Newcastle disease virus expressing H5 hemagglutinin gene protects chickens against Newcastle disease and avian influenza

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Newcastle disease virus (NDV)-expressing avian influenza virus (AIV) hemagglutinin (HA) of subtype H5 was constructed by reverse genetics. A cloned full-length copy of the genome of the lentogenic NDV strain Clone 30 was used for insertion of the ORF encoding the HA of the highly pathogenic AIV isolate A/chicken/Italy/8/98 (H5N2) in the intergenic region between the NDV fusion and hemagglutinin-neuraminidase (HN) genes. Remarkably, two species of HA transcripts were detected in cells infected with the resultant NDVH5. In a second recombinant (NDVH5m), a NDV transcription termination signal-like sequence located within the HA ORF was eliminated by silent mutations. Consequently, NDVH5m produced 2.7-fold more full-length HA transcripts, expressed higher levels of HA, and also incorporated more HA protein into its envelope than NDVH5. NDVH5m stably expressed the modified HA gene for 10 egg passages and both recombinants were found innocuous after intracerebral inoculation of 1-day-old chickens. Immunization of chickens with NDVH5m induced NDV- and AIVH5-specific antibodies and protected chickens against clinical disease after challenge with a lethal dose of velogenic NDV or highly pathogenic AIV, respectively. Remarkably, shedding of influenza virus was not observed. Furthermore, immunization with NDVH5m permitted serological discrimination of vaccinated and AIV field virus-infected animals based on antibodies against the nucleoprotein of AIV. Therefore, recombinant NDVH5m is suitable as a bivalent vaccine against NDV and AIV and may be used as marker vaccine for the control of avian influenza.

vaccine | recombinant Newcastle disease virus | reverse genetics | fowl plague

The highly pathogenic (HP) avian influenza virus (AIV) H5N1 currently circulating in Asia caused the death of >150 million birds, and infected 161 people killing 86 of them (www.who.int/csr/disease/avian_influenza/country/cases_table_2006_02_02/en). Because of the severity of this threat, several countries are taking steps to vaccinate their entire poultry population. Because the best way of preventing H5 infections of humans is to control the infection in birds, vaccination of birds at risk may constitute a successful intervention strategy in controlling fowl plague or avian influenza (AI). However, vaccination may lead to undetected circulation of virus among birds, resulting in severe restrictions in trade of live poultry or poultry products. Several vaccines developed over the past two decades to protect poultry against HPAIV, specifying hemagglutinins (HAs) H5 or H7, are based on inactivated whole-virus vaccines. Apart from the challenge of setting up a robust diagnostic test for differentiating vaccinated from infected animals, these vaccines have to be administered by labor-intensive and expensive parenteral injections. In view of the large undertaking to vaccinate billions of poultry in some parts of the world, development of efficacious vaccines that could be administered by mass application routes, such as spray or drinking water, is highly needed. Thus, an improved vaccine for controlling AI should be (*i*) efficacious in reducing virus transmission, (*ii*) genetically close to the circulating virus, (*iii*) serologically distinguishable from the WT virus, (*iv*) applicable by mass administration, and (*v*) inexpensive.

To develop an improved vaccine against AI, recombinant DNA technology was used to generate vectored, subunit, or DNA vaccines (1). Although several of these vaccines have been shown experimentally to protect against AI (1), only a fowlpox-vectored vaccine with H5 gene insert is commercially available. Although this vaccine, in principle, enables a differentiation between infected and vaccinated birds by serological tests, no such test system so far has been developed for mass application. Moreover, the recombinant fowlpox-H5 vaccine requires individual parenteral administration. A recently described adenovirus expressing the HA of AIV (2) also provided full protection when injected but only partial protection when given intranasally. Because of the advent of reverse genetics for specific manipulation of nonsegmented negative-strand RNA viruses (3), HA genes derived from various influenza A viruses had been inserted into members of this order, including Rinderpest virus (4), vesicular stomatitis virus (5), and Newcastle disease virus (NDV) (6). The potential use of mass applicable recombinant poultry vaccines, such as infectious laryngotracheitis virus (ILTV) (7, 8) and NDV (9) to simultaneously immunize chickens against AIV and ILTV or AIV and NDV recently has been investigated.

Newcastle disease (ND) is an economically important disease of poultry, which is controlled by routine vaccination in many parts of the world. In the United States alone, close to 9 billion birds are vaccinated annually by spray or drinking water with live lentogenic vaccine strains. NDV belongs to the genus *Avulavirus* within the family *Paramyxoviridae* (10). The negative stranded RNA genome of NDV that recently has become amenable to genetic manipulations (11–13) encodes six genes arranged in the order: 3′-nucleoprotein (NP); phosphoprotein; matrix- (M); fusion- (F); hemagglutinin-neuraminidase- (HN); polymerase protein-5′. To exploit the potential of NDV as a vaccine vector suitable for mass vaccination, the H7 of a low-pathogenic AIV has been incorporated between the phosphoprotein and M genes of a Hitchner B1 strain. However, the recombinant exhibited only poor protection of 40% of the immunized chickens against lethal challenges of AIV or NDV (9).

Here, we report the construction of a recombinant NDV based on a commercially available lentogenic live vaccine strain that efficiently protected chickens against lethal challenges of AIV and NDV and reduced AI virus transmission. Furthermore, we demonstrate the potential of an NP-ELISA in conjunction with the

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Abbreviations: AI, avian influenza; AIV, avian influenza virus; Ct, threshold cycle; F, fusion-protein; HA, hemagglutinin; HI, hemagglutination inhibition; HN, hemagglutinin-neuraminidase; HP, highly pathogenic; M, matrixprotein; NDV, Newcastle disease virus; NP, nucleoprotein; p.c., after challenge; p.i., after immunization.

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NDV-vectored AI vaccine for detection of circulating AIV, even in vaccinated animals.

Results

Construction of Recombinant NDV Expressing the H5 Gene of AIV. To evaluate the potential use of a recombinant NDV as a vaccine vector for mass immunization against AI and Newcastle disease, the ORF of AIV H5 was cloned between the NDV F and HN genes. The recombinant NDVH5 was recovered from transfected BSR-T7/5 cells (14) and propagated in embryonated specific pathogen-free eggs (15). After three passages, the titer of NDVH5 was similar to that of the parental virus Clone 30, amounting to 10^9 $TCID_{50}$/ml. The presence of the inserted H5 gene was confirmed by RT-PCR, and correct transcription was verified by Northern blot analyses (see Fig. 2). Although the H5 ORF in NDVH5 and AIV H5N2 is identical, the H5 ORF in recombinant NDV is flanked by ≈270 nucleotides of noncoding sequences of the HN gene. Therefore, the sizes of the NDVH5 and AIV H5 mRNAs correlate with the expected sizes of ≈2 kb and ≈1.7 kb (see Fig. 2). However, in NDVH5-infected cells, a second transcript of ≈1.1 kb representing ≈40% of total HA mRNA was observed (see Fig. 2). Interestingly, a less-abundant transcript of ≈1 kb accounting for ≈8% of total HA mRNA also was present in WT AIV-infected cells. The sequence at nucleotides position 1,021–1,029 of the H5 ORF showed a high homology to the transcription termination/polyadenylation signal of NDV and AIV (Table 1). To avoid any premature transcription termination, in a full-length clone, NDVH5m, silent mutations were introduced to alter this transcription termination signal-like sequence (Fig. 1 and Table 1). Northern blot analysis demonstrated that NDVH5m produced only the 2-kb H5 transcript (Fig. 2), which confirms that the short transcript in NDVH5 terminates prematurely. As a result of the modification, NDVH5m produced ≈2.7-fold more full-length HA than NDVH5. Furthermore, the transcription termination signal-like sequence in NDVH5 seems to undergo selective pressure, because this region had been altered within 5 egg passages, whereas it remained stable in NDVH5m over 10 passages (Table 1). Interestingly, the transcription termination signal-like sequence is located within the coding region for the cleavage site of the HA protein, so that the short H5 transcripts most likely encode only the HA_1 region. Hybridization with probes specific for NDV F and HN genes confirmed correct transcription of the flanking NDV genes, with no size difference to corresponding transcripts from the parental NDV Clone 30 (Fig. 2). Furthermore, only a slight reduction in the transcription level of the HN gene was observed as a result of the insertion of the H5 ORF (Fig. 2).

The AIV H5 Protein Is Incorporated into the Envelope of NDVH5 Particles. Previous studies showed that incorporation of foreign proteins into the envelope of unrelated viruses can occur passively

Table 1. Similarity of transcription termination sequences of NDV, AIV, and NDVH5 recombinants

Genome region	Virus	Egg passage	Nucleotide/amino acid sequence*
Transcription	NDV	—	aga aaa aa
termination	AIV	—	gga aaa aa
H5 ORF†	NDVH5	3	aga aaa aaa *Arg Lys Lys*
nucleotides	NDVH5	5	aga a**g**a aaa *Arg* ***Arg*** *Lys*‡
1021–1029	NDVH5m	10	agg aa**g** aa**g** *Arg Lys Lys*

*Amino acid sequence in italic.
†AIV/A/chicken/Italy/8/98 (H5N2); accession no. AJ305306.
‡Differences to sequence of accession no. AJ305306 are in bold.

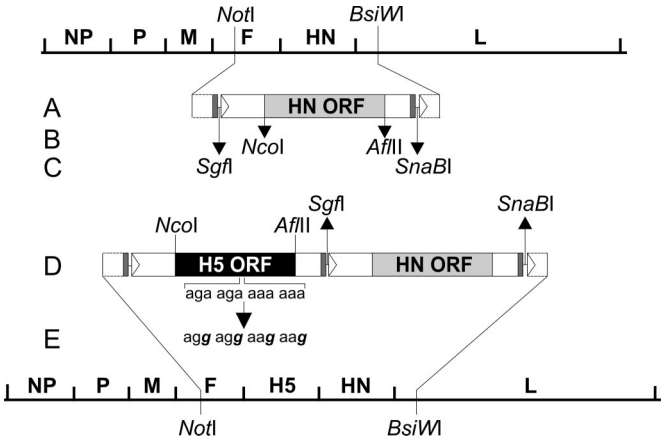

Fig. 1. Construction of recombinant NDV expressing AIV H5. Transcription control signals are marked by a triangle for transcription start and a gray rectangle for transcription stop sequences. The HN ORF of NDV was substituted by the H5 ORF of AIV, and the HN gene was inserted subsequently (steps A–C). Recombinant NDVH5 (step D) contains the authentic HA sequence, whereas recombinant NDVH5m carries an HA in which the cleavage site sequence was altered by silent mutation (step E). P, phospoprotein; L, polymerase protein.

in the absence of specific incorporation signal (16). Because the efficiency of passive incorporation mainly depends on the expression level of the heterologous proteins, we determined whether the HA protein expressed by NDVH5 recombinants was properly cleaved and incorporated into the envelope (Fig. 3). In immunoblot analysis of cells infected by either recombinant, the AIV H5-specific antiserum detected three proteins of ≈70, 50, and 25 kDa, presumably representing the uncleaved HA_0 and the cleaved HA_1 and HA_2 proteins (Fig. 3*A*). Thus, HA expressed by the NDV recombinants is correctly processed by proteolytic enzymes. The total amount of HA protein produced in cells infected with NDVH5 was remarkably less than that in NDVH5m-infected cells, and the HA_2 protein in NDVH5-infected cells was barely visible. As expected, no reactivity could be detected in NDV Clone 30-infected cells, whereas all three HA protein species were detected in AIV H5N2-infected cells. Because the AIV-specific antiserum had been raised against whole virus, other proteins presumably representing NP, NA, and M also were detected in AIV-infected cells (Fig. 3). Interestingly, immunoblot analyses of purified virions indicated that the HA protein was incorporated efficiently into the envelope of

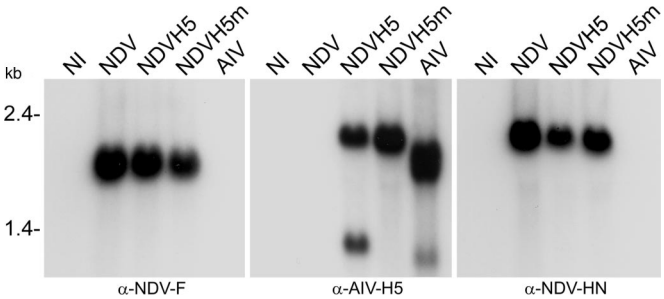

Fig. 2. Northern blot analyses of transcripts produced by NDV recombinants. Total RNA of chicken embryo fibroblast cells infected with NDV Clone 30, NDVH5, NDVH5m, or AIV H5N2, and noninfected cells (NI) were prepared 8 h after infection. RNA was separated in denaturing agarose gels, transferred to nylon membranes, and hybridized with ^{32}P-labeled gene-specific antisense cRNAs of NDV-F, AIV-H5, and NDV-HN. Six micrograms of the respective RNA were subjected to Northern hybridization, except for the AIV lane in the center blot in which only 2 μg were loaded to avoid an excessive AIV signal. Sizes of RNA markers (kilobases) are indicated on the left.

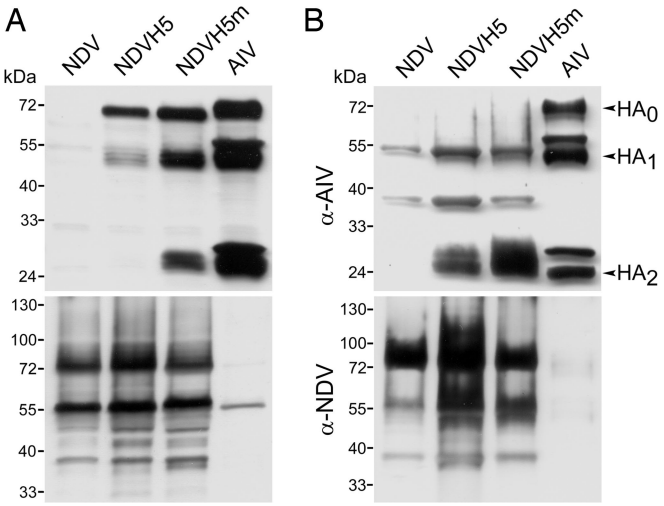

Fig. 3. Western blot analyses of NDV recombinants expressing AIV H5. Lysates of infected cells (*A*) or purified virions (*B*) of NDV Clone 30, NDVH5, NDVH5m, or AIV H5N2 were incubated with either an AIV subtype H5-specific antiserum (α-AIV) or a NDV-specific antiserum (α-NDV). Binding was visualized by chemiluminescence after incubation with peroxidase-conjugated secondary antibodies. Locations of marker proteins are indicated on the left, and the uncleaved (HA_0) and processed forms (HA_1 and HA_2) of AIV hemagglutinin are indicated on the right. In AIV-infected cells or virions, additional viral proteins corresponding to NP, NA, and M also are detectable.

both recombinants (Fig. 3*B*). Although more NDVH5 protein (1.5-fold) was subjected to immunoblot analysis, the amount of HA_2 protein incorporated into NDVH5 still was considerably lower than in NDVH5m virions. Thus, NDVH5 produces and incorporates much less HA_2 protein, presumably due to the premature termination of transcription.

Purified virions of NDV, AIV H5N2, or recombinant NDVH5m were further examined by immunoelectron microscopy by using polyclonal antisera against NDV or AIV H5, followed by labeling with gold-tagged secondary antibodies. Newcastle disease virions exhibited staining only with the NDV-specific antiserum (Fig. 4*A*), and AI virions reacted only with AIV-specific antiserum (Fig. 4*F*). In contrast, the surface of NDVH5m virions was labeled with both antisera, demonstrating that its envelope contained H5 (Fig. 4*D*) in addition to homologous F and HN proteins (Fig. 4*C*). These results show that the envelope of recombinant NDV accommodated the foreign membrane protein.

Recombinant NDV Carrying AIV HA Protein Is Innocuous in Chickens. To quantitate virulence of an NDV isolate, the pathogenicity of the virus for 1-day-old chickens is assessed after intracerebral inoculation (17). The most virulent viruses yield indices close to the maximum score of 2.0, whereas lentogenic strains produce values close to 0.0. Because the HA is an important virulence determinant for AIV, the intracerebral pathogenicity indices for NDVH5 and NDVH5m were determined to evaluate whether expression of H5 of HPAIV alters NDV virulence. The resulting intracerebral pathogenicity index values were 0.0 for both recombinants, demonstrating that expression of the AIV H5 did not noticeably affect NDV virulence.

Recombinant NDV Expressing the AIV HA Protein Protects Chickens Against NDV and AIV Challenge. Because of the more promising expression level of H5 protein, only NDVH5m was tested in an animal experiment. Recombinant NDVH5m was administered to 25 3-week-old chickens at a dose of 10^6 mean embryo infectious doses (EID_{50}) per animal by oculonasal instillation.

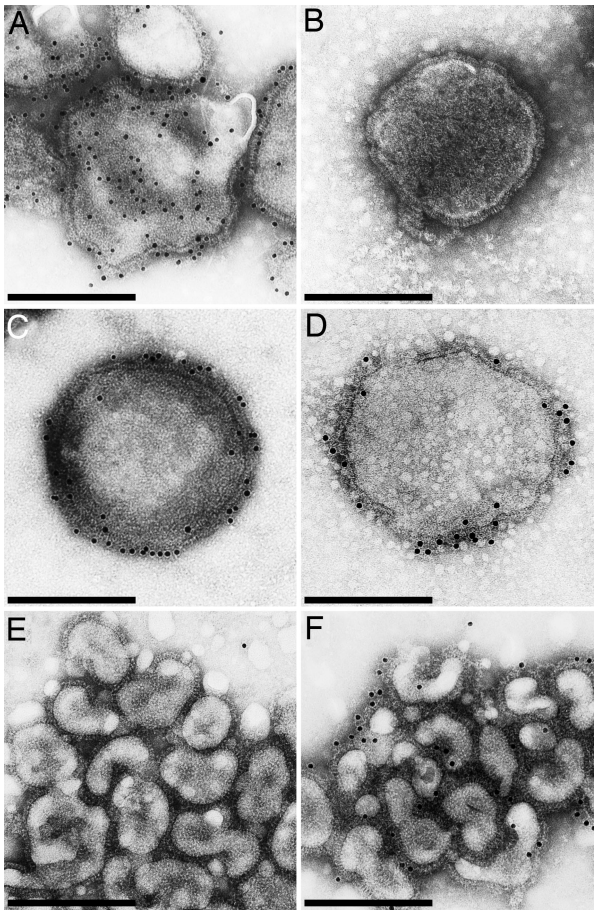

Fig. 4. Immunoelectron microscopy of purified virions of NDV Clone 30 (*A* and *B*), NDVH5m (*C* and *D*), and AIV H5N2 (*E* and *F*). Binding of antisera against NDV (*A*, *C*, and *E*) and AIV (*B*, *D*, and *F*) was detected with gold-tagged secondary antibodies, respectively. (Scale bars: 250 nm.)

During the observation period, all of the animals remained healthy without any adverse reactions or clinical signs. In the hemagglutination inhibition (HI) test, AIV H5-specific antibodies were first detectable on day 14 in 28% of the sera, and increased to 92% on day 21 after vaccination (Fig. 5*A*). An earlier onset of immunity against AIV could be detected in 96% of sera at 7 days after immunization (p.i.) by using an indirect immunofluorescence (IF) test (Fig. 5*A*).

To determine the protective effect of a single vaccination, a group of 5 and a group of 10 vaccinated animals, together with an appropriate number of naive animals, were subjected to lethal challenge with NDV and AIV, respectively. Because all animals had developed NDV-specific antibodies at high levels already after 14 d p.i. (mean HI titer of $2^{6.0}$), 100% of the vaccinated chickens were protected against the lethal NDV challenge, whereas all nonvaccinated control animals died within 4 days, exhibiting typical signs of Newcastle disease (Fig. 5*B*). Challenge infection with HPAIV H5N2 caused severe disease in nonimmunized chickens, with a mortality rate of 100% (Fig. 5*C*). In contrast, all animals of the NDVH5m immunized group survived the lethal challenge. Seven of 10 chickens remained completely healthy, whereas three animals exhibited only very mild transient respiratory symptoms. However, the resulting clinical score of 0.05 was minimal compared with the clinical score of 2.61 for the control group (Fig. 5*C*). To determine the effect of a second vaccination on protection against AI, the remaining 10 chickens received a second immunization on day 42 after the first

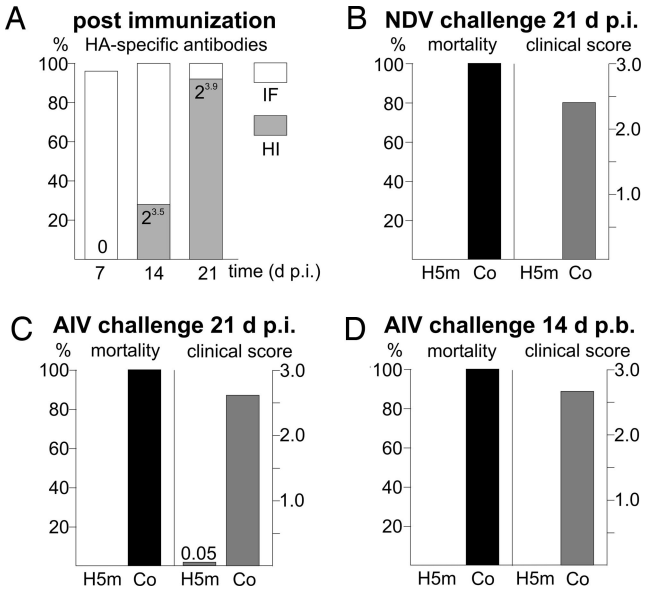

Fig. 5. Animal experiment. Development of HA-specific antibodies detected by HI test (*A*, gray bars) or indirect immunofluorescence (IF) (*A*, white bars) in sera of chickens immunized once with NDVH5m. The mean HI titers per group are given. Mortality rates and clinical indices of NDVH5m-immunized chickens (H5m) and control animals (Co) after challenge with velogenic NDV (*B*) or HPAIV H5N2 after one (*C*) and after two immunizations (*D*), respectively, are shown. The animals were observed daily for a period of 10 days for clinical signs and classified as healthy (0), ill (1), severely ill (2), or dead (3). A clinical index was calculated that represents the mean value of all chickens per group for this period.

vaccination and were challenged 2 weeks later. All animals were completely protected against clinical disease, whereas all control animals developed severe disease and died within 4 days, resulting in a clinical score of 2.66 (Fig. 5*D*).

NDV-AI Vaccine Reduces AIV Shedding. AIV shedding was monitored by real time RT-PCR. Viral RNA (vRNA) was detected in all challenged chickens of both nonimmunized groups on day 2 after challenge (p.c.). The threshold cycle (Ct) values ranged between 32.2–38.1 and 29.2–35.5, respectively (Table 2). In contrast, vRNA could be detected in only 11 of 160 swabs of

Table 2. Shedding of AIV challenge virus

Group	Time scale,* d p.c.	NDVH5m oro	NDVH5m cloacal	Control oro	Control cloacal
	AIV challenge 21 d p.i.				
AIV shedding†	2	2/10	1/10	9/9	7/9
	4	0/10	0/10	—‡	—‡
	8	3/10	0/10	—‡	—‡
	14	1/10	0/10	—‡	—‡
Ct range		36.6–39.1		32.2–38.1	
	AIV challenge 14 d p.b.				
AIV shedding†	2	2/10	0/10	5/5	4/5
	4	0/10	1/10	—‡	—‡
	8	0/10	0/10	—‡	—‡
	14	1/10	0/10	—‡	—‡
Ct range		37.5–39.6		29.2–35.5	

*The time scale discriminates between days (d) after immunization (p.i.), boost immunization (p.b.), and challenge infection (p.c.).
†AIV shedding determined by real-time RT-PCR of oropharyngeal (oro) and cloacal swabs; range of real-time RT-PCR threshold cycles (Ct) are given.
‡Investigations were prevented by the death of the animals.

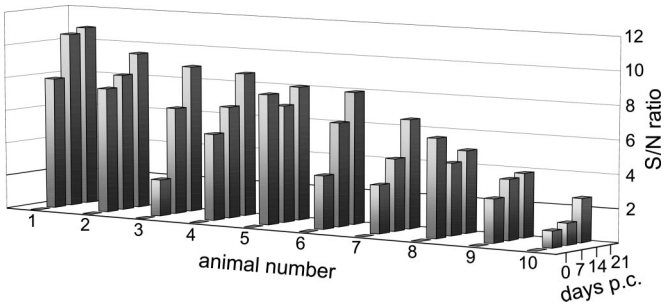

Fig. 6. Serological examinations by NP-ELISA. Sera of chickens were investigated by an indirect NP-ELISA at a dilution of 1:300. The obtained S/N (OD sample/OD negative control) ratios were plotted for sera of chickens immunized twice with NDVH5m collected before (0 d p.c.) and on day 7, 14, and 21 after AIV challenge (p.c.). The cutoff value of 2.0 is marked by a bold line.

immunized chickens with Ct values close to the detection limit. The vRNA in seven swabs from chickens of the once-immunized group had Ct values of 36.6–39.1, whereas vRNA Ct values of four swabs from animals vaccinated twice ranged between 37.5–39.6 (Table 2). However, virus isolation in eggs from all swabs of immunized chickens that had Ct values in real time RT-PCR was unsuccessful, even after repeated passaging, demonstrating that the detected Ct values may not be indicative of the presence of viable virus precluding any virus transmission.

NP-ELISA Differentiates AIV Infected from Vaccinated Animals. The most important disadvantage in preventive vaccination of poultry against AIV infection is the probability that vaccination could enable the virus to circulate undetected among birds. Because the recombinant NDV vaccine contains only the HA gene of AIV, an ELISA for specific detection of antibodies against the highly immunogenic NP was established to analyze the sera of vaccinated animals before and at different times after the challenge. Whereas antibodies against AIV NP were absent in sera of all animals before challenge infection, NP seroconversion could be detected in 90% and 100% of the chickens on day 7 and 21 after AIV challenge, respectively (Fig. 6). This result demonstrates that the NP-ELISA test permits differentiation of vaccinated from infected animals, thus allowing detection of any circulating AIV among vaccinated birds.

Discussion

AIV of subtype H5 and H7 may cause fowl plague, which has devastating consequences in the poultry industry. In particular, in geographic regions with few resources, vaccines with improved delivery and lower cost of administration are urgently needed. Various approaches to exploit the potential of different poultry vaccines for mass immunization against AI, including NDV, are currently underway (7–9). However, a recently described recombinant NDV expressing AIV H7, which had been derived from the lentogenic Hitchner B1 vaccine strain provided only a partial protection of 40% against a HPAIV challenge. Most likely this effect was due to the fact that the parental recombinant NDV already protected only 70% of the vaccinated chickens against NDV infection because of excessive attenuation. It is known that insertion of an additional gene exerts a further attenuating effect. In contrast, even after insertion of the HA gene into NDV Clone 30, NDVH5m protected all vaccinated chickens against lethal NDV and AIV challenges after an oculonasal administration, simulating a spray vaccination. This result demonstrates the great potential of NDVH5m for easy and cost-efficient mass immunization to protect poultry against NDV and AIV. Thus, NDVH5m alleviates the impracticability and high cost of par-

enteral administration associated with the inactivated AI whole virus vaccines.

Apart from the success of immunization by the parental NDV, the level of expression of the inserted gene was of paramount importance for the efficacy of NDVH5m. NDVH5m, in which the NDV gene end-like sequence at the cleavage site encoding region of the HA gene had been modified by silent mutations, produced 2.7-fold more HA mRNA than NDVH5, which expresses the authentic HA ORF. This modification resulted in higher levels of HA synthesis and incorporation and increased efficacy. Intriguingly, WT AIV H5N2 also produced a corresponding truncated HA_1 mRNA, albeit at only 8% of the level of total HA mRNA. Besides our H5N2 strain, 87% of 220 examined AIV H5 sequences deposited in GenBank contain the same transcription termination-like sequence. Assuming that the truncated mRNA, which encodes only HA_1, is translated into functional protein, the biological significance of the increased HA_1 production by these viruses remains to be determined.

One of the concerns of vaccination against AI is that current vaccines do not produce sufficient immunity to completely prevent infection and subsequent virus transmission, although recent experiments demonstrated that vaccination with inactivated vaccines may be able to reduce the spread of AIV within a flock (18). Immunization with NDVH5m also was sufficient to reduce shedding of infectious AIV after challenge to an extent that no viable virus could be demonstrated. The antigenic match between vaccine and challenge virus is one of the most decisive factors in determining the efficiency of the vaccine in preventing virus transmission. By reverse genetics, a new recombinant NDV carrying an HA derived from a currently circulating field strain could be generated in ≈4–5 weeks. This technique enables the construction of a tailor-made matching vaccine in a very short period.

A further concern is the probability that AIV could circulate undetected among vaccinated birds. This disadvantage is mainly due to the difficulty of tracking the circulating virus when conventional whole-virus vaccines are used. The ELISA described here, which is based on the detection of antibodies against the highly immunogenic NP, represents a simple test that could be used in conjunction with any recombinant vaccine lacking the NP as a vaccine component. It is also compatible with the agar-gel-immunoprecipitation test that is also used to trace AIV infection. The ability of this test to detect the presence of anti-NP antibodies in NDVH5m-vaccinated chickens demonstrates that detection and subsequent elimination of birds that become infected after vaccination is possible. The availability of such a robust test in conjunction with an inexpensive NDV-AI vector vaccine for mass application will undoubtedly help in adding vaccination as a serious tool to control AI in poultry and, consequently, minimizing the risk of its transmission to humans.

Because incorporation of the HA gene derived from a HPAIV into NDV may alter its virulence, we evaluated pathogenicity of the recombinants by intracerebral inoculation into 1-day-old chickens. An intracerebral pathogenicity index value of 0.0 was obtained for both NDV recombinants. This result demonstrates that the degree of virulence of the recombinants expressing the HA of AIV in chickens is indistinguishable from that of the parent that represents a currently used vaccine strain. The safety of NDV recombinants expressing protective antigens of respiratory human pathogens was recently demonstrated in two species of nonhuman primates after intranasal immunization (19). These data provide evidence that a NDV-based intranasal vaccine can potentially also be developed against AIV infection in humans. Considering the current threat of pandemic H5N1, this approach should seriously be considered.

Materials and Methods

Viruses and Cells. Recombinant NDV based on the vaccine strain Clone 30 has been described in ref. 13. The AIV isolate A/chicken/Italy/8/98 (H5N2) was kindly provided by I. Capua (Instituto Zooprofilattico Sperimentale delle Venetie, Padova, Italy). The velogenic NDV strain Herts 33/56 and the NDV Clone 30 vaccine (Nobilis) were obtained from Intervet, Boxmeer, The Netherlands. The viruses were propagated in 10-day-old embryonated specific pathogen-free chicken eggs. BSR-T7/5 cells stably expressing phage T7 RNA polymerase (14) were used to recover infectious NDV from cDNA. Primary chicken embryo fibroblasts and primary chicken embryo kidney cells were used for *in vitro* characterization of recombinant NDV.

Construction of Recombinant Viruses Expressing the AIV H5 Gene. The plasmid pflNDV-1, expressing the full-length antigenomic RNA of NDV Clone 30 (13) was used to introduce the AIV H5 gene. First, a NotI/BsiWI-fragment (nucleotides 4,953–8,852) of the Clone 30 genome was inserted into pUC18 plasmid (Fig. 1, step A). NcoI and AflII sites (underlined) were then introduced (Fig. 1, step B) by using primers MP1 (5′-gacaacagtcctcaaccatggaccgcgccg-3′) and MP2 (5′-ctggctagttgagtcaattcttaaggagttggaaagatggc-3′). The AIV H5 ORF, which has been amplified from plasmid pCD-HA5 (7) by specific primers containing NcoI or AflII sites (PH5F2: 5′-ccttccatggagaaaatagtgcttc-3′ and PH5R2: 5′-cctccttaagtataattgactcaattaaatgcaaattctgcactgcaatgatcc-3′), was used to substitute the HN ORF of Clone 30 after digestion with NcoI and AflII. In addition, SgfI and SnaBI sites were introduced into the intergenic region in front of the polymerase gene by using primers MP3 (5′-caaaacagctcatggtacgtaatacgggtaggacatgg-3′) and MP4 (5′-gtaagtggcaatgcgatcgcaggcaaaacagctcatgg-3′). Similar sites flanking the HN gene (Fig. 1, step C) were generated by using primers MP3 and MP5 (5′-gaaaaaactaccggcgatcgctgaccaaaggacgatatacggg-3′) for insertion of the HN gene behind the H5 ORF (Fig. 1, step D). The H5 sequence that resembles a transcription termination-like sequence of NDV was modified by silent mutations by using primer MPH5F2 (5′-ggaatgtccctcaaagaaggaggaagaagagaggactatttggggc-3′), as shown in step E of Fig. 1. Finally, the NotI/BsiWI-fragment of pflNDV-1 was substituted by a similar fragment obtained from steps D and E to create full-length clones NDVH5 or NDVH5m, respectively (Fig. 1). The length of the resulting clones (17,196 nt) is a multiple of 6, thus following the "rule of six" required for replication-competent NDV (20, 21). All mutagenesis reactions used the QuikChange II XL site-directed mutagenesis kit (Stratagene).

Transfection and Virus Recovery. To recover recombinant NDV expressing AIV H5, the full-length clones were transfected together with plasmids expressing the NP, phosphoprotein, and polymerase proteins into BSR-T7 cells by using Lipofectamine 2000 (Invitrogen) at a DNA:lipofectamine rate of 1:1.5. Virus propagation and confirmation of the recovery of infectious virus were carried out as described in refs. 13 and 15.

Northern Blot Analyses. Chicken embryo fibroblasts were infected with NDV Clone 30, NDVH5, NDVH5m, or AIV H5N2 at a multiplicity of infection of 10 and incubated for 8 h at 37°C. Total RNA of infected and noninfected cells was prepared (22), separated in denaturing agarose gels, transferred to nylon membranes, and hybridized with radiolabeled cRNAs as described in ref. 23. Plasmids containing the ORFs of AIV H5 or NDV F and HN were used for *in vitro* transcription of ^{32}P-labeled antisense cRNA (SP6/T7 Transcription kit; Roche Applied Science). Quantitation of mRNA was performed by using FLA3000 (FujuFilm) and Advanced Image Data Analyzer software (Ray-

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test Isotopen-Meβgeräte, Straubenhardt, Germany). For calculation of ratios between H5 transcripts, the RNA length, and the signals of the F mRNAs of NDV recombinants were considered.

Western Blot Analyses. Chicken embryo kidney cells were infected at a multiplicity of infection of 5 with NDV Clone 30, NDVH5, NDVH5m, or AIV H5N2 and incubated for 30 h at 37°C. Lysates of infected cells or virions purified by centrifugation through a continuous sucrose gradient (30–60%) were separated by SDS/PAGE and transferred to nitrocellulose filters (TransBlot SD cell; Bio-Rad). Blots were incubated with a polyclonal rabbit antiserum against NDV, or a polyclonal chicken antiserum against AIV H5 (Intervet). Binding of peroxidase-conjugated species-specific secondary antibodies (Dianova) was detected by chemiluminescence with SuperSignal West Pico Chemiluminescent Substrate (Pierce) on x-ray films (Hyperfilm MP; Amersham Pharmacia).

Immunoelectron Microscopy. Purified virions were adsorbed to formvar-coated copper grids for 7 min. The grids were washed four times with PBS containing 0.5% BSA (PBSB) followed by incubation with a polyclonal NDV- or AIV H5-specific antiserum for 45 min. After several washes with PBSB, the grids were incubated with gold-tagged protein A (PAG 10; Biocell Laboratories) or gold-tagged rabbit anti-chicken antibodies (RCHL 10; Biocell Laboratories) for another 45 min. After a final wash with PBSB, the virions were counterstained with phosphotungstic acid (pH 7.2), and examined with an electron microscope (Tecnai 12; Philips, Eindhoven, The Netherlands).

Animal Experiments. The safety of the NDV recombinants was assessed by determining the intracerebral pathogenicity indices according to the European Community Council Directive (17).

Vaccination experiments were carried out by oculonasal administration of 10^6 EID_{50} of NDVH5m to 25 3-week-old specific pathogen-free chickens. To evaluate protection after a single vaccination, five of the vaccinates together with 5 naive controls were challenged with $10^{5.3}$ mean embryo lethal doses (ELD_{50}) of NDV strain Herts 33/56 intramuscularly 3 weeks p.i. Likewise, 10 of the vaccinates and 9 control animals were challenged oculonasally with $10^{7.7}$ EID_{50} of the HPAIV H5N2. The remaining 10 chickens received a second immunization 6 weeks after the first vaccination and were subsequently subjected to a similar AIV challenge together with 5 naive control animals 2 weeks thereafter. After the immunizations and challenge infections, all birds were observed daily for a period of 10 days for clinical signs and classified as healthy (0), ill [one of the following signs: respiratory signs, depression, diarrhea, cyanosis, edema, or nervous signs (1)]; severely ill [more than one of the signs mentioned above (2)]; or dead (3). A clinical score was calculated that represents the mean value of all chickens per group for this period.

Analysis of Virus Shedding by Real-Time RT-PCR and Virus Isolation. Oropharyngeal and cloacal swabs were collected to analyze AIV shedding by real-time RT-PCR on days 2, 4, 8, and 14 p.c. RNA from swabs was prepared either automated by using the Nucleo Spin kit (Macherey & Nagel), or manually by using the viral RNA kit (Qiagen). For the detection of AIV shedding after challenge infection, the influenza A virus real-time RT-PCR method based on amplification of the M gene was used (24). Quality of RNA extraction and absence of inhibition factors during the RT-PCR were verified by a heterologous internal control (25). The duplex assay was performed on the MX3000p (Stratagene) cycler by using the one step RT-PCR kit (SuperScript III One-Step RT-PCR system with Platinum TaqDNA polymerase; Invitrogen). The temperature profile was 30 min at 50°C, 2 min at 94°C, followed by 42 cycles of 30 sec at 94°C, 30 sec at 57°C, and 30 sec at 68°C. For virus reisolation, the same volume as used for RNA preparation was inoculated into each of three 10-day-old embryonated specific pathogen-free chicken eggs.

HI, Indirect Immunofluorescence, and NP-ELISA. To determine the presence of NDV and AIVH5 antibodies, blood samples were collected at 0, 7, 14, and 21 d p.i. and p.c. and subjected to HI tests as described in the European Community Council Directive (17, 26). For assessing the presence of AIVH5 antibodies after immunization, the sera were additionally analyzed in a 1:100 dilution by indirect immunofluorescence on AIV-infected chicken embryo fibroblasts. Antibodies against AIV NP were detected by an indirect ELISA. For this purpose, a purified recombinant baculovirus-derived GST-NP fusion protein, encompassing the complete coding region of the AIV NP gene, was used as antigen. Sera diluted 1:300 in PBS containing 0.05% Tween 20 were investigated in duplicate. Binding of secondary POD-conjugated goat-α-chicken IgG (H+L) (Rockland Immunochemicals) antibodies was detected by a color reaction with *o*-phenylenediamine at 492 nm.

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