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A gene-based avian influenza vaccine in poultry¹

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ABSTRACT Highly pathogenic avian influenza A (HPAI) viruses, specifically H5N1 strains, cause widespread morbidity and mortality in domestic and wild bird populations, and recent outbreaks have resulted in severe economic losses. Although still largely confined to birds, more than 300 human cases resulting in deaths have been reported to the World Health Organization. These sporadic human cases result from direct transmission from infected birds; however, a sustained outbreak of HPAI H5N1 increases the potential for the emergence of a human pandemic strain. One approach to the containment of HPAI H5N1 is the development of vaccines for use in poultry. Currently, the majority of avian influenza vaccines for poultry are traditional whole-virus vaccines produced in eggs. Although highly efficacious, these vaccines are hindered by long production times, inflexibility in quickly altering anti-

genic composition, and limited breadth of protection. Newer vaccines with more efficient manufacturing processes, enhanced efficacy, and cross-protection against multiple strains would improve preparedness. Reverse genetics technology has provided one such method, and emerging gene-based vaccines offer another approach that reduces dependence on egg-based production and human exposure to pathogenic viruses. Gene-based vaccines also provide rapid manufacturing, enhanced precision and versatility, and the capacity to protect against a broad range of viral subtypes. Vectors for these vaccines include replication-defective viruses, bacterial vectors, and DNA. Here we review the features of gene-based vaccination that may facilitate the control of HPAI H5N1 in poultry, and highlight the development of a hemagglutinin-based multivalent DNA vaccine that confers protection in mice and chickens.

Key words: poultry, influenza, vaccine, gene based, deoxyribonucleic acid

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INTRODUCTION

Severe outbreaks of highly pathogenic avian influenza A (HPAI) H5N1 (HPAI H5N1) in birds throughout Asia, Africa, and Europe have resulted in human deaths, raising concern about its potential for adaptation to humans. In addition, the virus has affected the poultry industry, leading to culling of flocks and major economic losses. From 1997 to 2007, more than 250 million birds have been killed or culled, compared with only 23 million in the previous 40 yr (Capua and Alexander, 2004; Food and Agriculture Organization of the United Nations, 2006; Peiris et al., 2007). The economic impact of HPAI H5N1 outbreaks in Asia has exceeded \$575 million (World Health Organization, 2005). The

direct costs from the death or destruction of poultry and agriculture are amplified by indirect costs, which include a significant impact on tourism and commerce. Dissemination results primarily from the movement of the virus through infected poultry, poultry products, and contaminated fomites, but migratory birds have also served as secondary vectors for the virus, rapidly spreading HPAI to Asia, Europe, and Africa (McLeod et al., 2005). Historically, avian influenza viruses have rarely infected humans, although the HPAI H7 and H9 subtypes have increasingly caused human infections (Fouchier et al., 2004; Taubenberger et al., 2007). However, H5N1 viruses killed 6 out of 18 infected people in the Hong Kong severe acute respiratory syndrome outbreak in 1997 (Capua and Alexander, 2006; Cinatl et al., 2007b). In addition, there is evidence that US poultry workers, swine, swine farmers, and veterinarians have been infected with avian influenza virus (Gray, 2008). Such zoonotic infections raise the concern that human hosts may facilitate viral reassortment and the creation of new subtypes. It has long been hypothesized that swine serve as “mixing vessels” between human and avian influenza, as supported by the finding that

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the pig trachea contains receptors for both human and avian influenza viruses (Ito et al., 1998; Van Reeth, 2007). An exchange of genes between these 2 viruses might grant the highly pathogenic avian virus transmissibility in humans, which could potentially result in a novel pandemic strain. However, the speculation that swine serve as intermediates between human and avian influenza has yet to be proven; no human seasonal strain containing swine influenza genes has yet been isolated. Evidence suggests that there are strong barriers preventing the infection of pigs by influenza viruses in other species, and major genetic events would have to take place for an avian virus to gain transmissibility in humans via swine (Kuiken et al., 2006; Van Reeth, 2007).

Whether human pandemics have avian origins is debatable (Vana and Westover, 2008), but HPAI H5N1 poses a significant threat for a human pandemic as it continues to affect poultry populations. Presently, the most effective means of controlling a potential pandemic virus in humans is vaccination, but vaccines are also an effective tool in the control of avian influenza in poultry when combined with adequate biosecurity.

Influenza Viruses

Influenza viruses come in 3 different types: A, B, and C. Type A demonstrates the ability to infect a wide host range, potentially resulting in epidemics and pandemics. Type A influenza viruses have a large number of subtypes and are identified by their surface glycoproteins, consisting of 1 of 16 hemagglutinins (**HA**) and 1 of 9 neuraminidases. Hence, the H5N1 subtype contains H5 HA and N1 neuraminidase. An effective vaccine for influenza A must induce humoral immunity, particularly neutralizing antibodies, against these glycoproteins, primarily the HA component. Vaccines are subtype specific, although cross-reactivity between the strains of the same subtype is occasionally seen. However, vaccines generated against one subtype do not neutralize a different subtype.

Vaccine development for avian influenza is particularly challenging because of frequent, unpredictable changes in viral structure via antigenic drift and antigenic shift (Tosh and Poland, 2008). Antigenic drift refers to minor, gradual changes to the surface antigens through high rates of point mutations, a consequence of the error-prone RNA-dependent RNA polymerase of the virus. Antigenic shift is a drastic change that results from a reassortment of genes via the coinfection of 2 influenza viruses in one host (Capua and Alexander, 2006; van den Berg et al., 2008). In reassortment, the unique, segmented nature of influenza viruses allows for the mixing and matching of genes, causing major changes in the viral structure and potentially the creation of a novel subtype. In fact, direct transfer of virus from one host type to a different host type has been established in canine species with equine influenza H3N8 (Crawford et al., 2005). Antigenic drift is responsible

for seasonal epidemics of influenza, whereas antigenic shift is associated with more serious consequences, such as a pandemic, because no previous immunity exists for a novel subtype.

Current Vaccines

Because of the high propensity of influenza viruses for mutation, vaccines must continually be updated to keep pace with the variation in surface antigens. For a vaccine to be licensed by the Animal and Plant Health Inspection Service of the USDA, it must meet standards in 4 criteria: 1) purity (exclusively desired compounds, consistent in production), 2) safety (no harmful effects on the host or environment), 3) efficacy (quantified standards of protection), and 4) potency (protection in a variety of conditions, reasonable dosages; Myers and Morgan, 2003; Swayne, 2008). The majority of currently available influenza vaccines for poultry are inactivated whole-virus vaccines, which are produced by rendering a live virus harmless through a series of physical or chemical processes (Qiao et al., 2006; Cinatl et al., 2007a; Marangon et al., 2008). In these processes, a wild-type donor virus is coinfects in embryonated eggs with an egg-adapted influenza strain. Clones are then screened until a reassortant is identified that has the appropriate HA profile of the pathogenic strain, yet is adapted for growth in eggs. The seed strain is then rendered noninfectious by treatment with formaldehyde or β -propiolactone (Webby and Sandbulte, 2008). These vaccines elicit an immune response in a variety of poultry species and do not pose any dangers to the host because an inactivated virus cannot replicate; however, they must also be administered in multiple doses to achieve a neutralizing titer. So far, inactivated whole-virus vaccines have been shown in clinical trials to be very effective in protecting both poultry and humans against highly pathogenic influenza viruses, especially when administered with an oil-in-water adjuvant such as MF59 (Leroux-Roels et al., 2007; Bernstein et al., 2008). However, it is important to note that in poultry, the vaccine may only protect against disease, and birds may shed variable quantities of virus, depending on individual responses and the homology between the vaccine strain and the HA of the challenge strain. In 2004, the USDA approved the development of a vaccine bank to produce up to 40 million doses of inactivated H5N2, H5N9, H7N2, and H7N3 vaccines for poultry (Becker, 2004).

A serious concern with inactivated vaccines is the lack of efficiency in production and the exposure of laboratory personnel to pathogenic viruses. These vaccines use viruses that are grown in embryonated eggs, a process that requires large biocontainment facilities for highly pathogenic viruses. Manipulation and handling of these wild-type avian influenza viruses involve high levels of biosafety requirements, and it usually takes at least 6 mo to produce an inactivated vaccine by using this method (Liu et al., 2006; Wright, 2008). These

severely limited time constraints are a serious concern for preparedness for large outbreaks. Thus, research efforts have been accelerated to develop new production methods and alternative strategies to reduce production time while continuing to elicit safe, sufficient immune responses.

Reverse Genetics

Reverse genetics technology represents a substantial improvement in generating inactivated and live attenuated vaccine prototype strains. Reverse genetics was initially used to investigate influenza pathogenesis by the selective modification of genes to observe their effects on behavior and phenotype (Ahringer, 2006). This technique was used to attenuate viruses through directed mutation of internal genes or the removal of virulence factors in highly pathogenic strains (Subbarao and Katz, 2004). For virus attenuation, this method is more efficient, precise, and versatile than developing a seed strain via the traditional method of attenuation, which requires multiple passages of a virus in eggs until the virus loses virulence. Reverse genetics also enables the generation of replication-competent virus from cloned plasmid DNA (Subbarao et al., 2003; Subbarao and Katz, 2004; Song et al., 2008; Webby and Sandbulte, 2008). Such vaccines have been evaluated for immunogenicity and efficacy against avian influenza in swine, equines, and poultry (Quinlivan et al., 2005; Richt et al., 2006; Webster et al., 2006; van den Berg et al., 2008). These are not considered gene-based vaccines but traditional vaccines with an alternative production method, and therefore will not be discussed further. However, reverse genetics technology bridges the concept of traditional and modern gene-based vaccines.

GENE-BASED VACCINES

Gene-based vaccines differ from traditional vaccines in their ability to allow selective expression of viral gene products in immunized subjects, and they have emerged as a promising new approach to vaccination. An essential component is the vector that permits the delivery of a gene encoding the immunogen *in vivo* rather than injecting preformed virions or viral proteins, as is done conventionally. It aims to elicit immune protection by manipulating viral genes to maximize safety and immunogenicity while allowing for cell culture-based production. In some instances, production can be much more efficient; vaccines can be generated in as little as 4 mo and on a large scale. More important, shorter production times accelerate the process of vaccine deployment after a new pathogen is identified. The vaccines are designed to be free of contaminants and to maximize safety; in most cases, they cannot replicate within eukaryotic cells, and virulence genes can be removed to mitigate any risks posed by reassortment with field strains. In addition, gene-based vaccines can be tailored to target specific virus field strains because pro-

duction schedules allow for the incorporation of newly emerging threats (Swayne, 2008; Wright, 2008). Here, we briefly review 3 types of gene-based vaccines: virus vector-based vaccines, bacterial vector-based vaccines, and DNA vaccines.

Virus Vector-Based Vaccines

Virus vectors deliver genes encoding protective proteins to the vaccinated host, preferably in replication-defective viruses. Protective genes (e.g., HA for influenza A) along with enhancer-promoter and terminator regulatory sequences, are inserted into the viral vector nucleotide sequences. When the viral vector vaccine is injected, the inserted influenza proteins are expressed, and they induce cellular responses, humoral immune responses, or both to influenza virus. These vaccines are readily manufactured under good manufacturing processes. Currently, canarypox virus vector vaccines are licensed for use against rabies and leukemia in cats, as well as West Nile virus and influenza in horses (Poulet et al., 2007).

One viral vectored vaccine has been licensed for use in poultry. The Merial recombinant fowlpox virus AI-H5 (rFP-AI-H5; Merial Limited, Duluth, GA), also known as Trovac AI-H5, is administered in the hatchery, which improves biosecurity and quality control and allows for coadministration with other routinely used vaccines as well as for protection at a younger age (Bublöt et al., 2006; van den Berg et al., 2008). In addition, strategies for differentiating infected from vaccinated animals are readily used with existing serological tests (van den Berg et al., 2008). Although only licensed for chickens, this vaccine has also shown good immunogenicity and protection in other domestic avian and mammalian species, such as ducks and cats (Karaca et al., 2005; Steensels et al., 2007).

Other viral vector vaccines based on recombinant Newcastle disease viruses (**NDV**) and recombinant adenoviruses have been developed and may qualify for licensure. Newcastle disease is caused by avian paramyxovirus I and may result in a very high mortality rate in poultry, depending on the pathotype, but avirulent NDV strains are commonly used as live vaccines. Newcastle disease virus vaccines are produced at a lower cost and greater yield than inactivated vaccines, and can be administered through aerosol sprays, drinking water, or eye drops, reducing the cost of administration (Ge et al., 2007). Bivalent NDV-H5 vaccines engineered by reverse genetics have shown protection against lethal challenges of both NDV and HPAI (Veits et al., 2006; Ge et al., 2007).

Recombinant, replication-defective adenoviral (**rAd**) vectors have also been developed for avian influenza and other varieties of pathogens. A human rAd5 vector encoding an avian influenza H7 HA elicits protective immunity against avian influenza with a single dose in chickens. This vaccine can be injected *in ovo* in conjunction with another adenoviral vector encoding an

H5 HA, eliciting robust antibody responses against both H5 and H7 proteins (Toro et al., 2008). Replication-defective adenoviral-based vaccines efficiently stimulate cellular and humoral responses and allow the incorporation of multiple HA genes, raising the possibility of protection against multiple strains of AI. In fact, experimental DNA prime-rAd boost vaccines were found to induce stronger T-cell responses than cold-adapted live attenuated vaccines in mice, and to confer protection against a variety of influenza subtypes, including highly pathogenic H5N1 viruses (Lo et al., 2008). These findings suggest that viral gene-based vaccination may be able to elicit heterosubtypic immunity (**Het-I**). Heterosubtypic immunity denotes a broader range of immunity that protects against multiple subtypes of influenza A, rather than multiple strains of one subtype, and is the basis for the development of universal flu vaccines. Usually induced by infection with wild-type viruses, Het-I reduces morbidity and mortality and promotes rapid viral clearance. Comparative studies in mice have shown that an experimental DNA prime-rAd boost regimen may induce an Het-I response more readily than current licensed inactivated or live attenuated vaccines (Lo et al., 2008). Although this effect has been observed in mice, whether it can apply to other species, including humans, remains unknown.

One limitation of vectored virus vaccines is that maternal antibodies or preimmunity resulting from natural infections may neutralize the viral vector and possibly affect its efficacy. However, vaccines can be constructed with rare serotypes, chimeric viruses, or adjuvants to avoid or overcome maternal antibodies, and could be administered early in life to avoid other forms of preimmunity. Nonetheless, enhancing these vaccines relies heavily on choosing and developing the most appropriate vector to deliver viral proteins and elicit protective immunity in the host.

Bacterial Vector-Based Vaccines

In addition to viruses, recombinant bacteria have been investigated as a possible vector for influenza vaccines. The concepts are similar to virus vectors; genes from a target pathogen are inserted into bacteria, which are then used to deliver them into the host to stimulate an immune response. Bacterial vectors are advantageous because they are relatively inexpensive, well-suited for large-scale production, and can be administered orally (Shata et al., 2000; Parsa and Pfeifer, 2007). In addition, bacterial vectors inherently trigger an immune response and naturally stimulate the activity of antigen-presenting cells (Matzinger, 1994; Banchereau and Steinman, 1998; Paglia et al., 1998; Shata et al., 2000; Parsa and Pfeifer, 2007). With precise dosages and delivery, bacterial vectors may provide their own natural adjuvant, and could possibly be used in conjunction with viral vaccines to boost immune responses. Despite these various advantages, bacterial vectors may encounter more obstacles because of their

larger size, which limits gene transfer rates, as well as the large numbers of bacterial antigens that might compete with the recombinant vaccine antigen for immunogenicity. In addition, there is a risk of endotoxin and exotoxin release if the bacteria is not attenuated properly, which could result in mild reactions, such as food poisoning, or severe adverse events, such as septic shock (Parsa and Pfeifer, 2007). These reactions can be reduced by choosing strains lacking particular toxins or by counteracting them with antibiotics. Examples of common bacterial vectors are *Salmonella*, Bacille Calmette-Guerin, *Listeria monocytogenes*, and *Shigella*. For avian influenza, attenuated salmonella vectors expressing universally conserved avian influenza ion-channel proteins M2 or M2e have recently been shown to provide broad protection against low-pathogenic influenza challenges (Hargis et al., 2008; van den Berg et al., 2008). However, this vector did not prevent mortality in lethal challenges of HPAI. Although bacterial vectors have potential for gene-based vaccine delivery, their advanced development has proven more challenging than the development of DNA or viral vectors, and few have been used in poultry.

DNA Vaccines

Deoxyribonucleic acid vaccines have received much attention since they were first reported to induce protective immune responses. Viral genes are cloned into eukaryotic expression vectors and inserted into DNA plasmids, which are used to transform *Escherichia coli*. The resulting DNA is purified and administered intramuscularly or intradermally. The DNA is then processed by antigen-presenting cells that produce the viral proteins with their own transcription or translation mechanisms. The viral proteins are then processed through the proteasome and presented on MHC class I and II molecules, where they are detected by the immune system, triggering both cellular and humoral immune responses. Encoding highly conserved sequences from influenza, such as the nucleoprotein or M2, may induce cross-reactive responses against different kinds of influenza viruses. Deoxyribonucleic acid vaccines are safer than conventional vaccines because they are noninfectious and do not replicate in mammalian cells. There is no risk of contracting disease from the vaccine, and negative responses to other vectors are avoided, including preimmunity to the vector. Unlike virus vectors, there are no immune responses against a DNA plasmid vector. This also means that the vaccine can be administered safely and effectively in multiple doses to boost immunity until desired levels are achieved. In addition, the manufacture of DNA plasmid within *E. coli* is fast and efficient, and allows the production of highly pure and stable vaccines to be scaled up (Cinatl et al., 2007a; van den Berg et al., 2008). Deoxyribonucleic acid vaccine technology is expensive relative to other vaccines because the doses required to elicit immunity are relatively large, but ongoing research is addressing

concerns about both the strength of immunogenicity and the cost-effectiveness. However, it is clear that an effective DNA vaccine offers several significant advantages in its great versatility, safety, stability, ease of production, storage, and delivery.

Deoxyribonucleic acid vaccines are already currently available for commercial use against other types of pathogens. Wyeth Fort Dodge Laboratories (Fort Dodge, IA), in conjunction with the Centers for Disease Control and Prevention (Atlanta, GA), developed a DNA vaccine to protect horses from West Nile virus, and this became the first DNA vaccine licensed by the USDA (Holl and Redding, 2005; Dauphin and Zientara, 2007). Shortly afterward, another DNA vaccine developed by Swiss-based Novartis Animal Health (Basel, Switzerland) received approval to protect salmon against infectious hematopoietic necrosis virus (Lorenzen and LaPatra, 2005; Vical Inc., 2005). In addition, the Merial Memorial Sloan-Kettering Cancer Center (New York, NY) and the Animal Medical Center (New York, NY) developed a DNA vaccine that is licensed to treat and prevent canine melanoma (Bergman, 2007; Merial, 2007). Regarding influenza, DNA vaccines elicit protective responses in several different animal models, such as the mouse, chicken, and ferret; however, no DNA vaccines are currently licensed for protection against influenza virus.

A Polyvalent DNA Vaccine for Avian Influenza

We have recently developed an effective trivalent DNA vaccine that elicits broad protection against different H5N1 sublineages in poultry (Rao et al., 2008a). It encodes HA proteins from 3 different H5N1 strains: A/Indonesia/05/2005, A/Anhui/1/2005, and A/Vietnam/1203/2004 (Rao et al., 2008b). The vaccine was developed by first comparing and evaluating different DNA vaccine regimens in mice. High-level expression and immunogenicity vectors were used to encode HA from 10 different strains of influenza virus. In this study, various monovalent and multivalent vaccines conferred protection against a lethal strain of H5N1 A/Vietnam/1203/2004. In chickens, a multivalent H5 HA DNA vaccine elicited broad, robust responses against matched and unmatched challenge strains with as little as two 5- μ g doses. This vaccine can be administered intradermally or subcutaneously with the CO₂-powered, needle-free Agro-Jet device, developed by MIT Canada Inc. (2008). The vaccine effectively generates an immune response that neutralizes the avian influenza virus in chickens and prevents productive replication in the host (Rao et al., 2008b). Although DNA vaccines are generally considered an expensive strategy, the reduction of effective dose (<5 μ g), along with the capability of the Agro-Jet to deliver rapid repeated injections make this DNA vaccine cost-effective and practical for use in poultry.

Conclusions

Major outbreaks of highly pathogenic avian influenza and the imminent threat of a pandemic highlight the need for effective vaccines as a means for control and prevention. The vast majority of current vaccines are inactivated whole-virus vaccines produced in eggs. This production method is limited by the availability of embryonated eggs and by its lack of versatility in quickly altering antigenic composition to respond effectively to a rapidly changing field virus. Thus, newer vaccines with more efficient production technologies are needed for a more timely response to severe outbreaks. Gene-based vaccines have emerged as viable candidates to meet these challenges. Not only can they be produced at a much faster rate and on a larger scale by using safe cell-based production methods, but they can also exhibit the potential for enhanced efficacy against a broader range of influenza strains by stimulating both cellular and humoral responses. New gene-based technologies include replication-defective viral and bacterial vectors and DNA vaccines. Each demonstrates advantages over conventional vaccines, but they continue to face specific challenges, such as variable immunogenicity and cost of production, which may limit their widespread use. A multivalent DNA vaccine that elicits protection in both mice and chickens at low doses now shows promise, and it represents an attractive candidate for licensure and use in poultry. Gene-based vaccine technologies represent an advance in animal influenza vaccine development that provide a viable alternative to existing egg-based and inactivated viral vaccines.

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REFERENCES

- Ahringer, J. 2006. Reverse genetics. WormBook doi/10.1895/wormbook.1.47.1. <http://www.wormbook.org> Accessed July 2008.
- Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature* 392:245-252.
- Becker, A. L. 2004. USDA funds avian flu vaccine bank for poultry. <http://www.cidrapbusiness.net/cidrap/content/influenza/avian-flu/news/nov0504vaccine.html> Accessed July 2008.

- Bergman, P. J. 2007. Anticancer vaccines. *Vet. Clin. North Am. Small Anim. Pract.* 37:1111–1119.
- Bernstein, D. L., K. M. Edwards, C. L. Dekker, R. Belshe, H. K. Talbot, I. L. Graham, D. L. Noah, F. He, and H. Hill. 2008. Effects of adjuvants on the safety and immunogenicity of an avian influenza H5N1 vaccine in adults. *J. Infect. Dis.* 197:667–675.
- Bublot, M., N. Pritchard, D. E. Swayne, P. Selleck, K. Karaca, D. L. Suarez, J. C. Audonnet, and T. R. Mickle. 2006. Development and use of fowlpox vectored vaccines for avian influenza. *Ann. N. Y. Acad. Sci.* 1081:193–201.
- Capua, I., and D. J. Alexander. 2004. Avian influenza: Recent developments. *Avian Pathol.* 33:393–404.
- Capua, I., and D. J. Alexander. 2006. The challenge of avian influenza to the veterinary community. *Avian Pathol.* 35:189–205.
- Cinatl, J. Jr., M. Michaelis, and H. W. Doerr. 2007a. The threat of avian influenza A (H5N1). Part IV: Development of vaccines. *Med. Microbiol. Immunol. (Berl.)* 196:213–225.
- Cinatl, J. Jr., M. Michaelis, and H. W. Doerr. 2007b. The threat of avian influenza A (H5N1). Part I: Epidemiologic concerns and virulence determinants. *Med. Microbiol. Immunol. (Berl.)* 196:181–190.
- Crawford, P. C., E. J. Dubovi, W. L. Castleman, I. Stephenson, E. P. Gibbs, L. Chen, C. Smith, R. C. Hill, P. Ferro, J. Pompey, R. A. Bright, M. J. Medina, C. M. Johnson, C. W. Olsen, N. J. Cox, A. I. Klimov, J. M. Katz, and R. O. Donis. 2005. Transmission of equine influenza virus to dogs. *Science* 310:482–485.
- Dauphin, G., and S. Zientara. 2007. West Nile virus: Recent trends in diagnosis and vaccine development. *Vaccine* 25:5563–5576.
- Food and Agriculture Organization of the United Nations. 2006. Should wild birds now be considered a permanent reservoir of the virus? *FAO AIDE News* 40:1–11. <http://www.fao.org/docs/eims/upload/209858/AVIbull040.pdf> Accessed July 2008.
- Fouchier, R. A., P. M. Schneeberger, F. W. Rozendaal, J. M. Broekman, S. A. Kemink, V. Munster, T. Kuiken, G. F. Rimmelzwaan, M. Schutten, G. J. Van Doornum, G. Koch, A. Bosman, M. Koopmans, and A. D. Osterhaus. 2004. Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. *Proc. Natl. Acad. Sci. USA* 101:1356–1361.
- Ge, J., G. Deng, Z. Wen, G. Tian, Y. Wang, J. Shi, X. Wang, Y. Li, S. Hu, Y. Jiang, C. Yang, K. Yu, Z. Bu, and H. Chen. 2007. Newcastle disease virus-based live attenuated vaccine completely protects chickens and mice from lethal challenge of homologous and heterologous H5N1 avian influenza viruses. *J. Virol.* 81:150–158.
- Gray, G. C. 2008. Pandemic influenza planning: Shouldn't swine and poultry workers be included? 2008 Poultry Sci. Assoc. Keynote Symp. Poultry Sci. Assoc., Savoy, IL. (Abstr.)
- Hargis, B. M., S. L. Layton, D. R. Kapczynski, K. Cole, M. M. Cox, Y. M. Kwon, L. R. Berghman, K. Liljebjelke, and W. J. Bottje. 2008. Development and evaluation of a potential universal *Salmonella*-vectored avian influenza vaccine. 2008 Poultry Sci. Assoc. Keynote Symp. Poultry Sci. Assoc., Savoy, IL. (Abstr.)
- Holl, S., and J. Redding. 2005. USDA issues license for West Nile virus DNA vaccine for horses. http://www.aphis.usda.gov/lpa/news/2005/07/wnvdna_vs.html Accessed July 2008.
- Ito, T., J. N. Couceiro, S. Kelm, L. G. Baum, S. Krauss, M. R. Castrucci, I. Donatelli, H. Kida, J. C. Paulson, R. G. Webster, and Y. Kawaoka. 1998. Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. *J. Virol.* 72:7367–7373.
- Karaca, K., D. E. Swayne, D. Grosenbaugh, M. Bublot, A. Robles, E. Spackman, and R. Nordgren. 2005. Immunogenicity of fowlpox virus expressing the avian influenza virus H5 gene (TRO-VAC AIV-H5) in cats. *Clin. Diagn. Lab. Immunol.* 12:1340–1342.
- Kuiken, T., E. C. Holmes, J. McCauley, G. F. Rimmelzwaan, C. S. Williams, and B. T. Grenfell. 2006. Host species barriers to influenza virus infections. *Science* 312:394–397.
- Leroux-Roels, I., A. Borkowski, T. Vanwollegem, M. Drame, F. Clement, E. Hons, J. M. Devaster, and G. Leroux-Roels. 2007. Antigen sparing and cross-reactive immunity with an adjuvanted rH5N1 prototype pandemic influenza vaccine: A randomised controlled trial. *Lancet* 370:580–589.
- Liu, M. A., B. Wahren, and G. B. Karlsson Hedestam. 2006. DNA vaccines: Recent developments and future possibilities. *Hum. Gene Ther.* 17:1051–1061.
- Lo, C. Y., Z. Wu, J. A. Misplon, G. E. Price, C. Pappas, W. P. Kong, T. M. Tumpey, and S. L. Epstein. 2008. Comparison of vaccines for induction of heterosubtypic immunity to influenza A virus: Cold-adapted vaccine versus DNA prime-adenovirus boost strategies. *Vaccine* 26:2062–2072.
- Lorenzen, N., and S. E. LaPatra. 2005. DNA vaccines for aquacultured fish. *Rev. Sci. Tech.* 24:201–213.
- Marangon, S., M. Cecchinato, and I. Capua. 2008. Use of vaccination in avian influenza control and eradication. *Zoonoses Public Health* 55:65–72.
- Matzinger, P. 1994. Tolerance, danger, and the extended family. *Annu. Rev. Immunol.* 12:991–1045.
- McLeod, A., N. Morgan, A. Prakash, J. Hinrichs, and Food and Agriculture Organization of the United Nations. 2005. Economic and social impacts of avian influenza. <http://www.fao.org/avianflu/documents/Economic-and-social-impacts-of-avian-influenza-Geneva.pdf> Accessed August 2008.
- Merial. 2007. Merial's new vaccine treats deadly cancer in dogs. http://us.merial.com/merial_corporate/news/press_releases/03-26-2007_melanoma_consumer.asp Accessed July 2008.
- MIT Canada Inc. 2008. Agro-Jet. <http://www.mitcanada.ca/products/products.html> Accessed July 2008.
- Myers, T. J., and A. P. Morgan. 1997. Policy and guidance for licensure of avian influenza vaccines in the United States. Pages 373–378 in *Proceedings of the 4th International Symposium on Avian Influenza*, Athens, GA. D. E. Swayne and R. D. Slemons, ed. Georgia Center for Continuing Education, Athens.
- Paglia, P., E. Medina, I. Arioli, C. A. Guzman, and M. P. Colombo. 1998. Gene transfer in dendritic cells, induced by oral DNA vaccination with *Salmonella typhimurium*, results in protective immunity against a murine fibrosarcoma. *Blood* 92:3172–3176.
- Parsa, S., and B. Pfeifer. 2007. Engineering bacterial vectors for delivery of genes and proteins to antigen-presenting cells. *Mol. Pharm.* 4:4–17.
- Peiris, J. S., M. D. de Jong, and Y. Guan. 2007. Avian influenza virus (H5N1): A threat to human health. *Clin. Microbiol. Rev.* 20:243–267.
- Poulet, H., J. Minke, M. C. Pardo, V. Juillard, B. Nordgren, and J. C. Audonnet. 2007. Development and registration of recombinant veterinary vaccines. The example of the canarypox vector platform. *Vaccine* 25:5606–5612.
- Qiao, C., G. Tian, Y. Jiang, Y. Li, J. Shi, K. Yu, and H. Chen. 2006. Vaccines developed for H5 highly pathogenic avian influenza in China. *Ann. N. Y. Acad. Sci.* 1081:182–192.
- Quinlivan, M., D. Zamarin, A. Garcia-Sastre, A. Cullinane, T. Chambers, and P. Palese. 2005. Attenuation of equine influenza viruses through truncations of the NS1 protein. *J. Virol.* 79:8431–8439.
- Rao, S., L. J. DeTolla, D. Perez, D. Smith, W. P. Kong, T. Tumpey, A. Garcia-Sastre, C. J. Wei, Z. Y. Yang, and G. J. Nabel. 2008a. A gene-based vaccine: Multivalent HA DNA vaccination confers long-term protection in mice and chicken. 2008 Poultry Sci. Assoc. Keynote Symp. Poultry Sci. Assoc., Savoy, IL. (Abstr.)
- Rao, S., W. Kong, C. Wei, Z. Yang, M. Nason, D. Styles, L. DeTolla, E. Sorrell, H. Song, H. Wan, G. Ramirez-Nieto, D. Perez, and G. Nabel. 2008b. Multivalent HA DNA vaccination protects against highly pathogenic H5N1 avian influenza infection in chickens and mice. *PLoS One* 3:e2432.
- Richt, J. A., P. Lekcharoensuk, K. M. Lager, A. L. Vincent, C. M. Loiacono, B. H. Janke, W. H. Wu, K. J. Yoon, R. J. Webby, A. Solorzano, and A. Garcia-Sastre. 2006. Vaccination of pigs against swine influenza viruses by using an NS1-truncated modified live-virus vaccine. *J. Virol.* 80:11009–11018.
- Shata, M. T., L. Stevceva, S. Agwale, G. K. Lewis, and D. M. Hone. 2000. Recent advances with recombinant bacterial vaccine vectors. *Mol. Med. Today* 6:66–71.
- Song, J. M., Y. J. Lee, O. M. Jeong, H. M. Kang, H. R. Kim, J. H. Kwon, J. H. Kim, B. L. Seong, and Y. J. Kim. 2008. Generation and evaluation of reassortant influenza vaccines made by reverse genetics for H9N2 avian influenza in Korea. *Vet. Microbiol.* 130:268–276.

- Steensels, M., S. Van Borm, B. Lambrecht, J. De Vriese, F. X. Le Gros, M. Bublot, and T. van den Berg. 2007. Efficacy of an inactivated and a fowlpox-vectored vaccine in Muscovy ducks against an Asian H5N1 highly pathogenic avian influenza viral challenge. *Avian Dis.* 51(Suppl.):325–331.
- Subbarao, K., H. Chen, D. Swayne, L. Mingay, E. Fodor, G. Brownlee, X. Xu, X. Lu, J. Katz, N. Cox, and Y. Matsuoka. 2003. Evaluation of a genetically modified reassortant H5N1 influenza A virus vaccine candidate generated by plasmid-based reverse genetics. *Virology* 305:192–200.
- Subbarao, K., and J. M. Katz. 2004. Influenza vaccines generated by reverse genetics. *Curr. Top. Microbiol. Immunol.* 283:313–342.
- Swayne, D. E. 2008. Avian influenza vaccines and therapies for poultry. *Comp. Immunol. Microbiol. Infect. Dis.* doi:doi:10.1016/j.cimid.2008.01.006.
- Taubenberger, J. K., D. M. Morens, and A. S. Fauci. 2007. The next influenza pandemic: Can it be predicted? *J. Am. Med. Assoc.* 297:2025–2027.
- Toro, H., D. C. Tang, D. L. Suarez, J. Zhang, and Z. Shi. 2008. Protection of chickens against avian influenza with non-replicating adenovirus-vectored vaccine. *Vaccine* 26:2640–2646.
- Tosh, P. K., and G. A. Poland. 2008. Emerging vaccines for influenza. *Expert Opin. Emerg. Drugs* 13:21–40.
- van den Berg, T., B. Lambrecht, S. Marche, M. Steensels, B. S. Van, and M. Bublot. 2008. Influenza vaccines and vaccination strategies in birds. *Comp. Immunol. Microbiol. Infect. Dis.* 31:121–165.
- Van Reeth, K. 2007. Avian and swine influenza viruses: Our current understanding of the zoonotic risk. *Vet. Res.* 38:243–260.
- Vana, G., and K. M. Westover. 2008. Origin of the 1918 Spanish influenza virus: A comparative genomic analysis. *Mol. Phylogenet. Evol.* 47:1100–1110.
- Veits, J., D. Wiesner, W. Fuchs, B. Hoffmann, H. Granzow, E. Starick, E. Mundt, H. Schirrmeier, T. Mebatsion, T. C. Mettenleiter, and A. Romer-Oberdorfer. 2006. Newcastle disease virus expressing H5 hemagglutinin gene protects chickens against Newcastle disease and avian influenza. *Proc. Natl. Acad. Sci. USA* 103:8197–8202.
- Vical Inc. 2005. Vical announces marketing approval of DNA vaccine for salmon. Goliath, The Gale Group, Cengage Learning, Farmington Hills, MI. http://goliath.ecnext.com/coms2/summary_0199-4481412_ITM Accessed August 2008.
- Webby, R. J., and M. R. Sandbulte. 2008. Influenza vaccines. *Front. Biosci.* 13:4912–4924.
- Webster, R. G., R. J. Webby, E. Hoffmann, J. Rodenberg, M. Kumar, H. J. Chu, P. Seiler, S. Krauss, and T. Songserm. 2006. The immunogenicity and efficacy against H5N1 challenge of reverse genetics-derived H5N3 influenza vaccine in ducks and chickens. *Virology* 351:303–311.
- World Health Organization. 2005. Avian influenza: Assessing the pandemic threat. <http://www.who.int/csr/disease/influenza/H5N1-9reduit.pdf> Accessed June 2008.
- Wright, P. F. 2008. Vaccine preparedness—Are we ready for the next influenza pandemic? *N. Engl. J. Med.* 358:2540–2543.