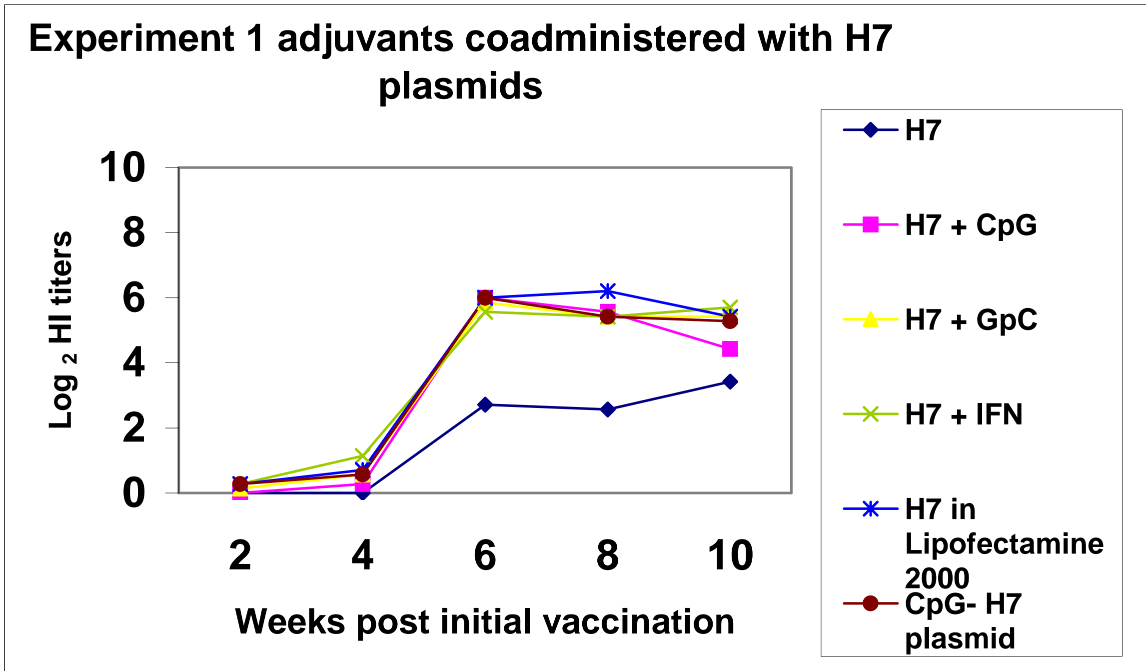


Figure 5.

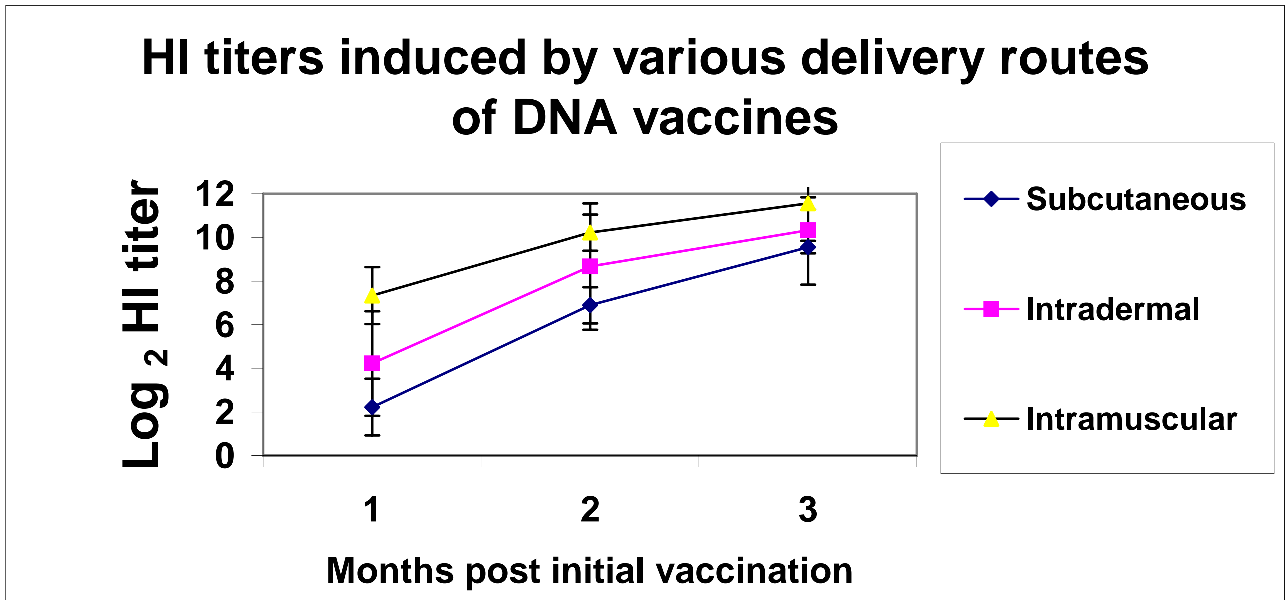


Figure 6.

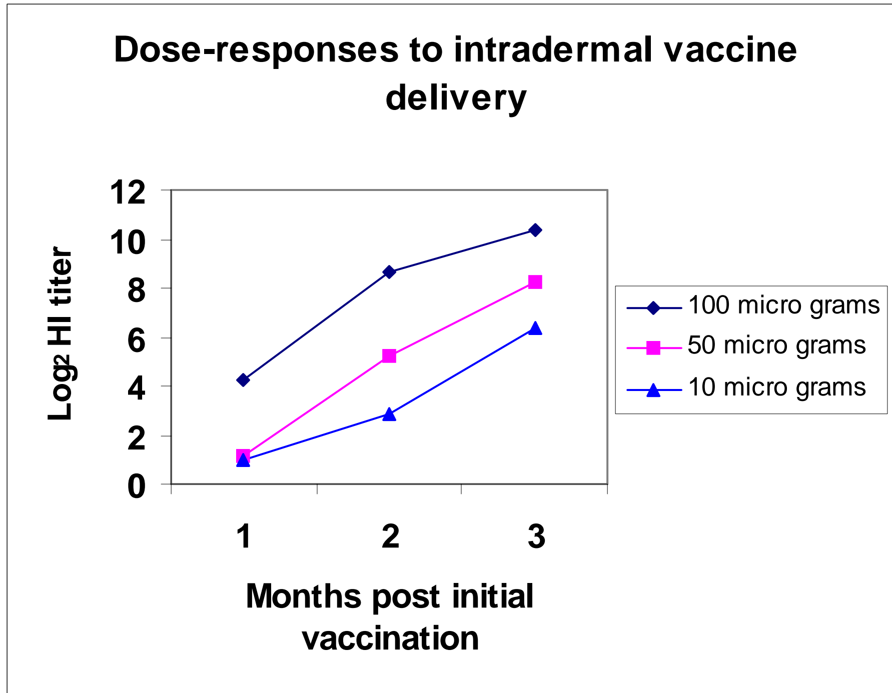


Figure 7.

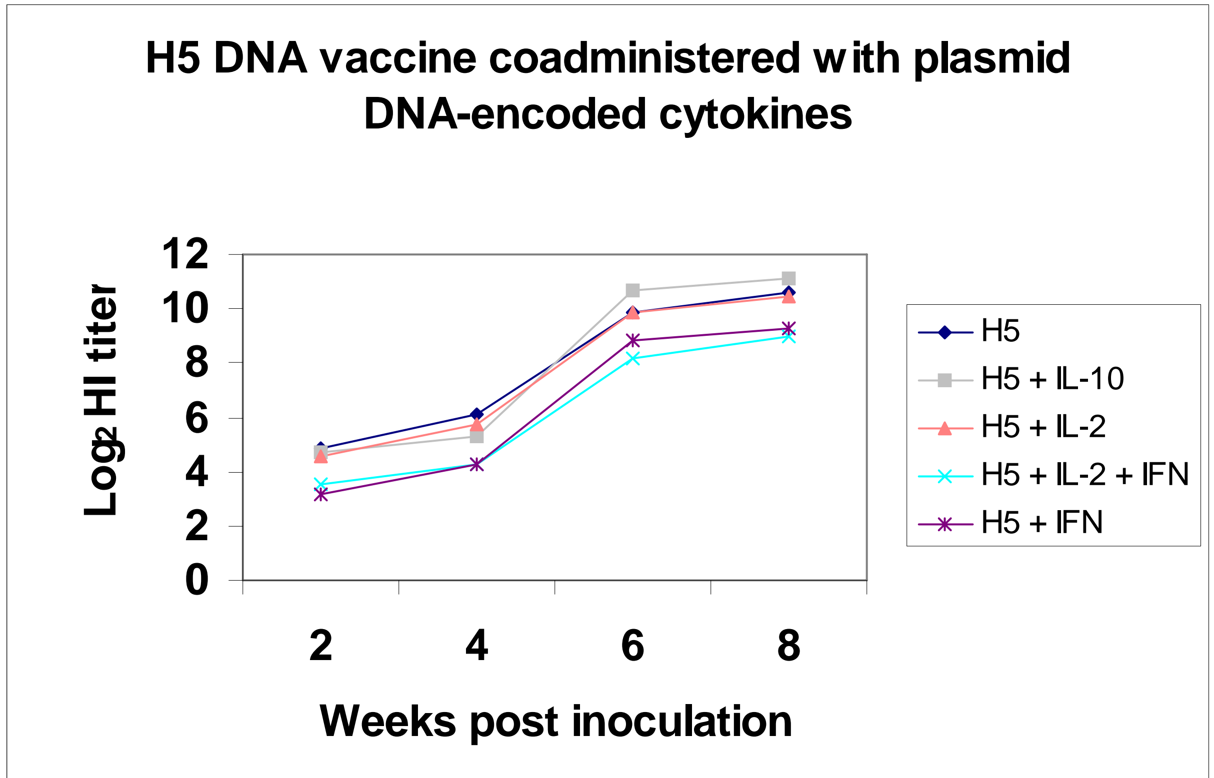
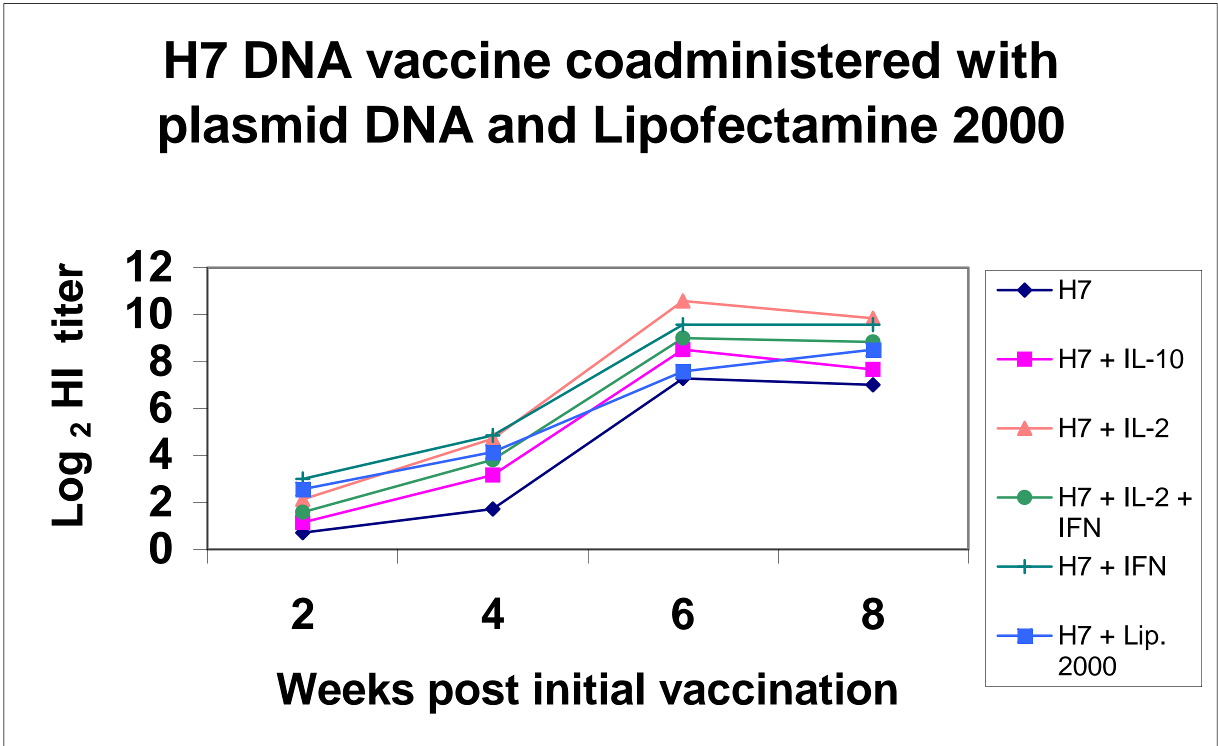


Figure 8.



REFERENCES

1. Abbas A. K. and A. H. Lichtman. 2003. Cellular and Molecular Immunology. Independence Square West. Philadelphia, PA.

2. Braun D., I. Caramalho, and J. Demengeot. 2002. IFN-α/β enhances BCR-dependent B cell responses. International Immunology. 14:411-419.

3. Capua, I. and D. Alexander. 2004. Avian Influenza: recent developments. Avian Pathology. 3:393-404.

4. Choi. J., and Y. S. Choi. 1998. IL-10 interrupts memory B cell expansion in the germinal center by inducing differentiation into plasma cells. Eur. J. Immunol. 28:508-515.

5. Chow Y., W. Huang, W. Chi, Y. chu, and M. Tao. 1997. Improvement of hepatitis B virus DNA vaccines by plasmids coexpressing hepatitis B surface antigen and interleukin-2. J. Virology. 71:169-178.

6. Dalloul, R.A., H.S. Lillehoj, M. Okamura, H. Xie, W. Min, X. Ding, and R.A. Heckert. 2004. *In vivo* effects of CpG oliogodeoxynucleotide on *Eimeria* infection in chickens. Avian Diseases. 48:783-790.

7. Fouchier R. A. M. , V. Munster, A. Wallensten, T. M. Bestebroer, S. Herfst, D. Smith, G. F. Rimmelzwaan, B. Olsen, and A. D. M. E. Osterhaus. 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J. Virol. 79:2814-2822.

8. Fynam E. F., R. G. Webster, D. H. Fuller, J. R. Haynes, J. C. Santoro, H. L. Robinson. 1993. DNA vaccines: Protective immunizations by parenteral, mucosal, and gene-gun inoculations. Proc. Natl. Acad. Sci. 90: 11478-11482.

9. Klinman D.M., K. M. Barnhart, and J. Conover. 1999. CpG motifs as immune adjuvants. Vaccine. 17:19-25.

10. Krieg A., A.K. Yi, J. Schorr, and H.L. Davis. 1998. The role of CpG dinucleotides in DNA vaccines. Trends Microbiol. 6:23-7.

11. Lamb, R. A., and R. M. Krug. 2001. Orthomyxoviridae: the viruses and their replication. p. 1487-1532. *In* D. M. Knipe, P. M. Howley (ed.), Fields Virology, fourth ed. Lippincott Williams & Wilkins, Philadelphia, Penn.

12. Lee C.W., D.A. Senne, and D.L. Suarez. 2003. Development of hemagglutinin subtype-specific reference antisera by DNA vaccination of chickens. Avian Diseases. 47:1051-1056.

13. Li J., X. Liang, Y. Huang, S. meng, R. Xie, R. Deng, and L. Yu. 2004. Enhancement of the immunogenicity of DNA vaccine against infectious bursal disease virus by co-delivery with plasmid encoding chicken interleukin-2. Virology. 329:89-100.

14. Manam S., F.J. Ledwith, A.B. Barnum, P.J. Troilo, ClJ. Pauley, L.B. Harper, T.G. Griffiths II, Z. Niu, L. Denisova, T.t. Follmer, S.J. Pacchione, Z. Wang, C.M. Beare, W.J. Bagdon, and W.W. Nichols. 2000. Plasmid DNA vaccines: Tissue distribution and effects of DNA sequence, adjuvants and delivery methods on integration into host DNA. Intevirology. 43:273-281.

15. Miyamoto T., H.S. Lillehoj, E.J. Sohn, and W. Min. 2001. Production and characterization of monoclonal antibodies detecting chicken interleukin-2 and the development of an antigen capture enzyme-linked immunosorbent assay. Vet. Immunol Immunopath. 80:245-257.

16. Mo C. W., Y. C. Cao, and B. L. Lim. 2001. The in vivo and in vitro effects of chicken interferon α on infectious bursal disease virus and Newcastle disease virus infection. Avian Diseases. 45:389-399.

17. Moore K. W., R. de Waal Malefyt, R. L. Coffman, and A. O'Garra. 2001. Interleukin-10 and the interleukin-10 receptor. Annu. Rev. Immunol. 19:683-763.

18. OIE. 2004. Highly pathogenic avian influenza. *In* manual of diagnostic tests and vaccines for terrestrial animals, 5th edition.

19. Proietti E., L. Bracci, S. Puzelli, T. Di Pucchio, P. Sestili, E. De Vincenzi, M. Venditti, I. Capone, I. Seif, E. De Maeyer, D. Tough, I. Donatelli, and F. Belardelli. 2002. Type I IFN as a natural adjuvant for a protective immune response: Lessons from the influenza vaccine model. J. Immunol. 169:375-383.

20. Rami A. D., H. S. Lillehoj, M. Okamura, H. Xie, W. Min, X. Ding, and R. A. Heckert. 2004. *In vivo* effects of CpG oligodeoxynucleotide on *Eimeria* infection in chickens. Avian Diseases. 48: 783-790.

21. Rothwell L., J. R. Young, R. Zoorob, C. A. Whittaker, P. Hesketh, A. Archer, A. L. Smithe, and P. Kaiser. 2004. Cloning and characterization of chicken IL-10 and its role in the immune response to *Eimeria maxima*. J. Immunol. 173:2675-2681.

22. Rothwell L., A. Hamblin, and P. Kaiser. 2001. Produciton and characterization of monoclonal antibodies specific for chicken interleukin-2. Vet. Immunol. Immunopath. 83:149-160.

23. Schijns V. E. C. J., K. C. Weining, P. Nuijten, E. O. Rijke, and P. Staeheli. 2000. Immunoadjuvant activities of *E. coli*- and plasmid-expressed recombinant chicken IFN-α/β, IFN-γ, and IL-1β in 1-day- and 3-week-old chickens. Vaccine. 18:2147-2154.

24. Sekellick M. J., A. F. Ferrandino, D. A. Hopkins, and P. I. Marcus. 1994. Chicken interferon gene: cloning, expression, and analysis. Journal of Interferon Research. 14:71-79.

25. Stone H., B.Mitchell, and M. Brugh. 1997. *In ovo* vaccination of chicken embryos with experimental Newcastle disease and avian influenza oil-emulsion vaccines. Avian Diseases. 41:856-863.

26. Suarez D. L. and S. Schultz-Cherry. 2000a. The effect of eukaryotic expression vectors and adjuvants on DNA vaccines in chickens using an avian influenza model. Avian Diseases. 44:861-868.

27. Suarez, D. L. and D. E. Senne. 2000b. Sequence analysis of related low-pathogenic and highly pathogenic H5N2 avian influenza isolates from united states live bird markets and poultry farms from 1983-1989. Avian Diseases. 44:356-364.

28. Suarez D.L. 2000c. Evolution of avian influenza viruses. Vet. Microbiol. 74:15-27.

29. Sundick R. S., and C. Gill-Dixon. 1997. A cloned chicken lymphokine homologous to both mammalian IL-2 and IL-15. J Immunol. 159:720-725.

30. Swayne, D. E., and D. A. Halvorson. 2003. Influenza. p. 135-160. *In* Y. M. Said, H. J. Barnes, J. R. Glisson, A. M. Fadly, L. R. McDougald, D. E. Swayne (ed.), Diseases of Poultry. Iowa State University Press, Ames, IA.

31. Villegas P. 2003. Avian virus diseases laboratory manual. University of Georgia, Athens, GA.

32. Vleugels B, C. Ververken, and B.M. Goddeeris. 2002. Stimulatory effect of CpG sequences on humoral responses in chickens. Poultry Science. 81:1317-1321.

33. Yu D., F.G. Zhu, L. Bhagat, H. Wang, E.R. Kandimall, R. Zhang, and S. Agrawal. 2002. Potent CpG oligonucleotides containing phosphodiester linkages: in vitro and in vivo immunostimulatory properties. Biochem. Biophys Res Comm. 297:83-90.