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≪Review≫

Developments in Avian Influenza Virus Vaccines

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The serologically diverse influenza A viruses, although transmissible among various susceptible species, mostly infect avian species. Avian influenza viruses (AIV) are also notorious for adapting to mammalian species, including humans. Although eradication of commercial birds infected with AIV is the preferred method of control, the ever presence of potential avian migratory, reservoir species makes worldwide spread inevitable and vaccine development a high priority for poultry. Live, attenuated vaccine strategies are of concern because of the potential of AIV to mutate, through point mutations and/or reassortment of their segmented genome. Both live and the safer, killed vaccines are of concern because of competition of natural and vaccine antigens in critical diagnostic assays. Subunit vaccines, which allow for protein distinction for diagnostic purposes, may consist of purified AIV protein or genes that encode individual viral proteins. Most vaccines have targeted the virus hemagglutinin protein, which is responsible for induction of the most effective neutralizing antibodies. Gene vaccines that include plasmid DNA and viral vector delivery of AIV genes allow for endogenous *in vivo* amplification of protein within cells. While fowlpox virus vectors have been licensed and proven to be efficacious even in field situations, other viral vectors that target the respiratory tract are in experimental development with promising practical application for poultry. Maximum exploitation of vectored vaccines may incorporate enhancing immune molecules.

Key words: attenuated, Avian influenza virus, inactivated, recombinant, vaccines

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Introduction

Respiratory pathogens are highly contagious, and respiratory viruses are the most difficult of pathogens to control. The respiratory tract provides an efficient mechanism to aerosolize the environment with easily transmissible viral particles. These airborne particles can easily be inhaled by a new, susceptible host. Several distinctly different respiratory viruses, recognized decades ago, continue to be concerns for the poultry industry. Infectious laryngotracheitis viruses (ILV) within the Herpesviridae family are relatively large viruses with DNA genomes. The other economically important respiratory viruses of poultry have RNA genomes. Newcastle disease virus (NDV) and avian metapneumonia virus are in the Paramyxoviridae family, infectious bronchitis viruses (IBV) are the original prototype of the Coronaviridae family and the notorious avian influenza viruses (AIV) are members of the Orthomyxoviridae family. Among the respiratory pathogens in poultry, AIVs are not only serious avian pathogens, these viruses present unique concerns because of their close association with human influenza viruses and their potential to be zoonotic. In contrast, NDV may also infect humans, but infection is not a zoonotic concern because it is not known to spread between people and neither IBV

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nor ILV infect humans. Influenza viruses, as a group, have similar replication strategies, genetics and rather amorphous morphology. Avian influenza was initially known as "fowl plague," a characteristic respiratory problem described in poultry in the 19th century. "Fowl plague" was one of the first diseases shown to be caused by a filterable agent, which was the initial term for viruses, referring to them as etiologic agents that were smaller than the recently recognized bacterial pathogens. Although pathogenicity in poultry is highly variable, strains of AIV are categorized as either high pathogenic avian influenza (HPAI) or low pathogenic avian influenza (LPAI) in poultry.

All influenza viruses are in the Orthmyxoviridae family, have segmented genomes and have common replication strategies. Within the Orthomyxoviridae, there are three distinct classifications of influenza viruses, A, B and C. Avian influenza viruses are all classified as A. Nucleotide sequencing of viral genomes have identified close relationships between human and avian influenza viruses. It is thought that the mammalian influenza A viruses mostly originate or have originated from AIVs (Webster *et al.,* 1992). Strains of AIV have been transmitted directly to humans resulting in clinically illness and mortality (Subbarao *et al.,* 1998; Subbarao and Shaw, 2000; Lin *et al.,* 2000; Butt *et al.,* 2005). Consequently, influenza A viruses with their extraordinary capacity for mutation are seemingly promiscuous respiratory viruses that connect mammals and birds in an intriguing ecological matrix. The necessity for the immune system of both birds and mammals to control infection and provide protection against subsequent infections is at the center of much of the continuing research efforts in influenza.

Basic Virology

In order to understand the complex nature of AIV, one must first understand the mechanism of variation and function of encoded proteins that characterize this group of viruses. Therefore, the basic biochemistry will be described in terms of genes encoding viral proteins, the function of viral proteins, mechanisms of transmission from host to host or cell to cell, and the factors that promote diversity. Genetic diversity provides the genetic material for selection of newly adapted virus expressing modified proteins. New adaptations allow for change in host preference, or even allow for the evasion of established immunity. The genome of influenza A viruses, composed of RNA, is represented as a composite of 8 segments of single stranded RNA. The genome segments are referred to as negative sense because they are complimentary to mRNA that is translated by ribosomes into the encoded viral proteins. The 8 linear segments of the genome vary in size from approximately 900 to 2300 nucleotides. The 6 largest fragments (1 to 6) each encode one open reading frame (ORF) and each are transcribed into one functioning mRNA. The smallest two genomic segments are each used as templates to make 2 mRNAs that are translated into 2 proteins (Lamb and Krug, 2001). Within the viral particle, the 8 RNA segments are coated with numerous copies of the nucleocapsid protein (NP). Each nucleocapsid includes three polymerase proteins that are responsible for synthesis of viral RNA, and an RNA genome coated with NP. The eight nucleocapsids make up the core of the viral particle.

The viral RNA polymerases are notorious for making errors and thus generating mutations (Holland *et al.,* 1982). In the case of influenza, the three polymerase proteins synthesize three types of viral RNA; 1. full-length negative strand, 2. full-length positive strand and 3. functional mRNA. Negative sense, genomic RNAs serve as templates for the transcription of the mRNA translated by the ribosomes to make the viral proteins (Lamb and Krug, 2001). The negative sense segments will also serve as templates for the synthesis of full-length positive sense RNA that in turn serves as a template to generate more negative strand genomic segments. The viral mRNAs are synthesized as truncated complements of the negative sense genomic segments.

The 8 nucleocapsids within viral particles (also called virions) are surrounded by a bilipid membrane structure that originates from the host cell as the virus buds or leaves the cell (Fig. 1). Three proteins are embedded into the bilipid membrane envelope, the hemagglutinin protein (HA), neuraminidase protein (NA), and ion channel protein (M 2). The HA and the NA, anchored within the membrane, are responsible for the commonly used type or serotype description. The matrix protein (M 1), lying between the core and membrane, is critical for assembly, acting to tether together the nucleocapsids and membrane bound proteins. The M2

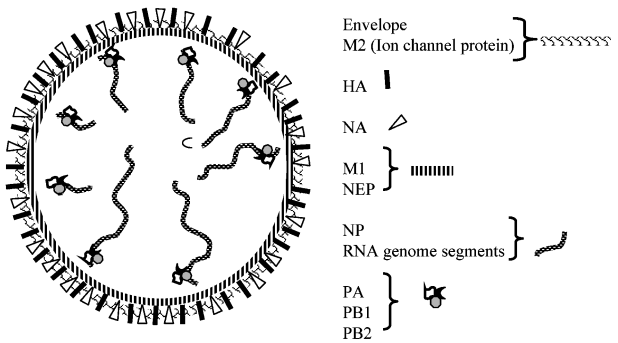

Fig. 1. **Schematic of an avian influenza viral particle**

protein, a channel for hydrogen ions, is responsible for regulating the pH environment of the virus, or specifically the HA. Two additional proteins have been characterized, the non-structural protein (NS 1) and the nuclear export protein (NEP), once called NS2 (Inglis and Almond, 1980; O'Neill *et al.,* 1998). NS1 has been found only in infected cells and not in virions. However, NEP, once thought to be present only in the infected cell was shown by Yasuda *et al.* (1993) to be associated with the M1 and to be a component of the viral particle and hence a structural, not a nonstructural, protein (Yasuda *et al.,* 1993).

The HA is the protein of greatest importance in defining the virus type with respect to antibody protection. Its name is logically derived from its ability to agglutinate erythrocytes. Prior to infection, the HA protein is proteolytically cleaved resulting in two subunit proteins that remain non-covalently associated with each other (Garten *et al.,* 1981; Bosch *et al.,* 1981). Cleavage of the whole HA into the external amino end, HA1, and the membrane bound or anchored carboxyl end, HA2, protein is required for virus to successfully infect a cell. Cleavage of the HA requires cellular proteases. The type of cell protease required for HA varies with the amino acid cleavage site of the HA. The protease requirements may also define the pathogenesis of the infecting virus (Ohuchi *et al.,* 1989; Vewy *et al.,* 1992; Perdue *et al.,* 1996; Garcia *et al.,* 1996). It had been shown that strains of AIV that are HPAI and capable of invading tissues beyond the respiratory tract had cleavage sites that were recognized by a broader range of proteases. In contrast, the cleavage of the less or non invasive LPAI virus HA was shown to be limited to proteases that were more restricted, as in the respiratory tract (Nicholls *et al.,* 2007). The LPAI viruses grown in cell culture generally require the addition of trypsin to cleave the HA and facilitate infection. Perdue *et al.* (1996) found that insertion of nucleotides encoding an arginine-lysine near the proteolytic site of a LPAI strain generated a virus that was capable of infecting cell culture without trypsin and was also pathogenic in chickens.

Antibodies specific for HA1 that prevent binding to the host cell receptor have been shown to be most effective in neutralizing virus. Membrane receptors on the host cell that interact with HA1 contain sialic acid sugars (Lamb and Krug, 2001). After binding to the cell receptor, the viral particle is engulfed into host cell endosomal vesicles. The HA2 is responsible for entry from the endosomes into the cytoplasm. The acid environment of endosomes, rather than destroying the virus, provides a favorable environment for the HA2 protein to modify its shape, allowing for exposure of previously hidden hydrophobic amino acids at the amino end of the protein (Luneberg *et al.,* 1995; Durrer *et al.,* 1996; Carr *et al.,* 1997; Carr and Kim, 1993; Kim *et al.,* 1998). This exposed hydrophobic peptide is able to interact and fuse with the cellular bilipid membrane of the endosomal vesicle. Fusion of the viral and cellular membranes results in entry of the virus into the host cytoplasm, where the nucleocapsids within the virion are released and make their way to the nucleus (Lamb and Krug, 2001). Within the nucleus, the negative sense genomes are used by the associated viral polymerases to synthesize viral mRNA. Influenza viruses have the unique distinction among RNA viruses of requiring nuclear, rather than cytoplasmic, functions for synthesis of viral mRNA. Influenza viruses take advantage of host nuclear functions to synthesize and process mRNA. Viral endonuclease activity encoded within the polymerase complex excises the 5′ 10 to 15 nucleotides of host mRNA along with the host mRNA 5′ attached caps (Krug *et al.,* 1979; Caton and Robertson, 1980). Unlike positive sense RNA viruses and the non-segmented RNA viruses with capped mRNA, influenza viruses do not encode for proteins that generate capping structures. The 5′ capped host mRNA fragments are used to prime or initiate transcription of viral mRNA. The virus further utilizes nuclear machinery to process viral mRNA by splic-

ing viral mRNAs of the two smallest genome segments (Lamb *et al.,* 1981; Lamb and Choppin, 1981; McCauley *et al.,* 1982). The splicing process allows mRNAs transcribed from segments 7 and 8 to function as two messages, the whole form and spliced, shortened form. Messenger RNA from segment 7 encodes both the M1 and M2, respectively. The NS 1 is translated from whole mRNA generated from segment 8, whereas the NEP (formerly NS2) is translated from spliced mRNA of segment 8. NEP, as its name suggests, shuttles the RNA from the nucleus to the cytoplasm (O'Neill *et al.,* 1998). The M1 has been shown to be associated with the NEP (Akausu *et al.,* 2003; Artz *et al.,* 2004). NS1, the only viral protein not found in the viral particle, has anti-IFN activity and has been associated with virulence in chickens (Garcia-Sastre *et al.,* 1998; Li *et al.,* 2006).

Epizoology

Although the HAs of AIV all function similarly in virus cell binding and entry, the determinants (or epitopes) that induce neutralizing antibodies differ with each type, such that neutralizing antibody of one type will not neutralize another AIV type. Virus neutralization or receptor binding is also defined by neutralization of hemagglutination activity or hemagglutination inhibition (HI), the basis for the traditional, most convenient method to determine serotype. Sixteen HA types have been defined. Virus with the 16th type was only published in 2005 (Fouchier *et al.,* 2005). Although secondary in importance to HA, the NA also houses determinants that induce antibody neutralization of virus. The NA cleaves sialic acid (neuraminic acid) from the glycosylated proteins. It was long thought that since the virus bound to sialic acid, the cleavage facilitated release of virus from cells surfaces (Palese *et al.,* 1974; Palese and Compans, 1976). However, recent studies would indicate that the NA has a role in the initiation of infection (Matrosovich *et al.,* 2004b; Ohuchi *et al.,* 2006). Antibodies to NA inhibit or neutralize neuraminidase activity (Webster and Campbell, 1972; Aymard-Henry *et al.,* 1973). Nine distinct NA types have been described based on induction of humoral immunity that inhibits neuraminidase function (Van Deusen *et al.,* 1983). The lay term "bird flu," as used by the media today, generally refers to a particular strain that is represented by the HA5 hemagglutinin and the NA 1 neuraminidase. The H5N1 type is well-known as being responsible for numerous outbreaks in birds, as well as being responsible for "crossing over" from chickens resulting in human mortality (Claas *et al.,* 1998). H5N1 has been isolated from poultry throughout much of the world. As a zoonotic, noncontagious human pathogen, H5N1 was reported by the World Health Organization to have caused morbidity in 272 persons with 166 fatalities between 2003 and early 2007. Countries or regions with affected persons included China, Southeast Asia, West Africa, and the Middle East (World Health Organization, 6 February, 2007).

Surveys have been conducted to determine the range of AIV in wild birds. The avian host range of AIV is incredibly broad. AIV has been isolated frequently from ducks, geese and swans (Anseriformes) and gulls, terns and waders (Charadriiformes), which are thought to be critical reservoirs for AIV although most avian species seem to be susceptible to these viruses (Slemons *et al.,* 1974; Slemons and Easterday, 1977; Panigraphy *et al.,* 1992; Allwright *et al.,* 1993; Alexander, 2000; Olsen *et al.,* 2006). AIV has also been isolated from avian species that include ratites, pheasants, quail, teal, pelicans, cormorants, passerines, psittacines and poultry. While the origins of most, if not all, influenza viruses are likely from wild life, AIV infection is rarely associated with clinical illness and mortality in their apparent natural hosts. These LPAI viruses are primarily enteric, readily shed in fecal material (Slemons *et al.,* 1974; Slemons and Easterday, 1977; Slemons and Easterday, 1978). It is of interest that the H5N1 is an exception to tissue preference in that it prefers the respiratory tissues of wild birds (Brown *et al.,* 2006). Whereas viruses representing all 16 HA and 9 NA are isolated as LPAI forms from wild birds, only H7 and H5 types have been implicated in the transition from typically LPAI strains to HPAI strains in poultry. Highly pathogenic AIV, as indicated by high mortality, has been defined in experimental studies with intravenous inoculation with infected allantoic fluid (Brugh, 1997; Hooper and Selleck, 1997). As might be expected, the degrees of pathology and mortality are complex and do not always completely correlate. It is an interesting question as to how many ultimate distinct HA types will be eventually described. Even

within a type, for example H5, antibodies may not be cross-reactive. With the increase in surveillance, the number of unique isolations and the number of avian species that serve as hosts for the various influenza types will be expected to increase. The NA would seem from the perspective of antibody epitopes to be more stable since the number of known NA types has not increased in recent years.

Ultimate genetic relationships of AIV strains depend on the nucleotide sequence analyses of genome segments. Phylogenetic comparisons of isolates allow determination of origins of newly isolated strains. These studies have been facilitated by the use of reverse transcriptase PCR and rapid nucleotide sequencing facilities. Garcia *et al.* (1996) sequencing HA of 18 H5N2 strains found that none were identical and mutations were not restricted to specific regions. However, comparisons did identify two lineages of these strains. Although nucleotide sequences of more conserved genes of various isolates are also often compared, the NA and HA sequences are most often used for such studies. For example, using information from the NA and HA genes, an H5N1 isolate from poultry in Germany was shown to be closely related to isolates of H5N1 circulating at the time in wild birds (Weber *et al.,* 2007). This is not to discount the importance of the genes encoded by the remaining 6 segments which are much more conserved.

All influenza A viruses share the same repertoire of viral proteins and genome segments, which seem to be readily exchanged when multiple strains infect the same host cell to produce viable chimeric progeny (Richman *et al.,* 1977; Desselberger *et al.,* 1978; Hayashida *et al.,* 1985). The segmented nature of the genome provides a ready mechanism for genetic reassortment of information between two viruses infecting the same cell (Fig. 2). Genetic shift refers to large simultaneous changes in information. The progeny of dual infection may theoretically be any combination of segments of the contributing parents. Viral RNA dependent RNA polymerases are notorious for generating and accumulating mutations, which are regularly incorporated into newly synthesized viral genomes and available to be packaged into progeny virus (Holland *et al.,* 1982). Virus with errors that either do not affect virus replication or improve the viability will predictably survive in progeny virus. The incorporation of mutations from polymerase errors is referred to as genetic drift.

Viruses adapt to their environment by selecting for characteristics that are most favorable for survival. It should not be surprising that avian and human viruses have receptors that specialize in the host for which they have been adapted. Recent studies have indicated that the AIV prefer the α-2,3-linked-sialic acid as receptor which is abundant

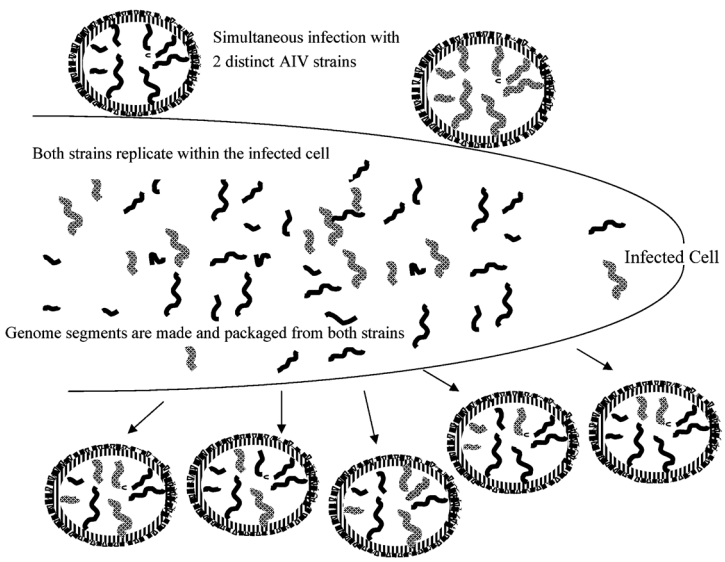

Fig. 2. **Schematics illustrating reassortment of AIV genome segments (genetic shift)**

throughout the respiratory tract of chickens (Matrosovich *et al.,* 2004a; Thompson *et al.,* 2006). In contrast, this receptor is found mainly in the lower respiratory tract of humans. It follows that whereas the virus generated in the upper respiratory tissues of chickens is readily transmitted to other hosts, transmission is more problematic when replication is restricted to tissues deep within the lungs, as with the avian virus in humans. The human adapted strains that readily transmit from human to human prefer the α-2,6-linked sialic receptor that is, in fact, abundant on cells throughout human respiratory tissues. Therefore, the human adapted virus is easily transmitted from the respiratory tract of one person to another.

Immune Responses

The preferred strategy for controlling AIV is to eliminate potential exposure to all AIV through adequate biosecurity. Overall, biosecurity precautions in commercial operations are successful in preventing infections in poultry. Outbreaks of AIV more often occur in live bird markets, with less security and risks of exposure to wild-birds or other potential sources for AIV exposure. When AIV is identified in a flock, the flock is destroyed as are poultry lying in defined distances from the center of the outbreak. Currently, vaccines are not encouraged for use in the United States unless HPAI is a known problem. Vaccines then are easily justified when birds are at risk of AIV infection, during or following known viral activity in a region. There are convincing arguments that the presence of LPAI strains creates an environment for evolution of HPAI (Halvorson, 1997). Halvorson (1997) showed that vaccine control of mildly LPAI reduced the risk of HPAI infection. Flocks with established AIV immunity reduce the circulation of AIV in the live markets and in commercial operations. Presumably, LPAI evolve into to HPAI, as through genetic drift or point mutations. Therefore, just minimizing the viral loads and activity of the LPAI within a flock discourages the appearance of HPAI. An alternative explanation, that is not mutually exclusive, could be that enhancement and maintenance of immunity through vaccines to AIV may inhibit the potential influx of new viruses.

Criteria for practical use of avian vaccines include cost, safety, and efficacy. The classical approach is to concentrate on developing vaccines that induce antibodies that neutralize viral infection or attachment to the infecting host cell, that is humoral immunity (Fig. 3). A prospective vaccine is administered to the chicken and the antibody response of collected sera is evaluated for antigen reactivity, as with an ELISA, for virus neutralization and, most importantly, for *in vivo* protection from challenge infection. With the exception of the last, the process of evaluation is biased for detecting antibodies. Although B cell induction resulting in antibody production implicates T helper ($CD4^+$) lymphocyte responses, vaccine studies rarely consider adaptive T cell immunity. Cellular immunity following antigen exposure includes virus specific $CD8^+$ T lymphocytes that remove host cells infected with virus. Adaptive cellular immunity is more difficult to assess than humoral immunity, but likely plays significant roles in providing protective immune responses to challenge virus in nature and in effective vaccines (Fig. 3). T lymphocyte stimulation by virally in-

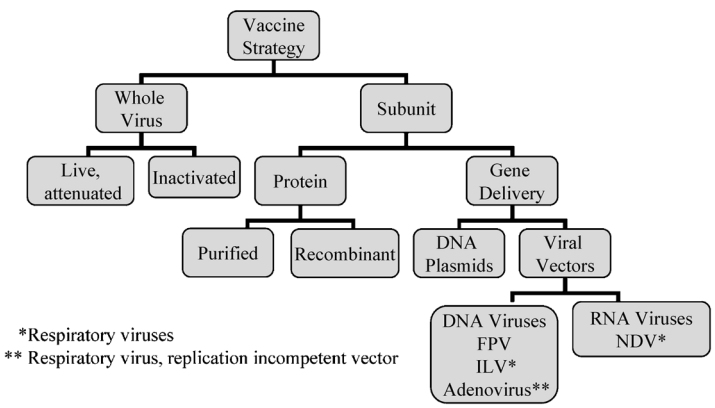

Fig. 3. **Flow chart of selected, published vaccine strategies**

fected cells or cellular immunity depends on the recognition of viral peptides in association with the major histocompatibility proteins (MHC). In spite of the likely contributions to the overall effectiveness of vaccine protection against challenge infection, $CD8^+$ T lymphocyte responses in chickens have only been characterized for basic science curiosity and not for routine vaccine evaluation (Thacker *et al.,* 1995; Seo and Collisson, 1997; Seo *et al.,* 1997; Collisson *et al.,* 2000; Seo *et al.,* 2000; Seo and Webster, 2001; Pei *et al.,* 2003). Seo and Webster (2001) applied the experiences from IBV studies to identify AIV specific $CD8^+$ T cells from H9N2 infected birds, demonstrating cross-protection of $CD8^+$ T lymphocyte from chickens infected with H9N2. Adoptive transfer of $CD8^+$ T cells from AIV infected H9N2 were capable of protecting MHC compatible chickens against challenge infection with H5 N1. The presence of H9N2 infection associated with the HPAI H5N1 infections in Hong Kong market birds was suggested to explain the lack of HPAI pathogenesis in 1997 (Shortridge, 1999). Vaccines, such as attenuated, live or viral vectored vaccines, that would induce both viral specific antibodies and $CD8^+$ lymphocytes, would be predicted to be more efficacious and have a greater likelihood of providing some kind of cross-reacting immunity. Whatever the mechanism of protection, the ultimate test of a vaccine is *in vivo* protection against challenge virus.

The cleavage of the HA into two subunits (HA1 and HA2) generate the total of 11 potential AIV proteins that could contribute to variations in virulence and adaptive immunity, in particular, cellular immune responses. Neutralizing antibodies are made in response to the HA (specifically, the outer HA1 subunit) or the NA. The additional 9 viral proteins, or 10, if HA1 and HA2 are included as distinct, could be potential targets for adaptive cellular immunity. The HA2 was shown in mice to house protective cross-reactive epitopes (Kuwano *et al.,* 1988). In mammals, the NP is thought to house the major T cell epitopes. The antigenic determinants for T cell immunity, those that interact with the T cell receptors on either $CD4^+$ or $CD8^+$ T lymphocytes, are more conserved than the epitopes that react with neutralizing antibodies, which are the active players in the selection of HA or NA variation. T cell epitopes are not under the selective pressures of the epitopes associated with neutralizing antibodies. Antigenic determinants for T cell receptors, consequently more conserved among AIV strains, are reasonable targets for vaccine development.

Attenuated Vaccines

Early development of AIV vaccines concentrated on attenuated, live virus. Live vaccines, effective in the prophylactic control of many viral infections, amplify the amount of virus and viral proteins within host cells and present the entire range of viral antigenic determinants to the cellular and humoral arms of the adaptive immune system (Fig. 4). The 1971 turkey isolate, influenza A/turkey/oregon/71 virus, shown to be avirulent in chickens, was evaluated experimentally years ago as a potential vac-

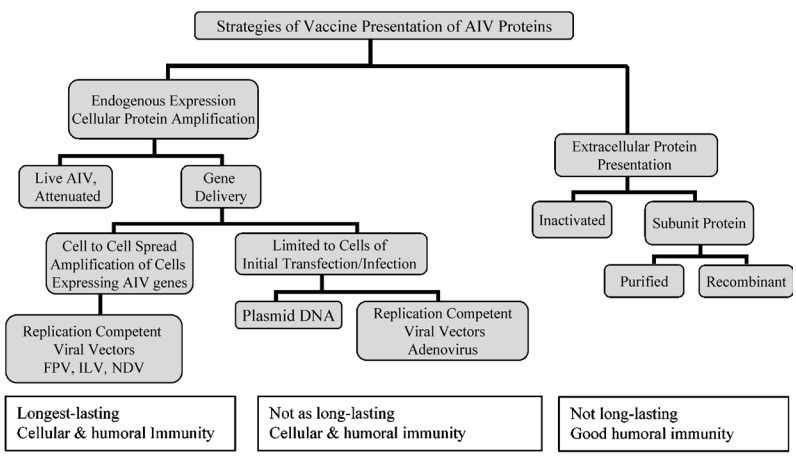

Fig. 4. **Immune responses of selected, published vaccine strategies**

cine in chickens (Butterfield and Campbell, 1978). Inoculated intratracheally with the live virus, chickens developed specific antibody. Virus from the infected chickens did not spread to cage mates, when they were in contact with naive chickens 4 days after infection. The stability of attenuation was demonstrated in studies that showed virus remained avirulent at least for 10 passages through chickens. Crawford *et al.* (1998) demonstrated that oral vaccination of chickens with live AIV resulted in protective immunity and prohibited cloacal shedding of virus inoculated by the oral route. Oral administration would primarily induce mucosal immunity and presumably be more relevant for a virus that targets the respiratory and enteric systems, entry sites for AIV infection.

Attenuation of virus candidates for vaccine use has relied mostly in the past on serendipity and those modifications responsible for attenuation are at most, poorly understood. While AIV proteins, except HA, and to a lesser extent NA, are relatively well conserved, small differences in any of the viral proteins could theoretically influence the ability for the virus to optimally replicate in a host and to cause disease. Mutations, especially in the HA, also can and do result in changes in the host range, such as adapting from wild birds to chickens or from any avian species to mammals, and can become more virulent, as when the proteolytic site or proteolytic enzyme preference is altered (Ohuchi *et al.,* 1989; Perdue *et al.,* 1996; Garcia *et al.,* 1996). The breakthrough development of reverse genetics methodology for Orthomyxoviruses provided the recombinant DNA tools to construct molecular clones of AIV, and modify the virus at the gene level. The methodology involves transfection of cultured cells with individual DNA plasmids representing each RNA genome segment (Lee *et al.,* 2005). Initially, in-order to provide the polymerase activity that is required for making viral RNA in cell culture, additional plasmids specialized in making functional viral polymerase activity were additionally incorporated into the reverse genetics system (Luytjes *et al.,* 1989). However, further modifications resulted in generation of viable virus from cDNA following transfection of only the 8 plasmids representing each segment (Hoffman *et al.,* 2000). The results are a powerful methodology that allows construction of recombinant virus that can be used for defining gene and protein function and potentially in constructing attenuated recombinant vaccines.

Attenuation required for safe vaccines has required more than the modification of the HA protein (Liu *et al.,* 2003). Modification of HA and other gene modifications, such as elimination of the NS1, which, when present, incapacitates cellular IFN, can provide safer live, AIV vaccines. Heterologous NA may be placed in constructed recombinant AIV providing a tool for differentiating between the vaccine and infecting field strains. Traditional, attenuated, live AIV vaccines have additionally not been a practical option for several reasons. The notorious capacity for cloacal shedding of viable AIV into the environment is a critical consideration when evaluating a candidate live vaccine and its efficacy in controlling against challenge infection. Furthermore, the potential for genetic drift through point mutations and genetic shift through reassortment of the AIV genome segments following infections with more than one virus provide mechanisms for generating novel, potentially more virulent virus. The capacity for reassortment of genome segments, which could overcome the effects of attenuated genes if a virulent virus simultaneously infected the same host should be addressed and methods to prevent reassortment should be developed. It has been strongly suspected that LPAI viruses left uncontrolled evolve into HPAI strains. Nucleotide sequencing has shown that determined genotypes of HPAI are disturbingly similar to those of LPAI. The attenuated strains could potentially similarly mutate. Enrichment of the genetic pool of AIV that could further alter species tropism has more than dampened enthusiasm for strategies that promote live vaccines. Overall, alternatives to live AIV based vaccines are currently preferred.

Inactivated Whole Virus Vaccines

The safer, inactivated whole AIV vaccines have been used with certain success in controlling infection with the homologous virus. With meticulous standardization, AIV, inactivated with beta-propriolactone and introduced i.m. to chickens in an oil emulsion mixture, was shown to induce immunity that protected against infection, disease and spreading (Wood *et al.,* 1985). In addition, following vaccination of turkeys with an inactivated virus and viral challenge, both the numbers of birds shedding

virus and the amount of virus were reduced (Brugh *et al.,* 1979; Karundakaran *et al.,* 1987; Donahue, 1997). However, protection in turkeys with inactivated vaccines was less effective than in chickens (Brugh *et al.,* 1979; Donahue, 1997). HI titers were consistently lower in turkeys than chickens in controlled studies using inactivated vaccine.

The duration of protection afforded by inactivated vaccines is limited (Donahue *et al.,* 1997; Cardona *et al.,* 2006). Following an outbreak of H 6N2 in California, three flocks were vaccinated with inactivated homologous virus. Cardona *et al.* (2006) showed that even after 2 inoculations when the inactivated vaccine induced specific antibody, protection lasted less than 21 weeks. Donahoe *et al.* (1997) demonstrated that HI activity could be relied upon for only 5 weeks. The limited duration of protection, especially compared with live vaccines may be related to the fact that inactivated vaccines do not amplify within the host. Therefore, the amount administered is the amount presented to the host. As with other types of vaccines that neither amplify virus nor viral protein within the host cells following inoculation, they are less effective at inducing adaptive cellular immunity.

A disadvantage to using whole homologous virus, whether inactivated or live, is the inevitable presence of antibodies to the HA, NA and NP, antigens used for diagnostic detection of AIV. The use of vaccine strains with homologous HA and heterologous NA types of challenging virus demonstrate the importance of HA type in protection, while leaving NA for diagnostic differentiation (Karundakaran *et al.,* 1987). McNulty *et al.* (1986) went even further in designing a strategy that did not interfere with HA diagnostic differentiation. They used homologous NA vaccine and challenge with heterologous HA. Although mortality and disease were reduced, birds were not protected against virus infection or shedding. Diagnostic antibodies to the conserved NP in any whole virus vaccine would not be expected to differentiate from the challenge virus.

Subunit Protein Vaccines

Subunit vaccines induce host immunity to selected individual AIV proteins, rather than whole virus. Traditional subunit vaccines consist of administration of proteins purified from virus. Subunit vaccines administered as purified proteins do not amplify within the host cell. Kodihalli *et al.* (1994) administered to turkeys highly enriched preparations of NP/HA proteins in an immunostimulating complex (ISCOM). The turkeys developed high AIV specific antibody and cellular immunity as identified by T cell proliferation and delayed-type hypersensitivity assays. Levels of HA and NA heterologous challenge virus and homologous challenge virus were decreased in the trachea and lungs of vaccinated birds. Recombinant technology further allowed for purifying viral proteins from systems that made large amounts of the desired protein. Baculovirus expressing only the AIV HA, either H7 or H5 was used to generate easily purified HA protein (Crawford *et al.,* 1999). The recombinant HA, H7 or H5, induced protective homologous immunity and reduced shedding. Because HA and NA in their natural forms are glycosylated, the eukaryotic recombinant systems with mechanisms for glycosylation, such as baculovirus, are preferred to the prokaryotic bacterial systems.

Gene delivery vectors, including DNA plasmids and viral vectors, function by expressing individual viral proteins of interest within host cells. These vaccine candidates are recombinant vectors that express mRNA. Therefore, subunit expression vectors amplify the viral protein(s) of interest within host cells. The HA is an obvious choice for subunit vaccines because it does induce neutralizing antibodies. The NA is a second choice, because although infection occurs, the replication of virus in dramatically reduced in the presence of specific immunity (Allen *et al.,* 1971; Rott *et al.,* 1974; Webster *et al.,* 1988). However, proteins with antigenic determinants that potentially have a more universal function in replication of AIV would be more conserved among AIV serotypes. A history of an epitope being conserved predicts genetic stability and resistance against genetic drift. In mice, the conserved ion channel M2 has also been shown to induce protective immunity and has become a target for vaccine development in mammals (Slepushkin *et al.,* 1995; Frace *et al.,* 1999; Livingston *et al.,* 2006). Viral antigens expressed by a gene delivery vector will also be processed into peptides for MHC presentation and induction of cellular immunity. Proteins may also be selected on the basis of inducing strong cellular immunity. The more conserved viral proteins are logical sources of induction of heterologous

cellular immunity, but could also be present on conserved regions of the otherwise variant HA and NA. An AIV protein being exploited for induction of cross-reactive immunity is the genetically stable NP, the most abundant AIV protein in the infected cell and viral particle. The NP is known to house cytotoxic T cell epitopes as demonstrated in mammals (Wysocka and Bennink, 1988). However, other AIV proteins may also be worthwhile choices for subunit expression vectors, especially for peptide presentation to and stimulation of T lymphocytes. It would be of interest to use the M2 for protection in chickens and determine the response of chickens to NP and M2.

DNA Plasmid Vaccines

In recent years, considerable effort has been devoted to engineering recombinant vectored AIV vaccines. These vaccines are safe since they present individual AIV antigens to the host in the absence of the complete AIV particle. They depend on the expression of genes carried within the vector not in proteins administered *in vivo.* The simplest are the DNA vaccines. These consist of DNA plasmids of bacterial (prokaryotic) origin. DNA plasmids are the tools used by the molecular biologist to manipulate genes and generate recombinant DNA. Virtually all molecular cloning techniques utilize plasmids for modifying and shuttling genes from one source to another. Even the cloning of RNA requires the generation of the DNA equivalent, which is inserted into a DNA plasmid. Although of bacterial origin, plasmids constructed for expression within eukaryotic cells carry promoter sequences that signal synthesis of mRNA within nucleated cells. These systems of eukaryotic expression have commonly been used in cell culture. It was remarkable when it was discovered that this tool for shuttling and manipulating DNA in bacteria and cell culture could be directly used for induction of immunity when administered *in vivo* (Benvenisty and Reshef, 1986; Wolff *et al.,* 1990; Acsadi *et al.,* 1991). The simple cellular administration of DNA encoding the gene of interest is referred to as transfection. If the DNA encodes the necessary sequences for transcription of mRNA, the transfected cells whether *in vivo* or *in vitro* become factories synthesizing antigen, thus amplifying the amount of protein that is available to induce immunity (Wolff *et al.,* 1990). One of the earliest reports of a successful DNA vaccine was the protection of chickens from challenge HPAI H7 following inoculation of a plasmids encoding the HA of H7 (Fynan *et al.,* 1995). They showed that 60% of the chickens receiving the DNA HA expression plasmid were protect as compared to only 3% that survived in the control birds.

Unlike inoculation of pathogen protein subunits, DNA expression plasmids typically induce T helper 1 immunity triggering $CD8^+$ lymphocyte responses rather than the T helper 2 immunity that consists of more vigorous antibody or B lymphocyte responses (Deck *et al.,* 1997). The DNA plasmid expression studies in mice have demonstrated that the NP is a potent inducer of cytotoxic T cells that cross-protect against influenza viruses with heterologous HA types (Ulmer *et al.,* 1993). Although most studies describing T cell responses to DNA vectors have been in mammals, a DNA plasmid expressing the NP of the avian coronavirus did induce a chicken $CD8^+$ T cell response that protected chicks from acute clinical illness (Seo *et al.,* 1997; Collisson *et al.,* 2000). Whereas the humoral responses include antibodies that neutralize virus HA binding, the induction of $CD8^+$ lymphocytes is valuable in eliminating infected cells, thus targeting antigenic determinants that are conserved. As mouse studies had indicted that DNA expression plasmids for the NP of AIV provided cross protection, Kodihalli *et al.* (2000) inoculated chickens with DNA plasmids expressing both HA (using H5 and H7) and the conserved NP and evaluated protection against AIV. This study indicated that birds were provided limited protection against the homologous or heterologous AIV. The methods used for presentation of the DNA and the number of times DNA was administered enhanced the vaccine efficacy. AIV specific antibodies are not necessarily detected after DNA vaccination, it seems that the B cells are primed such that vigorous antibody responses occur following the virus challenge (personal observations; Roh *et al.,* 2006). Protection against challenge infectious bursal disease virus (IBDV) in chickens was attributed to T cell responses rather than antibody (Kim *et al.,* 2004). Antibody responses may be enhanced to respectable levels with booster vaccine doses and subcutaneous routes (Lee *et al.,* 2006).

An unexpected benefit of DNA vectors is the

capacity for the DNA plasmids alone to enhance immunity. These bacterial derived plasmids can induce a level of cellular immunity even in the absence of expression of the viral protein (Pisetsky, 1996; Chu *et al.,* 1997; Manders and Thomas, 2000; Heeg and Zimmermann, 2000). The cellular immune system recognizes the bacterial DNA motifs (CpG) on the plasmid as foreign and responds by secreting cytokines. Cytosine, not methylated in bacteria, must be unmethylated in order to induce innate immunity. Furthermore, nucleotide motifs flanking CpG contribute to the successful or optimal induction of cytokines. Immunity to IBDV in chickens was significantly enhanced with the additional administration of CpG oligodeoxynucleotide motifs (Mahmood *et al.,* 2006). The effects of DNA plasmid-induced immunity is dependent on the viral antigen produced and the cross protective enhancing effect of the DNA itself.

Considering the amount of DNA required for multiple inoculations, a major disadvantage of using DNA plasmids as vaccines is that the process of preparing enough DNA is relatively expensive for routine use. Although the expense of DNA vaccines may prohibit its practical use in poultry operations, DNA expression vectors are valuable resources for comparing responses to antigens and are useful mechanisms to expand recombinant vectored applications. Novel modifications have been made to enhance the immune response to DNA vaccine candidates. Methods for improving *in vivo* transfection are continuing to be developed in order to enhance the efficacy of protein expression. Protection in chickens against IBDV was enhanced when the DNA plasmid vector was administered with liposomes, which promote cell fusion and entry (Li *et al.,* 2003). Because cytokines are natural regulators of immune cells, a promising modification has been to incorporate chicken cytokine genes into expression plasmids. Lillehoj *et al.* (2005) using *in ovo* inoculation demonstrated greater protection against coccidiosis when administered with IL-1, IL-2, IL-15 or IFN-γ genes. Whereas Roh *et al.* (2006) found that IFN-γ enhanced the splenocyte stimulation after DNA vaccination with IBDV, Hsieh *et al.* (2006) found no difference in protection against IBDV when the DNA vaccine was co-administration with or without an IFN-γ expression plasmid.

The general application of DNA vaccines will likely depend on greatly augmenting the response with less material. Modifications may incorporate genes expressing cytokines or other immune enhancing proteins that promote the interactions between the infected cell and the responding lymphocytes. The DNA vaccine, a potent stimulator of cellular immunity offers a strategy that could target cross-reactive AIV epitopes. DNA vaccines are safe because they are not infectious, do not encode the viral proteins necessary to be transmitted from cell to cell and are not capable of causing disease within the host. The strategies for enhancing DNA vaccine efficacy in chickens against AIV needs to be further investigated.

The safety of DNA vaccines is assured, in part, because they are not infectious. However, while the plasmid directed expression within host cells generates many copies of the viral antigen, the number of cells participating in the process is limited. Most viral vectors, in contrast, are replication competent so that they deliver genes into cells for expression of antigen, assure that more vector virus will be generated and assure that progeny vector will be transmitted to additional cells. Thus, both the antigen and the vaccine vector will be amplified within the chicken. Several viruses that are known to replicate in poultry have been modified for use as vehicles of delivery and protein expression for AIV genes. Most began as common viruses of poultry.

Viral Vectored Vaccine

Fowlpox virus (FPV), a large DNA virus, is thus far the most studied delivery viral vector for AIV. Fowlpox viruses replicate only in avian species and therefore, are not zoonotic and are safe for personnel (Beard *et al.,* 1991; Beard *et al.,* 1992; Skinner *et al.,* 2005). Fowlpox virus vectors expressing AIV proteins have been applied to field use. With more than 80 years of use, the poultry industry has experience with fowlpox vaccines in controlling for pox disease (Pastoret and Vanderplasschen, 2003; Skinner *et al.,* 2005). The poxviruses are among the largest of viruses with equivalently large genomes. The genomes of FPVs can handle large amounts of foreign material. While poxviruses have DNA genomes, unlike other DNA viruses, they replicate entirely in the cytoplasm not in the nucleus. Their large genomes encode multitudes of functional proteins that make them the most independent of all

viruses from their host cell. The genomes of FPV strains range between 260,000 and 309,000 nucleotides, whereas in comparison the entire genome of AIV with its 8 segments is about 13,500 nucleotides in length (Afonso *et al.,* 2000; Lamb and Krug, 2001). Genes of the poxvirus double stranded genome while allows transcript of mRNA from both directions encode a complex array of at least 260 potential proteins (Afonso *et al.,* 2000). Genes have been identified that encode 55 proteins that are present in the virus particle. Twenty-six genes were identified that are involved in making mRNA. Genes have also been described that encode homologues that metabolize nucleic acid, replicate and repair DNA, modify proteins and evade and/or mimic the host immune system.

In 1988, Taylor *et al.* reported that they had constructed a fowlpox vector for the HA of an HPAI virus. They further showed protection when the recombinant virus was administered to chickens and turkeys and challenged with the same or even heterologous HPAI virus. Tripathy and Schnitzlein (1991) also developed a recombinant fowlpox expressing the H5 HA. They inserted the HA into the fowlpox thymidine kinase gene. Chickens inoculated twice with the recombinant virus, each time by both the wing web puncture and comb scarification, developed antibodies that inhibited hemagglutation with the H5N9 (A/Ty/Wis/68) virus, although the sera did not inhibit the heterologous H4N8 (A/Ck/AL/75 virus. Homologous protection in challenge studies using a HPAI H5N2 was reported following vaccination with the fowlpox H5 vector. The vector infection resulted in typical fowlpox lesions that were more severe with the empty control virus not carrying the HA gene. Therefore, the insertion of the HA decreased the virulence of the vector. None of the vaccinated birds displayed signs of clinical illness.

Following a serious outbreak in Mexico in the late 1990's, the practical application of a fowlpox-HA vaccine was tested along with an inactivated HA vaccine. The fowlpox HA (A/Turkey/Ireland/1378/83 from an H5N8) recombinant was found experimentally to protect against chicken infection with the H5N2 Mexican strain (A/chicken/Queretaro/14588/19/95) dramatically reducing shedding and bird-to-bird transmission (Swayne *et al.,* 1997). In 1997, Garcia-Garcia *et al.* also reported field studies in Mexico with the fowlpox recombinant carrying the H5 from turkey//Ireland/1378/83 (H5N8). The Mexican field studies designed to determine risks by vaccinating 32,000 and 48,000 broilers resulted in no adverse effects on production (Garcia-Garcia *et al.,* 1997). It is interesting that although only 5% had serum HI titers, which were also qualitatively low, challenge studies indicated that 90 and 100%, respectively, of the birds were protected and there was no observed horizontal transmission. When regions are at a high risk of losses due to HPAI viruses, the use of this vaccine has been encouraged but only with special approval. It has been shown that protection is inconsistent when birds are inoculated with fowlpox vaccine prior to being given the recombinant fowlpox-HA vaccine (Swayne *et al.,* 2000). Prior exposure to fowlpox likely induces immunity that prevents subsequent successful use of the fowlpox-HA vector vaccine. The recombinant TROVAC-H5 fowlpox-HA vaccine has been licensed in the United States since 1998 (Bublot *et al.,* 2006). Advantages over the inactivated AIV vaccines, which have also been licensed for use, are ease of administration of a single dose sub-cutaneously, ease of monitoring of AIV infection and no adjuvant associated drop in performance. The duration of immunity from the recombinant vaccine lasted at least 20 weeks after a single administration (Swayne *et al.,* 1997). Recombinant fowlpox vaccines expressing appropriate Marek's and IBDV genes have now also been approved (Bublot *et al.,* 2006).

A universal concern of influenza virus infection left uncontrolled is the notorious capacity of these viruses to mutate and evolve, with the HA being under the most pressure for change. Even strains of the same serotype, such as H5, may evolve such that vaccines become ineffective. The long-term practical limitation of an AIV vaccine is its efficacy against heterologous AIV. Swayne *et al.* (2000) challenged chickens vaccinated with a fowlpox vector expressing the HA of A/turkey/Ireland/138/83 (H5N3) and challenged the vaccinated birds with the HPAI homologous virus and eight different HPAI H5 viruses isolated from various parts of the world. The morbidity and mortality of the vaccinated and challenged birds were zero. Three weeks after challenge, virus was detectable in oropharyngeal swabs of birds with 6 of the heterologous

H5 strains, but the numbers of birds with detectable viral titers and the level of titers were dramatically reduced in vaccinated birds challenged with 4 of these heterologous strains. The strains A/Chicken/Queretaro/14588–19/95 and A/Chicken/Pennsylvania/1370/83 caused illness in 90 and 100% of the vaccinated birds, similar to the unvaccinated controls, but the viral titers were about 100 fold less. Cloacal shedding was rarely detected in the vaccinated birds regardless of the strain. Titers of the vaccinated birds (1/10 from the Queretaro, also H5, strain) were at least one log lower than the unvaccinated controls for which titers ranged from 1.91 to 3.88 $\log_{10}$ EID_{50}/m*l*. The sequences of the HA amino acids of these nine H5 viruses were also compared in a phylogenetic tree. Rigorous analyses indicated that there was no correlation between the virus persistence and the sequence relatedness of the HA proteins. Either the sequence comparisons of the whole HA did not reflect the sequences impacting viral neutralization *in vivo* or other factors, such as cellular immunity, were contributing to protection of replication in the respiratory or enteric tissues. However, the fowlpox recombinant was highly efficacious for a wide variety of H5 strains regardless of origin and HA sequence differences. A concern in the exploitation of this vector is that the molecular biology of many of the putative fowlpox proteins have not been studied. Overall, the function and influence on cellular and host pathogenesis of many proteins are, at best, poorly understood.

In order to construct a fowlpox-HA vaccine that is more immunologically reactive, Mingxiao *et al.* (2006) incorporated the chicken IL-18 gene into a single vector with genes from both the H5 and H7 HA. Their fowlpox vector expressing IL-18, H5 and H7 induced protection in 10/10 birds in the absence of shedding as determined at 7 days post-challenge. Although the numbers were small, the incorporation of IL-18 appeared to reduce shedding. A difference in the incorporation of a cytokine in the fowlpox, as opposed to the DNA plasmid studies, which were administered with plasmids expressing cytokine genes is that the cytokines in the latter were encoded on separate vector molecules and may very likely not be expressed in the same host cells. The impact of cytokines may be more effective if assured of reaching the same cell as the viral gene as is the situation with the fowlpox-HA carrying the cytokine gene.

Respiratory Viral Vectors

As AIV in poultry is a respiratory pathogen, vectors that infect and present antigens to the respiratory tract are considered rational choices. In addition to targeting respiratory and enteric immunity, vaccine distribution in aerosols or drinking water are more efficient than s.c. or i.m. inoculatons. The Herpesviridae ILV is a respiratory pathogen of worldwide importance in poultry (Bagust *et al.,* 2000). Similar to other alpha herpesviruses, ILV establishes latency in the trigeminal ganglia (Bagust, 1986). Efficacious, attenuated vaccines, used to control ILV infection throughout the world, protect poultry against mortality and clinical illness, but not latency. These vaccines can be easily administered to large populations of chickens by aerosol or in water. Both humoral and cellular immunity have been shown to be induced by ILV and associated with immune protection (Fahey *et al.,* 1984, York and Fahey, 1990). Although the ILV DNA genome of more than 150,000 nucleotides in length is not as large as the genome of fowlpox viruses, it is still considerably larger than any RNA virus genome. The large genome size and identification of potential sites of foreign gene insertion make ILV an attractive candidate for AIV vaccine delivery. Genes of the ILV have been shown to be dispensable although deletion attenuates the virus *in vivo* (Fuchs *et al.,* 2000; Schitzlein *et al.,* 1995). Replacement of the UL50 gene encoding dUTPase activity with another gene resulted in complete *in vivo* attenuation (Fuchs *et al.,* 2000). The HA of A/chicken/Italy/8/98 with H5 specificity was engineered to replace the UL 50 to generate an ILV-H5 recombinant (Luschow *et al.,* 2001). After administering the recombinant vaccine by the intratracheal route, 23 of 23 birds developed H5 specific HI antibodies. Challenge studies with HPAI homologous H5 virus resulted in complete protection against observed clinical illness and death. Challenge studies with HPAI heterologous H5 virus resulted in protection against death and greatly reduced, although did not eliminate, observed clinical illness. Herpesviruses are notorious as a family in encoding proteins that function in immune or cellular manipulation. Although the understanding of the molecular biology of ILV lags behind many mammalian herpesviruses and the Marek's disease virus of poultry, the expanding un-

derstanding of the molecular biology and comparative homologues is critical for useful exploitation of this potential gene vector. To maximize its potential for gene delivery, more comprehensive research is needed on the functions of ILV genes, especially their role in pathogenesis and requirement for replication. Genes that are nonessential for replication or deleted to increase attenuation may be candidates for replacement by foreign proteins, such as AIV antigens.

Adenoviruses of both mammals and birds typically target the respiratory tract, thus inducing immunity at the site of avian influenza entry. Human adenoviruses have been developed as potential candidates for vaccines even in poultry (Gao *et al.,* 2006; Toro *et al.,* 2006). Full-length HA (H5) was inserted into a human recombinant replication-incompetent adenovirus vector (Gao *et al.,* 2006). This vector will bind to and enter the host cell, and express mRNA and protein from the delivered foreign gene. However, the vector virus does not encode the genes necessary to make more vector virus. The adenovirus-HA vector was given i.n. or subcutaneously (s.c.) to chickens prior to nasal challenge with a HPAI H5 virus. Birds given vector with HA were protected against clinical illness and mortality after HPAI H5 challenge infection and shedding was not observed in any vaccinated birds except at 2 days p.i. After vaccination but prior to challenge, HI antibodies were detected in sera from all birds given the vector by the s.c. route. Following *in ovo* inoculation, but not with i.n. inoculation, Toro *et al.* (2006) demonstrated protection in chickens against challenge homologous virus with the non-replicating human adenovirus-HA vaccine. The fact that human adenovirus can induce AIV immunity in poultry should encourage the adaptation of avian adenovirus vectors which replicate in the avian respiratory tract as a replication competent vector. Induction of mucosal immunity would provide protection at the sites of initial AIV entry. The avian adenovirus is a natural candidate because many strains successfully infect the respiratory tissues without causing disease (Yates *et al.,* 1976; McFerran *et al.,* 2000).

Two common poultry viruses with RNA genomes target the respiratory tissues. The paramyxovirus, Newcastle disease virus (NDV), and the coronavirus, infectious bronchitis virus (IBV) are candidates for delivery of foreign genes. Efficacious, traditional vaccines have been available for many years for NDV and IBV. The developed reverse genetics protocols have provided the tools for gene insertion into their RNA genomes, resulting in expression of foreign genes within host cells. Unlike the herpesviruses and adenoviruses with DNA genomes, these viruses replicate in the cytoplasm. A recombinant NDV carrying the influenza HA has been constructed and tested in poultry. Park *et al.* (2006) engineered chimera virus with the AIV HA1 of H7 inserted into the NDV genome. The HA1 sequences were flanked by oligonucleotides required for expression of the chimeric mRNA and fusion protein. The recombinant NDV-HA was shown to provide 90% protective immunity against AIV challenge with a HPAI-H7) strain and 100% protection against the velogenic NDV. Advantages of this approach to AIV vaccine development are the years of industry experience in using NDV vaccines, low cost of vaccine production and delivery, and a well-developed reverse genetics methodology, in addition to ready transmission to respiratory tissues.

Although molecular engineering of the coronavirus has lagged behind that of the previously described delivery system, including that of the paramyxovirus, recombinant IBV has been shown to express foreign proteins (Youn *et al.,* 2005). The green fluorescent protein gene inserted into the non-essential 5a open reading frame of the IBV genome expressed GFP and the entire gene 5 (a and b) can be deleted and used for recombinant gene delivery (Youn *et al.,* 2005; Casais *et al.,* 2005). An advantage to the use of IBV strains as vectors is that IBV vaccines are not only commonly, but routinely, used, their production is inexpensive and there is a large selection of IBV strains with distinct serotypes from which to chose for gene delivery. However, considerable, although feasible, modifications will need to be made for eventual utilization of IBV as an AIV vectored vaccine.

Challenges of Future Technology

With continuing breakthroughs in our understanding of those factors that promote protective immune responses and those that eliminate viral vector pathogenesis, viral vectors will be better refined for their efficacy and safety. Recombinant vaccines that express selected AIV proteins have

mostly concentrated on the HA. However, genes for NP, which are abundantly expressed during natural AIV infection, have also been incorporated into candidate vaccines. NP is appealing because it is highly conserved and likely to produce broad protection but it has been shown to induce cellular immunity. Other proteins may be considered, including NA and M2. A criterion will be to exclude AIV proteins that could be used for diagnostic differentiation between a natural infection and vaccine inoculation. An advantage of insertion of foreign genes, with gene replacement or without deletions, nearly always results in further attenuation. However, extreme attenuation can result in a vector that does not replicate or, of more concern, does not induce immunity.

A major thrust will be the expansion of potential molecules that when co-expressed with the HA will serve as molecular adjuvants that enhance protective immunity. An ever-increasing number of cytokines are being identified in chickens, most encoding well-characterized mammalian counterpart cytokines. Other potential immune modulating proteins are membrane bound accessory molecules that enhance lymphocyte interactions with APCs and other molecules that promote antigen presentation within APCs. Mammalian herpesvirus VP22 has been shown to facilitate spreading of DNA vectors in dendritic cells, which are the most effective cells for antigen presentation to lymphocytes (Oliveira *et al.,* 2001; Mwangi *et al.,* 2005). The immune responses of mice inoculated with a DNA vector with VP22 fused to the influenza NP was superior to the NP alone and protection was observed with an H1N1 or H3N2 challenge virus (Saha *et al.,* 2006). The Marek's disease virus also encodes a VP22 that similarly promotes the cell-to-cell spreading of antigen (O'Donnell *et al.,* 2002).

A consequence of any modification of the virus vector will be the potential for reduction in infectivity and/or presentation of antigens. Deletion and replacement of genes will likely impact viral replication, most likely creating a less viable virus. Although attenuation is desired, excess attenuation could result in essentially a nonviable virus. The optimal application of each vector system for gene delivery necessitates a thorough understanding of the molecular biology, especially in terms of gene function.

Viral vector technology has ushered in a new era in vaccine development. AIV is probably the most dreaded of zoonotic viruses today (Capua and Alexander, 2006). The seemingly uncontrolled spread of AIV throughout the world and the inevitable continuing evolution of the LPAI to HPAI viruses are issues that are compounded by the range of the susceptible reservoir of wild birds, many of which migrate long distances. The challenge to vaccine design is to enhance any broad immune response that could predictably protect the bird against any number of serotypes. Recombinant vectors are realistic tools for constructing vaccines with immune modulating genes.

Abbreviations: AIV-avian influenza virus; APC-centigera presenting cells; H-hemagglutinin in reference to serotypes; HA-hemagglutinin; HPAI-high pathogenic influenza infection; IBDV- infectious bursal disease virus; IBV-infectious bronchitis virus; IFN-interferon; IL- interleukin; ILV-infectious laryngotracheitis virus; i.m.-intramuscular; LPAI-low pathogenic avian influenza; M1-matrix protein; M2- ion channel protein; N-neuramindase in reference to serotype; NA- neuraminidase protein; NDV-Newcastle disease virus; NEP- nuclear export protein; NP- nucleocapsid protein; s.c.-subcutaneously.

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