

Novel avian influenza virus vaccines

W. Fuchs, A. Römer-Oberdörfer, J. Veits & T.C. Mettenleiter*

Institute of Molecular Biology, Friedrich-Loeffler-Institut, Südufer 10, 17493 Greifswald-Insel Riems, Germany

*Corresponding author: thomas.mettenleiter@fli.bund.de

Summary

Current vaccines against avian influenza (AI) virus infections are primarily based on classical inactivated whole-virus preparations. Although administration of these vaccines can protect poultry from clinical disease, sterile immunity is not achieved under field conditions, allowing for undetected virus spread and evolution under immune cover. Therefore, there is an urgent need for a robust and reliable system of differentiation between infected and vaccinated animals. Moreover, current AI vaccines must be administered individually, requiring the handling of excessively large numbers of animals, which makes it difficult to obtain high vaccine coverage. Consequently, AI vaccines conferring solid immunity that could be used for mass application would be advantageous. Several approaches are being pursued to improve existing vaccines and develop novel vaccines, all of which will be covered in this overview.

Keywords

Avian influenza virus – Genetic engineering – Recombinant technology – Reverse genetics – Vaccines.

Introduction

Avian influenza (AI) viruses (AIV) are classified into highly pathogenic and low pathogenicity AIV, depending on the severity of disease in affected species. Whereas low pathogenicity AIV (LPAIV) are ubiquitous, and represent part of the wild bird ecosystem, particularly in water birds, highly pathogenic AIV (HPAIV) are primarily found as causative agents of outbreaks of fowl plague in poultry (85). Although HPAIV outbreaks have occasionally occurred worldwide, they have, until recently, been restricted in geographic spread to the regional or, at most, national level. Since its first appearance in 1996, the HPAIV of the subtype H5N1 'Asia' has spread over three continents, causing the first nearly global panzootic of an HPAIV. Endemicity of HPAIV in poultry, as observed in several countries in Southeast Asia and Africa, as well as scattered outbreaks in domestic poultry in numerous other countries, prompted mass vaccination campaigns using commercially available vaccines and also led to increased efforts to develop novel vaccines with improved

characteristics. Although the first lines of defence against AI are surveillance, biosecurity, education, restrictions on movement, rapid and reliable diagnosis and the swift elimination of AI-infected poultry, vaccination can be an additional measure in a comprehensive control strategy. Vaccinating poultry not only enables the protection of chickens from clinical signs and death following challenge with HPAIV, but also reduces virus shedding (88). More importantly, it can prevent the spread of the notifiable LPAIV H5 and H7, both of which can spontaneously mutate into highly pathogenic forms, sometimes with only a single nucleotide alteration. Owing to this potential danger, the application of live virus vaccines based on low pathogenic viruses of the H5 and H7 subtype is not recommended. The development of new vaccines, especially for poultry, has received more attention since it was learned that HPAIV H5N1 can result in fatal cases in humans (see: www.who.int and 25). Several recent reviews on the development of AIV vaccines are available (12, 48, 84, 89, 93, 99, 105, 113, 116), and the authors of this paper re-examine this rapidly moving field, taking into account the most recent data and information.

Inactivated whole-virus vaccines

Historically, AIV strains used for inactivated vaccines have generally been based on LPAIV obtained from field outbreaks. The use of HPAIV for this purpose is limited, since this would require high-level biocontainment manufacturing facilities (84). Virus preparations are inactivated with beta-propiolactone and administered intramuscularly in an oil emulsion mixture (114). Homologous vaccines are prepared from virus specifying the same haemagglutinin (HA) and neuraminidase (NA) subtype as the field virus. The disadvantage of this is that these vaccines do not allow the detection of infection in vaccinated flocks (DIVA: differentiation between infected and vaccinated animals). The use of heterologous vaccines (7), containing the same HA subtype as the field virus but a different NA subtype, allows a DIVA approach by differentiating NA-specific serum antibodies. An important feature of inactivated whole-virus vaccines is their broad cross-protection against diverse field strains (88), based on the immune response against the different viral proteins, the high amount of antigen and the nature of the adjuvant. However, these vaccines have to be administered individually by injection, which is time-consuming and laborious, and increases the cost of vaccination.

Recently, it was shown that two different inactivated H5N9 vaccines protected chickens against challenge with an HPAIV H5N1 isolate from Thailand. This study demonstrated that such whole-virus vaccines can protect against morbidity and mortality and decrease virus shedding (4). Sakabe *et al.* (73) described the generation of an H7N7 vaccine virus with improved *in vitro* growth properties by co-inoculating LPAIV H7N7 and H9N2 isolates from migratory ducks into 10-day-old embryonated chicken eggs (ECE). The obtained allantoic fluid was mixed with antiserum against H9N2 and again propagated in ECE. After plaque cloning in Madin–Darby canine kidney cells, a reassortant H7N7 virus with high-growth potential was selected and used to prepare a novel inactivated vaccine which was highly efficacious against recent H7N1 HPAIV.

Attenuated live vaccines

Cold-adapted attenuated influenza vaccines have been developed for humans (1) and equines (54), but the use of attenuated live vaccines (especially of the H5 and H7 subtypes) in poultry is not recommended by the World Organisation for Animal Health or the Food and Agriculture Organization of the United Nations (FAO), since they may potentially mutate into HPAIV by

reassortment or mutation of the HA cleavage site. Moreover, like most inactivated vaccines, these live vaccines do not support an easy DIVA strategy.

Since the advent of reverse genetics for influenza virus (16, 45), and the development of entirely plasmid-based reverse genetic systems to rescue recombinant influenza virus, without the need for helper virus (17, 28, 29, 53), the timely generation of recombinant influenza viruses, according to the respective epidemiological situation, has now become possible. The system described by Hoffmann *et al.* (28, 29) uses eight bi-directional or ambisense vector plasmids, in which each influenza virus gene is flanked by promoters for cellular ribonucleic acid (RNA) polymerases Pol I and Pol II. This permits bi-directional transcription/replication and production of infectious influenza virus in transfected human kidney (293T) cells. The use of plasmid-based reverse genetics allows the safe and efficient generation of attenuated high-growth reassortant viruses, which derive the genes encoding the envelope proteins HA and/or NA from circulating influenza A viruses (39, 49, 96, 112) and the internal genes from vaccine donor strains, such as influenza A Puerto Rico/8/34 (PR8) (H1N1) or A/WSN 33 (H1N1).

To avoid the requirement for high-level biocontainment facilities, and to obtain high virus yields in ECE, the polybasic cleavage site of HPAIV H5 has been altered by deletion and/or mutation of basic amino acids, resulting in proteins specifying a monobasic cleavage site characteristic for LPAIV (43, 49, 96, 112). The resulting viruses were used as inactivated oil emulsion AI vaccines to immunise chickens (39, 43, 96, 112), ducks (39, 43, 49, 96) and geese (96). They provided effective protection from clinical disease and a significant reduction of virus shedding after challenge. A recombinant influenza virus derived from A/goose/Guandong/1996 (H5N1) and human influenza vaccine virus PR8 is available from a Chinese producer (64). Recently, Li *et al.* (41) described a similar recombinant in the PR8 background, specifying the HA and NA genes from the same donor virus. In this recombinant, in addition to the introduction of a monobasic HA cleavage site, the NA stalk region was replaced by the immunodominant epitope 5B19 of the S2 glycoprotein of murine hepatitis virus (MHV). Chickens immunised with this inactivated H5N1/PR8-5B19 vaccine virus were protected against challenge infection with different HPAIV H5N1 isolates, and produced a detectable antibody response against the MHV 5B19 epitope. This, however, would not allow detection of field virus infection in vaccinated animals.

Another promising approach is the development of influenza virus recombinants with truncated non-structural protein 1 (NS1). The NS1 is active in antagonising interferon response to infection, and its deletion results in stable attenuation (42). Thus, influenza

virus recombinants with truncated NS1 genes are safe and protective live vaccines (8, 69). Moreover, the absence of a serological response to NS1 after vaccination can be used as a differential diagnostic marker for infection, based on a test detecting anti-NS1 antibodies (103). Further candidates for live, genetically modified influenza vaccines based on reverse genetics are an NA-deficient virus (76) and an influenza virus with an atypical HA cleavage site, specifying a recognition motif for elastase that is resistant to activation during natural infection, but which can readily be activated under *in vitro* conditions to facilitate vaccine virus production (81). Attenuated influenza A viruses with other modifications of the HA cleavage site could also become candidates for live vaccines (80). Furthermore, recombinant AIV with deletions of the M2 gene may be suitable as live vaccines, since eliminating M2 leads to a virus that grows efficiently in cell culture, but only poorly in mice (109).

Using AIV as a vector for proteins from other viruses, e.g. the HN protein of Newcastle disease (ND) virus, has also been attempted, resulting in bivalent vaccines. Substituting the ectodomain of ND virus (NDV) HN (56) for the ectodomain of AIV NA resulted in a recombinant virus expressing the chimeric protein. Unfortunately, it was too attenuated to induce a satisfactory immune response.

Vector vaccines

Influenza viruses possess a limited number of immunogenic proteins, including the envelope glycoproteins HA and NA, matrix proteins M1 and M2, nucleoprotein NP and non-structural protein NS1. Of these, HA has been demonstrated to be the most relevant for inducing neutralising antibodies (90, 115). This situation facilitated the development of deoxyribonucleic acid (DNA), sub-unit and vector vaccines against mammalian and avian influenza viruses. Different chicken viruses have been used as vectors for the expression of AIV proteins. They include attenuated strains of DNA viruses, such as fowlpox (FP) virus (5, 6, 50, 78, 82, 87, 111) and infectious laryngotracheitis (ILT) virus (44, 106), as well as RNA viruses, such as NDV (23, 52, 56, 108; Fig. 1). In addition to these vectors, which can be used as live vaccines to protect poultry against two different infectious diseases of chickens simultaneously, viral vectors routinely used for transgene expression, such as replication-deficient adenovirus (22, 97, 98), alphavirus replicons (75), modified vaccinia virus Ankara (34, 107) and baculoviruses (13), have been successfully modified to express AIV antigens. Furthermore, *Salmonella* spp. have been used for surface expression of an influenza virus HA fusion protein with flagellin, or as carriers for HA expression plasmids (46, 94).

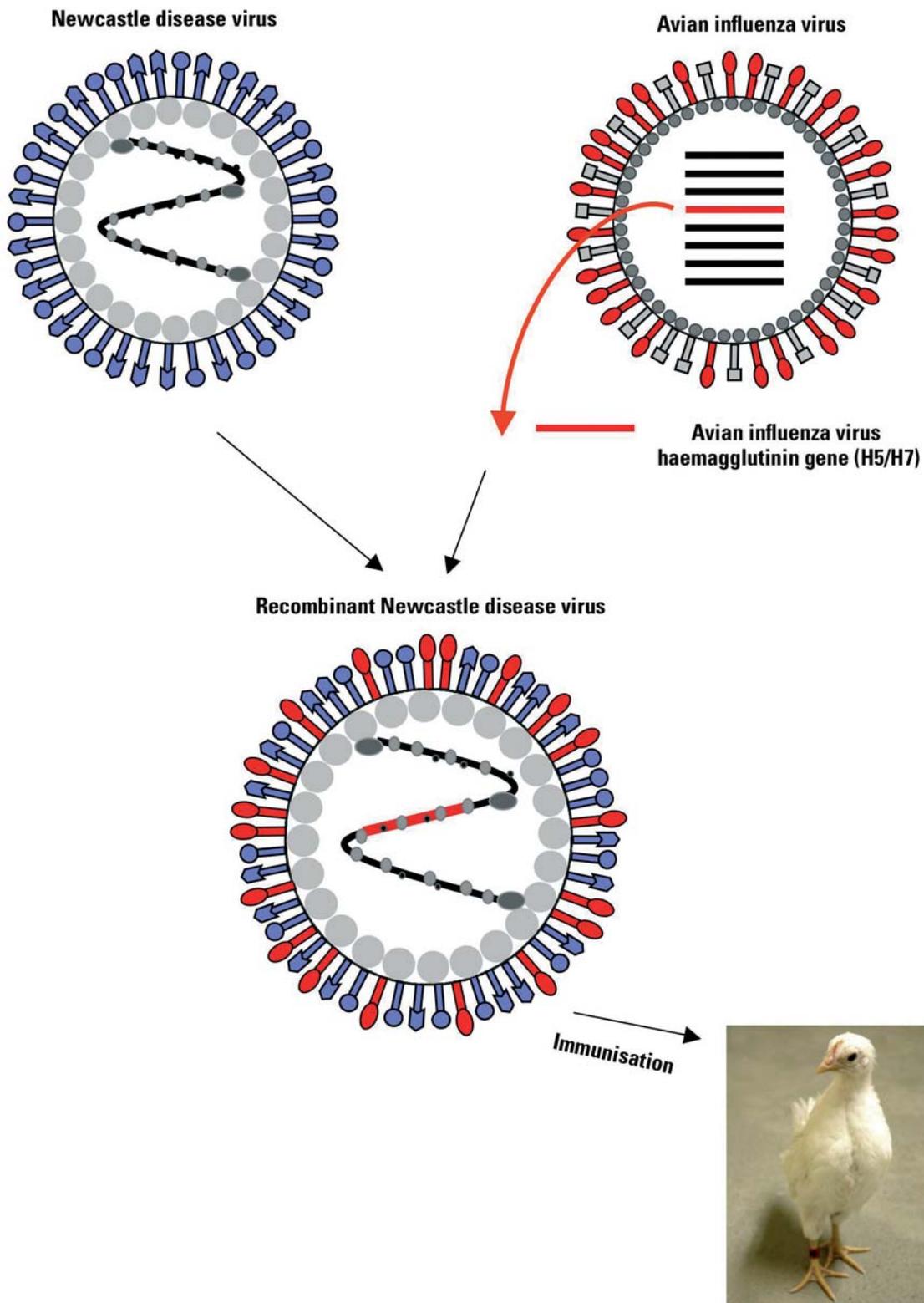
Replication-competent vector vaccines

Poxviruses

Attenuated but replication-competent viruses are probably the most economic vaccines, since they combine the immunogenic properties of protein and DNA vaccines and, due to their proliferation in the immunised animal, are efficacious even at low doses. Over the last few decades, many virus genomes have become accessible to reverse genetics and DNA manipulation technology, and directed deletion of virulence genes, as well as insertion of foreign genes, has become feasible. Poxviruses were among the first viral vectors used for the expression of heterologous proteins (55). Avian influenza virus genes were inserted into the genomes of attenuated FP virus (FPV), which were already in use as live-virus vaccines against FP in chickens and turkeys (66, 95, 101, 108). Non-essential regions of the FPV genome, such as the thymidine kinase gene locus (101), were used as insertion sites and the foreign proteins were expressed under the control of strong poxvirus promoters, for instance, the vaccinia virus H6 promoter (95). The considerable size of the FPV genome, of nearly 300 kilobase pairs, allowed not only insertions of single genes but also the simultaneous insertion of several genes, encoding, for example, HA and NA (66), or HA and NP (110).

Single vaccinations with approximately 10^5 infectious units of H5- or H7-expressing FPV recombinants protected chickens and ducks against lethal challenge infections with homologous or heterologous AIV of the corresponding subtypes (2, 6, 66, 87, 95, 101, 110), including recent Asian H5N1 isolates (5, 65, 82). However, like other AIV vaccines, HA-expressing FPV did not confer sterile immunity, as demonstrated by the re-isolation of HPAIV challenge virus from tracheal and cloacal swabs (5, 87, 95). Whereas co-expression of N1 subtype NA with H5 subtype HA apparently increased the efficacy and broadened the coverage of FPV-derived vaccines (65, 66), FPV-expressed NP did not detectably contribute to protection (110). Since NP proved to be dispensable for successful vaccination, NP-specific antibodies might be used in a DIVA strategy after immunisation of poultry with HA- or HA- and NA-expressing FPV. For that purpose, several enzyme-linked immunosorbent assay tests for NP-specific serum antibodies have been developed (15, 79).

Avian influenza virus vaccines based on fowlpox virus have been licensed for emergency use in the United States of America, and are already in use in Central America and East Asia. They can be produced economically on the chorioallantoic membrane of chicken embryos or in primary chicken cell cultures, and can be administered to

**Fig. 1****Construction of recombinant vector vaccines**

The gene of interest (in this case, the haemagglutinin gene) is isolated from avian influenza virus and inserted, bracketed by adequate transcriptional control elements, into the vector virus genome (in this case, Newcastle disease virus). The recombinant virus is then used to immunise the target species, resulting in immunity against both the viral vector and the gene donor virus

one-day-old chickens (100). However, to obtain optimal protection, individual subcutaneous vaccination (the wing-web method) is recommended. Since the natural host range of FPV is largely limited to chickens, to what extent FPV vector vaccines could be suitable for other species threatened by HPAIV remains to be evaluated in detail. Although HA-expressing FPV induced specific immune responses in cats (6), the protection of immunised turkeys was significantly less pronounced than that of chickens (95). Furthermore, it has been shown that, in chickens that had previously been immunised against FP, replication of HA-FPV was inhibited, and only insufficient protection against AIV ensued (86). It is possible that several of these problems could be overcome by the use of vectors based on other poxviruses. Recently, the highly attenuated modified vaccinia virus Ankara (MVA) has been engineered to express AIV H5 HA and shown to protect mice as well as chickens from H5N1 HPAIV challenge (34, 107). However, due to the severe replication defect of MVA, relatively high vaccine doses and booster immunisations might be required to achieve solid protection.

Herpesviruses

Like poxviruses, herpesviruses possess large, double-stranded DNA genomes that contain numerous genes which are not needed for virus replication in cultured cells, and which could be deleted or replaced by foreign DNA sequences. This also applies to the alphaherpesvirus which causes ILT in chickens (18). The ILT virus (ILTV) recombinants, which had been attenuated by deletion of the non-essential deoxyuridine triphosphatase (UL50) or UL0 genes, were used for insertion of the coding sequences of HA subtypes H5 and H7, or NA subtype N1 at the corresponding loci (18, 44, 57, 106). Interestingly, the expression of HA and NA under the control of the human cytomegalovirus major immediate-early (HCMV-IE) promoter could be significantly enhanced by the additional introduction of synthetic intron sequences into the 5'-non-translated region of the transgenes (57). These modifications might also be beneficial for DNA and other influenza vaccines requiring nuclear transcription and messenger RNA processing by cellular enzymes. A single ocular immunisation of chickens with 10^4 to 10^5 plaque-forming units of HA-expressing ILTV-recombinants reliably protected the animals from clinical symptoms after challenge with lethal doses of homologous HPAIV, including H5N1 Asia. However, the death of the animals was delayed, but not prevented, by immunisation with NA-expressing ILTV, although AIV-specific antibody responses were induced (19, 44, 57, 106). The efficacy of HA-expressing ILTV could be further enhanced by co-administration with an NA-expressing recombinant (57), which parallels the results obtained with other AIV vaccines. As described for NDV-derived vector vaccines (71), the level of subtype-specific protection achieved with

ILTV recombinants expressing H5 as the only AIV antigen apparently depends on the sequence homology between the HA proteins of the vaccine and the challenge virus (44, 57) to a much higher degree than observed with, for example, the inactivated AIV vaccines. However, ILTV vectors have been developed which permit the rapid substitution of transgenes by the HA genes of currently relevant AIV isolates. These updated recombinants are promising candidates for emergency vaccines against fowl plague, which, with respect to protection from disease and reducing AIV shedding, are as efficacious against homologous challenge as conventional inactivated vaccines or other vectored vaccines (57, 107). Furthermore, like other sub-unit, DNA or vectored AIV vaccines, HA-expressing ILTV mutants permit identification of AIV-infected animals in immunised populations by the detection of antibodies against, for example, NP, NA or NS proteins produced after infection.

Since chickens are not generally vaccinated against ILT, pre-existing immunity should not usually affect the efficacy of ILTV-based vector vaccines. Moreover, ILTV live vaccines can be produced in sufficient quantities in ECE or primary chicken cell cultures, and can be easily administered through sprays or drinking water (26).

One limitation of ILTV-based vector vaccines results from the narrow host range of this virus, which is almost restricted to chickens, and which barely replicates in other avian species, such as turkeys (26). In these species, AIV vaccines based on other viral vectors would be preferable. One candidate might be the apathogenic herpesvirus of turkeys (HVT), which has been used as a live vaccine against Marek's disease, and further developed as a vector expressing immunogenic proteins of NDV and infectious bursal disease virus (51, 102). Furthermore, HVT-based vaccines are suitable for *in ovo* vaccination of chickens (68). However, HVT recombinants expressing influenza virus proteins have not been described up to now.

Newcastle disease virus

Vaccination against ND with live vaccines is commonly practised, and immunisation is normally performed by spray or through drinking water. This offers good prospects for the development of bivalent NDV-AIV vaccines. After the development of reverse genetics systems for ND (58, 70), the NDV genome became amenable to targeted manipulation, e.g. the introduction of transgenes encoding AIV antigens (52). A first NDV-AIV recombinant expressing AIV H7 provided only partial protection (40%) from HPAIV challenge infection (90). This poor result was probably caused by overattenuation of the vector virus. After mutagenesis of the NDV fusion (F) protein cleavage site, and expression of a chimeric AIV H7 with the cytoplasmic domain of NDV F protein from a transgene

located between the phosphoprotein and matrix protein genes within the genome of the attenuated (lentogenic) NDV strain LaSota, 90% protection from infection with HA-matched HPAIV was achieved (56). A similar recombinant virus expressing AIV H5 is used in Mexico. An NDV LaSota-vectored vaccine expressing monobasic NDV F and AIV HA proteins was fully protective experimentally (24), and was in use in China in 2006 (105), but was apparently subsequently withdrawn for unknown reasons.

Other recombinant NDV based on LaSota-derived Clone 30 have been constructed and successfully used in challenge experiments. Genes encoding either H5 (108) or H7 (74) of HPAIV have been inserted between the NDV F and HN genes. Both vaccines conferred full clinical protection of immunised chickens against challenge with homologous HPAIV, and virus shedding was significantly reduced. Moreover, both proteins were incorporated into NDV particles, demonstrating that the NDV envelope is able to accommodate foreign proteins.

Optimal protection of immunised chickens requires a close match of the expressed AIV HA with that of circulating viruses (71), as is also known from human influenza vaccines. Therefore, continuous monitoring and occasional updating of the expressed AIV protein may be required. Nevertheless, recombinant NDV-AIV vaccines have two important advantages. They can be administered through a spray or drinking water, thus allowing the cost-efficient mass immunisation of poultry, and they allow DIVA diagnostics by the detection of antibodies against AIV antigens which are not expressed by the vector.

Replication-deficient vector vaccines

Adenoviruses

Promising carriers for AIV gene expression cassettes are replication-deficient human adenoviruses (22, 30, 97, 98). These viral vectors possess deletions of essential early genes (usually E1 and E3), but can be propagated to very high titres in trans-complementing cell lines, and foreign genes can easily be inserted, e.g. by site-directed Cre-*lox* recombination, or by mutagenesis of the plasmid-cloned virus genome in *Escherichia coli*. Defective adenovirus recombinants represent safe vaccines, and a single intramuscular or subcutaneous application of adenovirus particles containing H5 or H7 HA genes, under control of the HCMV-IE promoter, induced specific humoral and cellular immune responses and protected chickens, as well as mice, from lethal challenge infections with homologous HPAIV (22, 97, 98). An adenovirus vector containing an H5 gene was efficacious after *in ovo* vaccination of chicken

embryos, although efficacy was dependent on the degree of homology between the H5 of the vaccine and the challenge virus (97). Only limited protection of chickens could be achieved by intranasal application of the adenovirus preparations (97).

To improve the vaccines, transgene expression was enhanced by the use of synthetic HA genes with optimised codon usage (98). In addition, vectors expressing multiple HA genes, or HA together with NA, NP or M1, have been constructed to broaden the range of HPAIV strains covered by the vaccine (27, 30). Adenovirus-based AIV vaccines might become affordable for mass immunisation of poultry because of their suitability for automated *in ovo* administration. On the other hand, because of their safety, these non-replicating viruses are also candidate vaccines for protecting humans, in case of a pandemic caused by mutants or reassortants of current H5N1 HPAIV. Since their efficacy in humans might be affected by pre-existing immunity against the highly prevalent human adenoviruses, vector vaccines based on non-human adenoviruses have also been developed recently (72, 77).

Baculoviruses

Protein preparations from genetically engineered organisms expressing AIV proteins can also be used for immunisation. Subcutaneous injection of proteins, prepared from insect cells infected with recombinant baculoviruses expressing H5 or H7 subtype HA under baculoviral polyhedrin promoter control, is able to protect chickens from lethal challenge with different HPAIV isolates of the corresponding subtypes in a dose- and adjuvant-dependent manner. Double vaccination against both subtypes with mixed antigens was also possible (13). However, as with most other vaccination techniques, replication and shedding of the HPAIV challenge viruses were reduced, but not eliminated. Unlike HA, baculovirus-expressed NA induced only partial protection of chickens against HPAIV of the matching NA subtype N2 (91). To further improve the efficacy of recombinant HA, other approaches were evaluated, including combinations with M1, prime-boost vaccinations with baculovirus-derived protein and expression plasmid DNA, as well as baculovirus particles containing HA in their envelopes (38, 117). The HA-pseudotyped virions, together with suitable adjuvants, proved efficacious as mucosal vaccines for intranasal immunisation (59).

Virus-like particles

Baculoviruses

A recent advance in baculovirus-derived vaccines is the isolation of virus-like particles (VLP) (37, 62, 67). Such

membranous vesicles, containing matrix and envelope proteins of avian or mammalian influenza viruses, form spontaneously within and are released from insect cells (co-) infected with recombinant baculovirus(es) expressing the respective transgenes, either singly or in combination. Matrix protein M1 is sufficient to form VLP, which can then be purified by gradient centrifugation (37). The VLP were shown to possess similar sizes and properties to those of authentic influenza virus particles, including HA and NA activities (62). Subcutaneous, intramuscular or intranasal immunisation of different small mammals with VLP containing HA, NA and M1 (21, 62, 63), or only HA and M1 (67), induced neutralising antibodies and conferred protective immunity against challenge with homologous and heterologous influenza viruses of the matching HA subtype. Vaccination with VLP containing H5 and N1 from recent Asian HPAIV isolates protected mice and ferrets from lethal challenge with viruses from other H5N1 HPAIV clades (3, 47). Up until now, only very limited information has been available about the use of VLP for immunising poultry, although the first results of immunisation experiments indicate that VLP containing proteins of LPAIV H5N3 might be efficacious in ducks (60). In addition to VLP induced by recombinant baculoviruses, similar enveloped particles containing HA, NA and matrix protein M2 of H5N1 and H7N1 HPAIV were assembled in transfected mammalian cells around core particles, induced by co-expressed retrovirus Gag protein (92). However, immunogenicity of the purified VLP has been investigated only in mice so far. Thus, it remains to be seen whether VLP are generally suitable as vaccines for poultry, and whether the costs of vaccine production, as well as the effort required for individual administration, are acceptable.

Alphaviruses

Virus-like particles have also been produced on the basis of enveloped, positive-stranded RNA viruses belonging to the genus *Alphavirus* of *Togaviridae*, for instance, Venezuelan equine encephalitis virus (VEEV). The influenza virus HA (subtypes H1, H3 and H5) or NA (subtype N2) genes were inserted into the plasmid-cloned VEEV genome, instead of the open reading frame encoding the authentic capsid and envelope proteins (31, 61, 91). The transgenes were abundantly expressed after transfection of eukaryotic cells with *in vitro*-transcribed replicon RNA. Co-transfection with helper RNA encoding the alphavirus structural proteins led to the formation of RNA containing virus-like replicon particles (VRP) which could then be purified and used to infect a variety of mammalian and avian cells. In these cells, the RNA was again replicated and the transgenes were expressed, but no infectious viruses could be formed, due to the absence of the alphavirus structural protein genes. Thus, HA-expressing VRP represent safe, disabled infectious single cycle vaccines, which induced

humoral and cellular immune responses and protected mice against influenza virus challenge (31, 61). Virus-like replicon particles expressing H5 haemagglutinin also protected chickens from lethal infection with a Hong Kong H5N1 HPAIV isolate from 1997, after subcutaneous immunisation, whereas *in ovo* vaccination was only partially efficacious (75). Partial protection against homologous HPAIV challenge could also be achieved by vaccinating chickens with VRP expressing N2 neuraminidase (91).

Deoxyribonucleic acid vaccines

Inoculating mice with eukaryotic expression plasmids encoding influenza virus NP and HA provided the first information that DNA vaccines can induce specific antibodies and confer protective immunity (20, 104). Chickens were also protected from lethal infections with H5 or H7 HPAIV after a single vaccination with expression plasmids encoding HA of the matching subtype, even if the amino acid sequences differed by more than 10% between vaccine and challenge virus (32, 33). To enhance the efficacy of DNA vaccines against a broad range of H5 subtype influenza viruses, synthetic HA genes have been constructed, which encode consensus amino acid sequences, and employ codons that permit high expression levels (9, 35). Protection of chickens or mammals was improved by co-administering HA-expression plasmids with plasmids containing the NA, or the more conserved M or NP genes, of AIV (11, 36). Immunisation with a mixture of plasmids encoding H5 and H7 HA protected chickens against HPAIV of both subtypes (33). In contrast, DNA vaccines encoding only NA of the matching subtype, or the conserved NP, conferred only partial protection (33, 91). Thus, DNA vaccines, like most other influenza vaccines, should specify HA of the proper subtype. Fortunately, DNA vaccines originally generated to produce monospecific antisera in chickens are available for most of the 16 known HA subtypes (40), and might be suitable for immunising poultry against corresponding AIV strains. Since the immunogenic NP is not required for protection and, thus, does not need to be part of the vaccine, antibodies against NP can be useful markers for identifying AIV-infected animals in populations vaccinated with HA expression plasmids.

In addition to various AIV antigen types, different expression vectors, mostly using the strong HCMV-IE promoter/enhancer complex, different DNA doses and adjuvants, and elaborate application techniques, like gene gun delivery or *in vivo* electroporation, have been tested to optimise the efficacy of DNA vaccines (9, 11, 32, 33, 35, 83). However, despite positive results in laboratory experiments, their practical use for immunisation of poultry is still hampered by the high costs of DNA production and carriers such as gold particles, as well as

the necessity of individual, usually intramuscular, administration. Furthermore, most plasmids contain antibiotic resistance genes, which might be transferred to pathogenic bacteria. Several of these problems can be overcome with DNA vaccines suitable for oral or nasal spray delivery, by the use of synthetic microparticles or non-pathogenic bacteria as carriers (10, 14).

concepts gives testimony to the importance of this issue. Provided that politicians and public and funding agencies continue to support this venture, promising candidates could soon enter into development to become marketable products for use in the field, to combat not only H5N1 but AIV infections in domestic poultry in general.

Conclusion

Although several of the novel AIV vaccines are already in use, there is an urgent need to develop and bring to the market improved vaccines to allow cost-efficient mass vaccination and robust DIVA strategies. The multitude of approaches towards developing new vaccines and vaccine

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Nouveaux vaccins contre l'infection par les virus de l'influenza aviaire

W. Fuchs, A. Römer-Oberdörfer, J. Veits & T.C. Mettenleiter

Résumé

Les vaccins actuellement utilisés contre l'infection par les virus de l'influenza aviaire sont essentiellement des préparations classiques contenant du virus entier inactivé. Ces vaccins protègent effectivement les volailles contre la maladie clinique, mais dans les conditions de terrain ils ne permettent pas d'éliminer complètement le virus, de sorte que celui-ci peut continuer à se propager silencieusement sous cette couverture immunitaire. C'est pourquoi il est impératif de mettre au point rapidement un système robuste et fiable permettant de différencier les animaux infectés des animaux vaccinés. En outre, les vaccins actuels contre l'influenza aviaire sont conçus pour être administrés individuellement, ce qui requiert des manipulations excessivement nombreuses qui rendent difficile l'obtention d'une large couverture vaccinale. Il serait donc préférable de disposer de vaccins conférant une bonne immunité dans le cadre d'applications massives. Les auteurs présentent les différentes approches abordées actuellement pour améliorer les vaccins existants et mettre au point de nouveaux vaccins.

Mots-clés

Génétique inverse – Génie génétique – Technologie recombinante – Vaccin – Virus de l'influenza aviaire.

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Nuevas vacunas contra la infección por el virus de la influenza aviar

W. Fuchs, A. Römer-Oberdörfer, J. Veits & T.C. Mettenleiter

Resumen

Las vacunas actuales contra la infección por virus de la influenza aviar (IA) se basan principalmente en las clásicas preparaciones de virus enteros inactivados. Aunque la administración de esas vacunas puede proteger a las aves de la enfermedad clínica, en condiciones naturales no se logra una completa inmunidad estéril, lo que permite a los virus no detectados propagarse y evolucionar bajo "protección inmunitaria". De ahí la urgente necesidad de disponer de un sistema robusto y fiable para distinguir entre animales infectados y animales vacunados. Además, las actuales vacunas contra la IA deben ser administradas animal por animal, lo que, cuando hay que manejar un número demasiado alto de animales, hace difícil lograr una elevada cobertura de vacunación. Por este motivo sería ventajoso disponer de vacunas contra la IA que confirieran una fuerte inmunidad y pudieran aplicarse masivamente. Los autores pasan revista a las distintas líneas de trabajo que están en marcha para mejorar las vacunas existentes y obtener nuevas vacunas.

Palabras clave

Genética inversa – Ingeniería genética – Tecnología recombinante – Vacunas – Virus de la influenza aviar.



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