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A recombinant vesicular stomatitis virus replicon vaccine protects chickens from highly pathogenic avian influenza virus (H7N1)

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ABSTRACT

Highly pathogenic avian influenza viruses (HPAIV) of subtypes H5 and H7 cause fatal disease in poultry (fowl plague) but also have zoonotic potential. Currently commercially available vaccines often do not provide sufficient protection and do not allow easy discrimination between vaccinated and infected birds. Therefore, vaccination of domestic poultry against H5 and H7 HPAIV is not allowed in many countries, or is only possible after special permission has been provided. We generated a recombinant marker vaccine based on non-transmissible vesicular stomatitis virus (VSV) expressing the HA antigen of HPAIV A/FPV/Rostock/34 (H7N1) in place of the VSV G gene. This virus, VSV*ΔG(HA), was propagated on a helper cell line providing VSV G *in trans*. Since no progeny virus was produced after infection of non-complementing cells, the vector was classified as biosafety level 1 organism ("safe"). Chickens were immunized via the intramuscular route. Following booster vaccination with the same replicons high titers of serum antibodies were induced, which neutralized avian influenza viruses of subtypes H7N1 and H7N7 but not H5N2. Vaccinated chickens were protected against a lethal dose of heterologous HPAIV A/chicken/Italy/445/99 (H7N1). Secretion of challenge virus was short-term and significantly reduced. Finally, it was possible to discriminate vaccinated chickens from infected ones by a simple ELISA assay. We propose that VSV replicons have the potential to be developed to high-quality vaccines for protection of poultry against different subtypes of avian influenza viruses.

1. Introduction

Avian influenza viruses (AIV) are highly contagious respiratory pathogens that are endemic in aquatic birds worldwide [1]. Based on the major viral surface antigens hemagglutinin (HA) and neuraminidase (NA), AIV are classified into 16 different HA and 9 different NA subtypes that show no serological cross-reactivity and low sequence homology with each other. The segmented RNA genome of influenza viruses allows reassortments among different sublines to occur leading to several combinations of HA and NA subtypes. Thus, a highly diverse genetic pool of influenza viruses is present in wild bird populations.

AIV seem to be well adapted to aquatic birds and normally do not cause any disease in waterfowl. However, AIV replicate in the gastrointestinal tract of these birds and are shed into the environment in large quantities facilitating transmission to other species including domestic poultry and mammals. Furthermore, infectious virus may be transmitted by migratory birds over long distances. For reasons that are not completely understood, highly pathogenic AIV (HPAIV) may evolve from low pathogenic ones in domestic birds [2]. HPAIV are characterized by mutations in several genes, but mutations that affect proteolytic activation of the glycoprotein HA often play a dominant role [3]. In contrast to low-pathogenic AIV, HPAIV are readily cleaved by ubiquitously expressed subtilisin-like proteases resulting in rapid dissemination of HPAIV in infected animals [4,5]. Infections with HPAIV therefore cause fatal disease with high morbidity and mortality rates leading to tremendous economic losses. This was strikingly illustrated by the recent H5N1 outbreak in Asia, which spread to Europe and Africa. Though AIV are not easily transmitted to humans normally, several individuals that were exposed to high concentrations of H5N1 became infected and more than 60% of them died [6]. It is of major concern that H5N1 may eventually adapt to efficient replication in man. In addition, it cannot be ruled out that H5N1 may form reassortants with human influenza viruses, which may then be transmitted from

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person to person more easily. As the human immune system is completely naive towards HA antigens of H5 subtype, such reassortants may cause pandemics with probably millions of deaths. In 2003, an H7N7 outbreak in The Netherlands also affected humans indicating that HPAIV of this subtype also have zoonotic potential though they may be less pathogenic than H5N1 [7]. Thus, the effective control of HPAIV in poultry is not only important from the economic point of view but may also save human lives.

In addition to strict biosecurity measures, vaccination of domestic poultry would be a convenient way for AIV control, if reliable protective and safe vaccines would be available. At the moment, live attenuated, avirulent AIV are not accepted as potential vaccine candidates in most countries because of high risk of virus reversion to virulence due to antigenic drift and shift. Likewise, the general use of inactivated whole virus H5 and H7 subtype vaccines is prohibited in many countries. This policy is mainly based on two arguments. First, currently available inactivated H5 and H7 AIV vaccines may protect from clinical disease but may not prevent virus shedding from vaccinated and consecutively infected birds. Second, there is currently no standardized method available to discriminate infected from vaccinated animals. Therefore, immunization with these vaccines may lead to undetected spread of AIV, which results in severe trading restrictions for vaccinated birds. Moreover, it has been reported that vaccination may readily select for escape mutants if sterile immunity cannot be achieved [8].

Vector vaccines expressing influenza virus antigens provide an attractive alternative to conventional inactivated whole virus vaccines. Vector vaccines usually encode for only some influenza virus antigens and can therefore be used as marker or DIVA vaccines ("differentiating infected from vaccinated animals"). Moreover, vector vaccines also trigger cell-mediated immunity since antigenic epitopes will be presented by MHC-I molecules. To date, several recombinant fowlpox viruses expressing the H5 hemagglutinin have been generated [9–13], and one has been licensed and is currently used in Mexico. In addition, infectious laryngotracheitis virus (ILTV) and Newcastle disease virus (NDV) were used for expression of H7 or H5 hemagglutinin [14–17]. Though most of these vector vaccines were shown to provide protection, there are still concerns left with respect to their safety. Replication-competent viral vectors, in particular those based on RNA viruses, might mutate and revert to virulence. In this respect, replication-incompetent viral vectors based on human adenovirus type 5 represent an interesting alternative vaccine approach [18,19]. Likewise, single-cycle vesicular stomatitis virus (VSV) vector lacking the essential glycoprotein G gene was recently shown to induce protective immunity in mice against challenge with either highly pathogenic H5N1 [20] or A/WSN/33 (H1N1) [21]. Such replication-incompetent RNA replicons are promising vaccine candidates because they are safe and induce strong immunity by stimulating both humoral and cellular immunity. Furthermore, they do not induce neutralizing antibodies to the vector itself, and can be used in booster protocols [22]. VSV is not a naturally occuring avian pathogen thus excluding any pre-existing immunity in poultry populations to the vector itself. Despite these obvious advantages, VSV-based vectors have not been used for vaccination of poultry so far.

In this study, we used a single-cycle VSV vector expressing either HA or NP antigen of HPAIV H7N1 for immunization of chickens. Vaccinated animals were protected from lethal infection with heterologous HPAIV H7N1 and demonstrated significantly reduced virus shedding. In addition, immunized animals were easily distinguished from infected ones using a commercially available serological test. This study suggests that non-transmissible VSV replicons represent a promising vector system for vaccination of poultry.

2. Materials and methods

2.1. Cells

BHK-21 were obtained from the German Cell Culture Collection (DSZM, Braunschweig) and grown in Earle's minimal essential medium (EMEM) supplemented with 5% fetal bovine serum (FBS). BHK-G43, a transgenic BHK-21 cell clone expressing VSV G protein in a regulated manner, was maintained as described previously [23]. Madin–Darby canine kidney (MDCK) cells (type II) were provided by Kai Simons (MPI of Molecular Cell Biology and Genetics, Dresden) and cultured with EMEM and 5% FBS. Primary chicken embryo fibroblasts (CEF) were prepared from 10 days old specific pathogen-free (SPF) chicken embryos and maintained in McCoy's 5A/Leibovitz's L15 (1:1) medium containing 4% FBS.

2.2. Viruses

Avian influenza viruses A/FPV/Rostock/34 (H7N1) and A/chicken/Italy/445/99 (H7N1) were kindly provided by Wolfgang Garten (Institute of Virology, University of Marburg) and Ilaria Capua (Istituto Zooprofilattico Sperimentale delle Venezie, Padova, Italy), respectively. Avian influenza viruses A/duck/Potsdam/15/80 (H7N7) and A/teal/Föhr/Wv11378-79/03 (H5N2) were kindly provided by Martin Beer (Institute of Diagnostic Virology, FLI Riems). All viruses were propagated in the allantoic cavity of 10-day-old embryonated SPF chicken eggs. The 50% egg infectious dose (EID_{50}) was determined by infecting eggs in triplicate with serial virus dilutions. Titers were calculated according to the Spearman–Kärber method [24]. Infectious titers of A/FPV/Rostock/34 (H7N1) were determined by plaque assay on MDCK cells. Recombinant modified vaccinia virus Ankara expressing T7 phage RNA polymerase (MVA-T7), a kind gift of Gerd Sutter (Paul-Ehrlich-Institut, Langen, Germany), was propagated and titrated on primary CEFs [25].

2.3. Plasmid constructs

A cDNA encoding the full-length antigenomic (positive-strand) vesicular stomatitis virus RNA (strain Indiana; GenBank accession number J02428) was assembled in the pUC18 plasmid. The cDNA was placed under control of the T7 promotor sequence and was followed by the hepatitis delta ribozyme and the T7 terminator sequences according to a previous report [26]. Unique MluI and BstEII restriction enzyme sites were introduced upstream and downstream of the VSV glycoprotein G ORF, respectively. An additional transcription unit comprising the consensus transcription start sequence, XhoI and NheI restriction sites, and a transcription stop sequence were introduced into the G–L intergenic region [27]. The enhanced green fluorescent protein (eGFP) gene was amplified from the pEGFP-N1 plasmid (Clontech) by *Pfu* PCR and cloned into the XhoI and NheI sites resulting in the plasmid pVSV*. The HA open reading frame of A/FPV/Rostock/34 (GenBank accession number M24457) was amplified from the plasmid pTM1-HA(H7) (kindly provided by Wolfgang Garten, University of Marburg) and cloned into the MluI and BstEII restriction sites of pVSV* thereby replacing the VSV G gene. The resulting plasmid was designated pVSV*ΔG(HA). The NP gene of A/FPV/Rostock/34 (GenBank accession number M21937) was amplified by RT-PCR using total RNA from virus-infected MDCK cells as template and cloned into the MluI and BstEII restriction sites of pVSV* resulting in the plasmid pVSV*ΔG(NP). The N, P, and L genes of VSV were amplified by RT-PCR and cloned into the pTM1 plasmid downstream of the T7 promotor and the encephalomyocarditis virus IRES sequence [28].

2.4. Generation of single-cycle VSV vector vaccines

BHK-G43 cells were seeded in 6-well plates and maintained in EMEM medium with 5% FBS until they reached 90% confluence. The cells were treated with mifepristone (10^{-9} M) for 6 h to induce VSV G expression [23], and were infected for 1 h at 37 °C with MVA-T7 using an m.o.i. of 5 pfu/ml. Subsequently, the cells were transfected with a set of plasmids including the antigenomic plasmid (5 μg), pTM1-N (2.5 μg), pTM1-P (1.5 μg), and pTM1-L (0.5 μg) using Lipofectamine™ 2000 (Invitrogen) as transfection reagent. Six hours post transfection, the cells were washed twice with medium and maintained in fresh medium with 5% FBS and mifepristone (10^{-9} M) for 48 h. The cell culture supernatant was added to fresh mifepristone-treated BHK-G43 cells in 6-well plates and incubated for 24 h at 37 °C. Successful rescue of infectious virus was monitored by fluorescence microscopy for detection of GFP-expressing cells. To remove vaccinia virus, the cell culture supernatant of positive wells was passed through a 0.22 μm pore size filter. The VSV replicons were propagated on mifepristone-induced BHK-G43 cells. To determine infectious virus titers, confluent BHK-21 grown in 96-well microtiter plates were inoculated in duplicate with 40 μl of serial ten-fold virus dilutions for 1 h at 37 °C. The wells received additional 60 μl of EMEM and were incubated for 20 h at 37 °C. The infectious titers were calculated based on the number of GFP-expressing cells/well and expressed as fluorescence-forming units per milliliter (ffu/ml).

2.5. Immunofluorescence analysis

Primary chicken fibroblasts were grown on 12-mm-diameter cover slips for 48 h and inoculated with either VSV*ΔG(HA), VSV*ΔG(NP), VSV*ΔG, or A/FPV/Rostock/34 (H7N1) for 1 h at 37 °C using an m.o.i. of 3 ffu(pfu)/cell. Eight hours after infection, the cells were fixed with 3% paraformaldehyde for 20 min. For detection of NP antigen the cells were permeabilized with 0.2% Triton X-100 for 5 min. The cells were incubated for 60 min with either a rabbit polyclonal antibody directed against A/FPV/Rostock/34 (H7N1) (1:1000; kindly provided by W. Garten, Marburg) or a monoclonal antibody directed against influenza virus NP antigen (1:500; Serotec). The primary antibodies were detected with FITC-conjugated secondary antibodies directed against the IgG fraction of the respective species (1:500; Sigma). Conventional epifluorescence was performed using a Zeiss axiovert 2 microscope.

2.6. Cell surface biotinylation, immunoprecipitation, and Western blot analysis

Chicken fibroblasts were grown on 6-well dishes to confluence and infected with either VSV*ΔG(HA), VSV*ΔG(NP), VSV*ΔG, or A/FPV/Rostock/34 (H7N1) for 1 h at 37 °C using an m.o.i. of 3 ffu(pfu)/cell. Eight hours following infection, the cells were washed twice with ice-cold PBS and incubated for 20 min at 4 °C with 250 μl of sulfo-NHS-LC-biotin (0.5 mg/ml; Pierce) in PBS. The labeling reaction was stopped by incubating the cells with 0.1 M glycine/PBS. The cells were lysed in 600 μl of NP40 lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, and protease inhibitor mixture) and insoluble material was removed by centrifugation (16,000 × *g*, 4 °C, 30 min). For immunoprecipitation of HA antigen, 500 μl of clarified cell lysate were incubated at 4 °C overnight with 50 μl of protein A-Sepharose (50% slurry; Sigma) and 2 μl of polyclonal rabbit anti-H7N1 serum with agitation. The beads were washed three times with NP40 lysis buffer and antigen was eluted by incubating the beads in 2× SDS sample buffer for 10 min at 95 °C. The immunoprecipitates were separated by SDS-PAGE under reducing conditions (0.1 M dithiothreithol), transferred to nitrocellulose membranes by semi-dry blotting, and incubated overnight with blocking reagent (Roche). After incubating the membrane for 60 min with a biotinylated streptavidin-peroxidase complex (1:2000; GE Healthcare), antigens were visualized by enhanced chemiluminescence (SuperSignal, Pierce). The light emission was recorded with a supercooled CCD camera (Chemi-Doc System, BioRad).

For detection of NP antigen, infected chicken fibroblasts were directly lysed in 500 μl of hot (95 °C) SDS sample buffer 8 h p.i. without prior biotinylation. Chromosomal DNA was sheared by several passages through a 21-gauge needle attached to a 1 ml-syringe. The cell lysates were separated by SDS-PAGE under non-reducing conditions, blotted to nitrocellulose membrane, and subsequently incubated with monoclonal anti-NP antibody (1:100; Serotec), biotinylated anti-mouse IgG (1:1000; Sigma), and streptavidin-peroxidase complex (1:2000) to finally allow detection of NP antigen by chemiluminescence.

2.7. Animal experiments

Specific pathogen-free embryonated chicken eggs (VALO®, Lohmann LSL-LITE) were purchased from Lohmann Tierzucht GmbH, Cuxhaven, Germany. Birds were raised under isolation conditions according to the animal welfare guideline. Water and feed were provided *ad libidum*. Three weeks after hatch, 60 birds were randomly divided into 4 groups of 15 chickens/group, and immunized intramuscularly according to the following regimen: 0.25 ml of EMEM medium (group 1), 0.25 ml of BHK-G43 cell culture supernatant with 2×10^7 ffu of VSV*ΔG (group 2), 0.25 ml of BHK-G43 cell culture supernatant with 2×10^7 ffu of VSV*ΔG(HA) (group 3), 0.5 ml of cell culture supernatant with both VSV*ΔG(HA) and VSV*ΔG(NP), 2×10^7 ffu each (group 4). Three weeks after primary vaccination, chickens were immunized a second time with the same vaccines using the same dose and route.

Two weeks after booster vaccination, 10 birds of each group were transferred to isolation units under BSL-3 conditions and challenged oculonasally with 10^7 EID_{50} per animal of HPAIV A/chicken/Italy/445/99 (H7N1). The infected animals were surveyed daily for clinical signs of disease, which were scored as follows: healthy (0), peri-ocular swelling (1), severely ill (2), and dead (3). Birds were assessed "severely ill" if they demonstrated at least two of the following symptoms: respiratory distress, ruffled feather, apathy, anorexia, diarrhea, cyanosis of the exposed skin, comb and wattles, oedemas of the face and/or head, and nervous signs. Five birds of each group were left unchallenged and were surveyed for three weeks for detection of any side effects due to vaccination. All chickens were analyzed by immunohistochemistry for detection of influenza NP antigen.

2.8. Serological tests

Blood samples were collected from chickens at days 0, 14, 28 p.i. and at day 21 p.c. To test for hemagglutination inhibition (HI) activity, 25 μl of two-fold serially diluted serum samples were dispensed in U-bottom 96-well microtiter plates and incubated for 60 min at room temperature with 25 μl of AIV (4 HAU). Thereafter, each well received 50 μl of freshly prepared chicken erythrocyte suspension (1%). Following incubation for 60 min at 4 °C the HAI titer was determined by the reciprocal dilution causing complete inhibition of erythrocyte agglutination.

For detection of anti-NA (N1 subtype) antibodies a commercially available competitive ELISA test was used according to the manufacturer's instructions (IDVET, Montpellier, France).

To test for the presence of virus neutralizing (VN) antibodies, serum was heat inactivated at 56 °C for 30 min and subjected to serial two-fold dilutions using cell culture medium as diluent. Equal volumes of diluted serum and influenza virus (100 $TCID_{50}$)

were combined and incubated in microtiter plates (100 μl/well) in quadruplicates. After incubating the samples for 1 h at room temperature, 50 μl of MDCK cell suspension (2×10^5 cells/ml) were added and incubated at 37 °C for 48 h. The cells were fixed with 3% paraformaldehyde in PBS for 20 min, permeabilized with 0.2% Triton X-100 for 5 min, and subsequently incubated with a monoclonal anti-NP antibody (1:1000, Serotec), and a peroxidase-linked rabbit anti-mouse IgG (1:500, Dako), each for 1 h at room temperature. Finally, infected cells were stained with AEC peroxidase substrate (1.7 mM 3-amino-9-ethylcarbazole and 0.1% H_2O_2 in 50 mM sodium acetate buffer [pH 5.0]). The reciprocal serum dilutions at which 50% of the wells were protected from virus infection (ND_{50}) were calculated according to Spearman–Kärber [24].

2.9. Analysis of virus shedding by RT-PCR and virus isolation

Oropharyngeal and cloacal swabs were collected from chickens on days 0, 2–9, 11, 14, and 21 p.c., placed in 3 ml of sample media, and stored as described previously [29]. RNA was extracted from swab samples using the Nucleospin Multi 96 Virus kit (Macherey-Nagel). For detection of viral RNA, a real-time RT-PCR based on amplification of the viral matrix protein gene [30] was performed as duplex assay using a heterologous internal control [16,29,31]. To correlate RT-PCR results with infectious virus titers, samples showing threshold cycle (Ct) values between 29 and 42 were used for virus reisolation in 10-day-old embryonated SPF chicken eggs [29]. Since samples with Ct values higher than 38 failed to yield infectious virus after two egg passages, only Ct values up to 38 were regarded as indicative for the presence of infectious virus.

2.10. Statistical analysis

Statistical analysis was performed using the paired Student's *t*-test, and $p < 0.05$ was considered significant.

3. Results

3.1. Generation of VSV*ΔG vectors expressing H7N1 antigens

For generation of a non-transmissible VSV replicon vaccine, we replaced the G gene in the VSV genome by either the hemagglutinin (HA) or the nucleoprotein (NP) gene of H7N1 HPAIV A/FPV/Rostock/34 (Fig. 1). Both vectors, VSV*ΔG(HA) and VSV*ΔG(NP), were engineered to express GFP from an additional transcription unit downstream of HA and NP, respectively (Fig. 1), to ease detection and titration of the recombinants. A VSV replicon that expressed GFP but neither AIV antigen was generated as a vector control (VSV*ΔG). All VSV replicons were successfully propagated on a helper cell line that provided the VSV G protein *in trans*

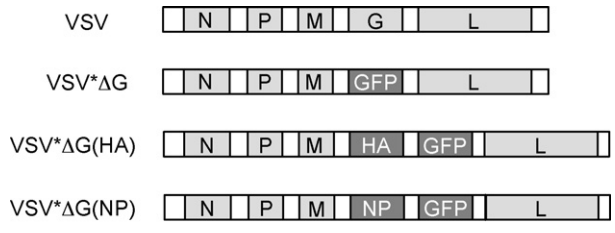

Fig. 1. Genome maps of recombinant VSV vectors. The VSV genome encodes for the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and the RNA polymerase (L). The open reading frame encoding glycoprotein G was replaced in the parental VSV genome by the open reading frame for enhanced green fluorescent protein (GFP), which resulted in the vector VSV*ΔG (the asterisk denotes for GFP). VSV*ΔG(HA) and VSV*ΔG(NP) vectors were generated by replacing VSV G with the HA and NP genes of A/FPV/Rostock/34 (H7N1), respectively. These vectors expressed GFP from an additional trancription unit placed into the G–L junction.

[23]. Titers of more than 10^8 infectious units per milliliter were achieved with this system (see also Fig. 3B).

To study VSV vector-driven expression of AIV antigens, we infected primary CEFs with VSV*ΔG(HA), VSV*ΔG(NP), and VSV*ΔG, respectively, using a multiplicity of infection of 3 ffu/cell. Eight hours post infection, the cells were fixed and analyzed by indirect immunofluorescence (Fig. 2A). HA antigen was detected on the cell surface while NP antigen was primarily detected in the nucleus of infected cells. CEF infected with the control vector VSV*ΔG did not bind either antibody. After immunoprecipitation of cell surface biotinylated HA from VSV*ΔG(HA)-infected CEF lysates with polyclonal rabbit anti-AIV (H7N1) serum, two bands of 50 kDa (HA_1) and 25 kDa (HA_2) were detected (Fig. 2B). In HPAIV (H7N1)-infected cells, not only the HA subunits but also NA antigen were identified at the cell surface. By Western blot analysis, NP antigen appeared as a single band of approximately 56 kDa in total lysates of VSV*ΔG(NP) or A/FPV/Rostock/34 (H7N1)-infected cells, whereas no signal was obtained with VSV*ΔG or mock-infected cells (Fig. 2C). Since a polyclonal antibody was used, additional protein species were also detected in lysates of H7N1-infected CEF. Together, these data indicate that the transcomplemented VSV*ΔG vectors were able to efficiently infect primary chicken fibroblasts to drive high-level expression of AIV antigens. The recombinant antigens showed correct subcellular localizations and HA was subjected to posttranslational cleavage as expected.

3.2. HA does not substitute for VSV G functions

Similar to VSV G, influenza virus HA exhibits receptor-binding and pH-dependent fusion activity and has been previously shown to be incorporated into VSV virions [32]. Therefore, we could not exclude in the first place that substitution of VSV G by HA would result in a replication-competent virus. To address this point, BHK-G43 cells, either pretreated with mifepristone for induction of VSV G expression or left untreated, were infected with transcomplemented VSV*ΔG(HA) and subsequently incubated with a neutralizing antibody to inactivate all the virus that had not entered the cells. Twelve hours post infection, GFP expression was detected by fluorescence microscopy indicating that infection was successful (Fig. 3A, first cycle). When the culture supernatant of these cells was inoculated with fresh cells, GFP fluorescence was subsequently detected only if cell culture supernatant from mifepristone-treated BHK-43 cells was used (Fig. 3A, second cycle). In contrast, cell culture supernatant from untreated BHK-G43 cells or from normal BHK-21 cells did not contain any infectious virus vector (detection limit of 10 ffu/ml). Even when VSV*ΔG(HA) was serially passaged (10 times) on BHK-G43 cells, autonomously replicating virus did not emerge (data not shown). These findings indicate that HA is unable to substitute for VSV G functions and that the phenotype of the vector is stable. Thus, VSV*ΔG(HA) propagation was only possible on transcomplementing BHK-G43 cells which allowed titers of 3×10^8 ffu/ml to be reached (Fig. 3B). VSV*ΔG(HA) was classified by the German Central Commisssion for Biosafety in biosafety level 1 category ("safe").

3.3. VSV*ΔG(HA) induces neutralizing antibodies in immunized chickens

Three weeks old SPF chickens were inoculated intramuscularly with 250 μl virus-free EMEM or medium containing 2×10^7 ffu of either VSV*ΔG(HA) or VSV*ΔG. A fourth animal group was vaccinated with a mixture of VSV*ΔG(HA) and VSV*ΔG(NP) containing 2×10^7 ffu of each vector. Three weeks after the first immunization the animals were boostered using the same vector vaccines, dose, and route. Blood was collected two weeks after each immunization, and serum was analyzed for hemagglutination inhibition

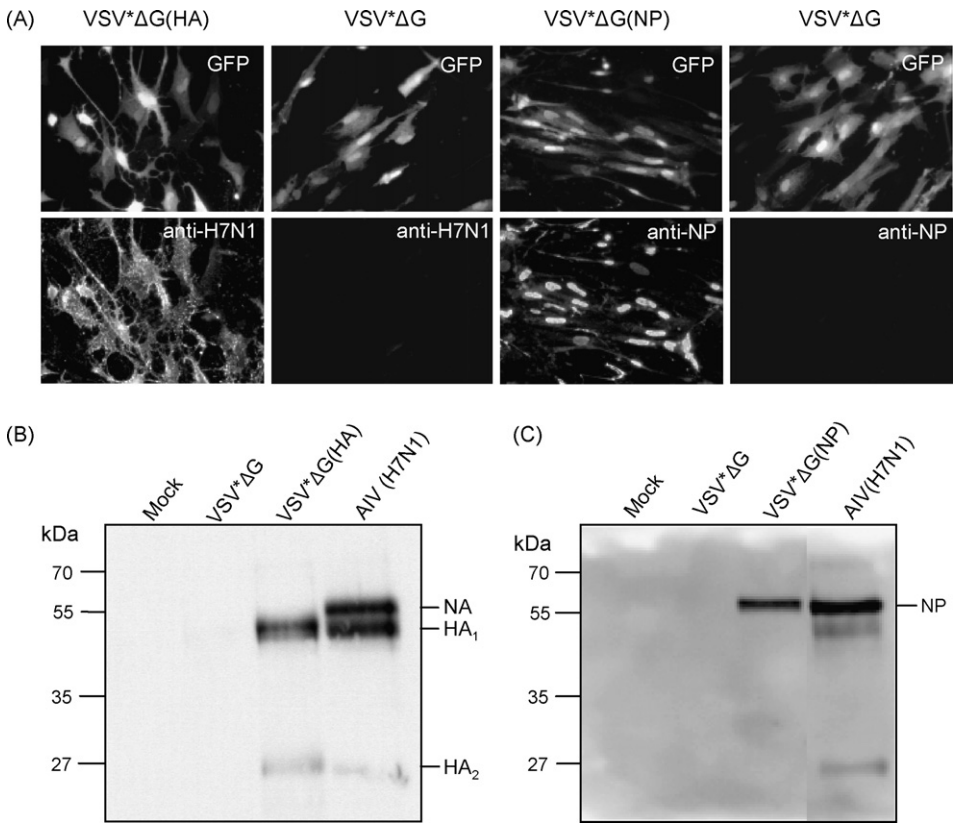

Fig. 2. VSV vector-driven expression of HA and NP antigens in primary chicken embryo fibroblasts (CEF). CEF were either infected with VSV*ΔG(HA), VSV*ΔG(NP) or VSV*ΔG at an m.o.i. of 3 infectious units/cell. (A) The cells were fixed with paraformaldehyde 8 h p.i. Cell surface HA was detected by indirected immunofluorescence with a polyclonal anti-AIV (H7N1) serum. For detection of NP antigen, cells were permeabilized with Triton X-100 and stained with a monoclonal anti-NP antibody. (B) At 8 h p.i., cell surface proteins were labeled with sulfo-NHS-LC-biotin. HA was immunoprecipitated from cell lysates, separated by SDS-PAGE under reducing conditions, and blotted onto a nitrocellulose membrane. Biotinylated HA antigen was detected with streptavidin-peroxidase by chemiluminescence. (C) Cell lysates were analyzed by Western blot using a monoclonal antibody directed to the influenza NP antigen.

(HAI) activity (Table 1). Control animals that had received VSV*ΔG or vehicle did not show any HAI activity. In contrast, sera collected from chickens 2 weeks after the first immunization with either VSV*ΔG(HA) or VSV*ΔG(HA) and VSV*ΔG(NP) showed low HAI titers against A/FPV/Rostock/34 (H7N1). HAI titers increased significantly after the second immunization reaching mean titers of 284 (HA group) and 320 HAI units (HA + NP group), respectively. This indicates that application of the same vector vaccine induced a clear booster effect. All immunized animals except of one in the HA + NP group responded to the recombinant VSV vaccines. Sera from boostered animals also showed significant HAI activity towards the heterologous strain A/chicken/Italy/445/99 (H7N1) and the more distantly related virus A/duck/Potsdam/15/80 (H7N7). However, lower HAI titers were detected with these strains com-

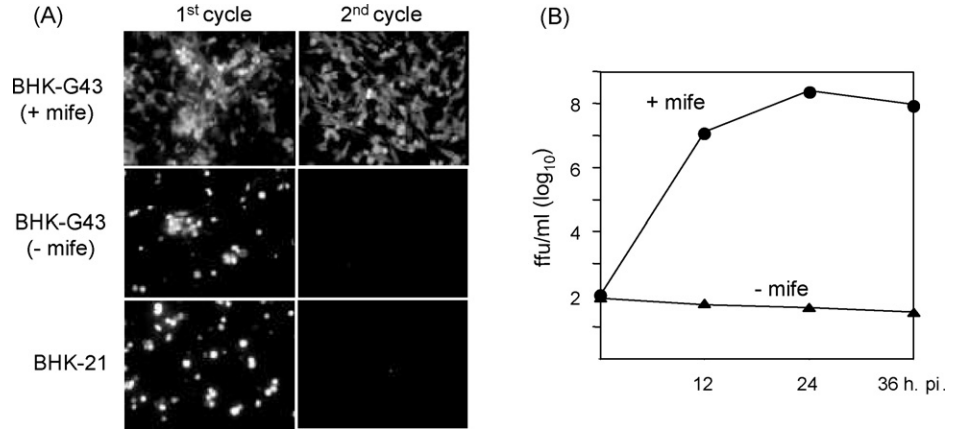

Fig. 3. VSV*ΔG(HA) requires trans-complementation by VSV G. (A) BHK-G43 cells (either pretreated with mifepristone for 6 h or left untreated) or BHK-21 cells were infected with VSV*ΔG(HA) using an m.o.i. of 0.1 (1st cycle). Viruses that did not enter the cells within 1 h were inactivated with a neutralizing antibody. Twelve hours after infection, the cell culture supernatant was used to infect fresh cells (2nd cycle). Cells were fixed 12 h p.i. and analyzed for GFP reporter expression by fluorescence microscopy. (B) BHK-G43 were treated for 6 h with mifepristone to induce VSV G expression or were left untreated. The cells were infected with VSV*ΔG(HA) using an m.o.i. of 0.05 and incubated in the presence (+mife) or absence (−mife) of mifepristone. At the indicated time points, aliquots of cell culture supernatant were sampled and titrated in triplicate on BHK-21 cells. Mean virus titers are shown. The experiment was repeated three times. Data from a representative experiment are shown.

Table 1
Serum antibody responses of chickens after immunization with VSV*ΔG replicons.

Antigen[a]	Average HAI units ($n = 10$) against[b]				
	A/FPV/Rostock/34 (H7N1)		A/chicken/Italy/445/99 (H7N1)	A/duck/Potsdam/15/80 (H7N7)	A/Teal/Föhr/Wv1378-79/03 (H5N2)
	1°	2°	2°	2°	2°
HA	22(7/10)	284(10/10)	270(10/10)	110(10/10)	<4(10/10)
HA + NP	38(9/10)	320(9/10)	157(9/10)	132(9/10)	<4(10/10)
GFP	<4(0/10)	<4(0/10)	<4(0/10)	<4(0/10)	<4(0/10)
Mock	<4(0/10)	<4(0/10)	<4(0/10)	<4(0/10)	<4(0/10)

[a] SPF chickens were immunized two times (3 and 6 weeks after hatch) by intramuscular route with 2×10^7 ffu of VSVΔG replicons expressing the indicated antigens.
[b] Blood was collected 14 days after each primary (1°) and booster immunization (2°). Sera were analyzed by hemagglutination inhibition tests using the indicated viruses. Mean HAI titers for ten birds of each group are shown. The number of birds out of ten showing titers higher than 4 HAI units are given in parentheses.

pared to the homologous A/FPV/Rostock/34 (H7N1). No HAI activity against A/teal/Foehr/Wv1378-79/03 (H5N2) was detected.

Serum antibodies capable to neutralize H7N1 in cell culture were not found in VSV*ΔG or mock-infected animals, but sera collected two weeks after the first immunization with VSV*ΔG(HA) showed low neutralization titers (Fig. 4). The ND_{50} values increased considerably following the second immunization with the same vector vaccine reaching mean ND_{50} values of 344. The humoral response was fairly variable between individual chickens with ND_{50} titers of 795, 798, and 501 as the highest and 145, 150, and 38 as the lowest. Sera of boostered animals also showed neutralization activity (mean ND_{50} value of 148) against the distantly related virus A/duck/Potsdam/15/80 (H7N7). Animals that had been vaccinated with both VSV*ΔG(HA) and VSV*ΔG(NP) produced lower neutralization titers against A/FPV/Rostock/34 (H7N1) and A/duck/Potsdam/15/80 (H7N7) than animals that had received only VSV*ΔG(HA). However, this difference turned out to be not significant ($p \geq 0.1$).

3.4. VSV*ΔG(HA) protects from a lethal dose of heterologous H7N1

Two weeks after booster vaccination, chickens were challenged with heterologous H7N1 HPAIV A/chicken/Italy/445/99 (10^7 EID_{50}) which was applied oculo-nasally. Mock-vaccinated or VSV*ΔG-vaccinated animals showed first symptoms of disease at the second day after challenge (Fig. 5). Typical symptoms included ruffled feathers, apathy, anorexia, diarrhea, zyanosis and necrosis of the combs and wattles, and haemorrhages at legs. All control birds died between days 3 and 5 post challenge. By immunohistochemical analysis NP antigen was detected in multiple organs including brain, heart, lungs, pancreas, spleen, intestine, and kidney, indicative for a systemic infection (data not shown). Most chickens that had received VSV*ΔG(HA) or VSV*ΔG(HA) with VSV*ΔG(NP) showed no signs of disease for the whole observation period (21 days post challenge). However, some animals in these two groups showed temporary oedema formation at their heads (Fig. 5.). All VSV*ΔG(HA)-vaccinated birds survived challenge with heterologous HPAIV A/chicken/Italy/445/99 (H7N1) while one bird of group 4 (HA + NP) died on day 7. This chicken was tested positive for NP antigen in heart and brain suggesting that it died due to AIV infection. The vaccinated birds which survived the challenge infection were exsanguinated at day 21 post infection. Various organs were analyzed by immunohistochemistry but NP antigen was not detected (data not shown). Vaccinated but unchallenged chickens were also tested negative in this respect.

3.5. Virus shedding from vaccinated birds

To determine whether challenge virus is shed from vaccinated chickens, oropharyngeal and cloacal swabs were collected from infected birds at daily intervals starting from day 2 post challenge. The samples were analyzed for the presence of AIV RNA segment 7 by real-time RT-PCR (Fig. 6) and isolation in embryonated chicken eggs. It was not possible to isolate infectious influenza virus from samples with Ct values higher than 38 (Table 2). A correlation of virus infectious titers and quantitative RT-PCR revealed that a ten-fold increase/decrease of EID_{50} led to a change in the Ct value by 3.3 (J. Veits, unpublished results). Oropharyngeal swabs collected from mock-vaccinated or VSV*ΔG-vaccinated birds gave rise to mean Ct values of 32 at day 2 p.c. and 20 at day 4. RT-PCR analysis of cloacal swab samples resulted in mean Ct values of 30 at day 2 and

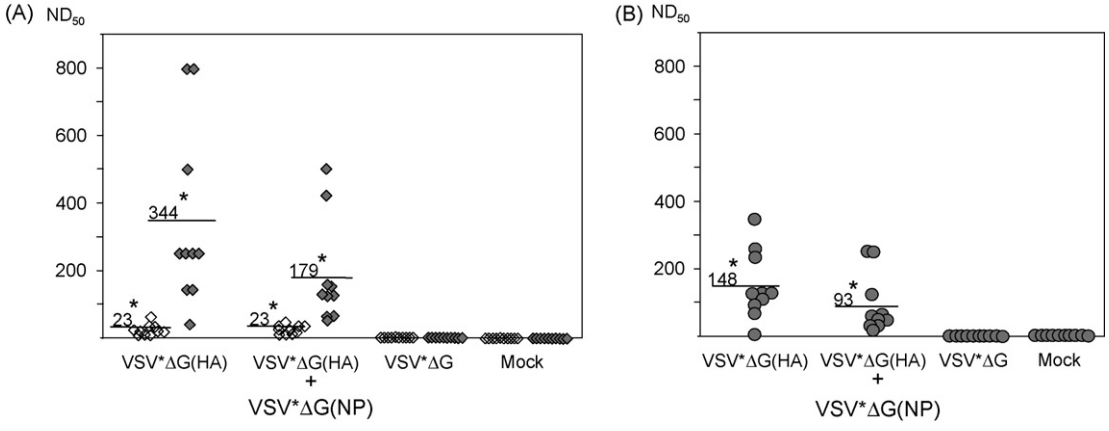

Fig. 4. Detection of neutralizing antibodies in serum of immunized chickens. Sera were prepared from SPF chickens 14 days after one (white symbols) or two (grey symbols) immunizations with the indicated VSVΔG replicons, and analyzed for their ability to inhibit infection of MDCK cells with (A) A/FPV/Rostock/34 (H7N1) and (B) A/Potsdam/15/80 (H7N7). Neutralizing titers are defined as the inverse serum dilutions causing complete virus neutralization in 50% of the wells (ND_{50}). Mean values of each group are indicated by horizontal lines. Significant differences ($p < 0.01$) compared to mock-vaccinated or VSV*ΔG-vaccinated birds are indicated by asterisks.

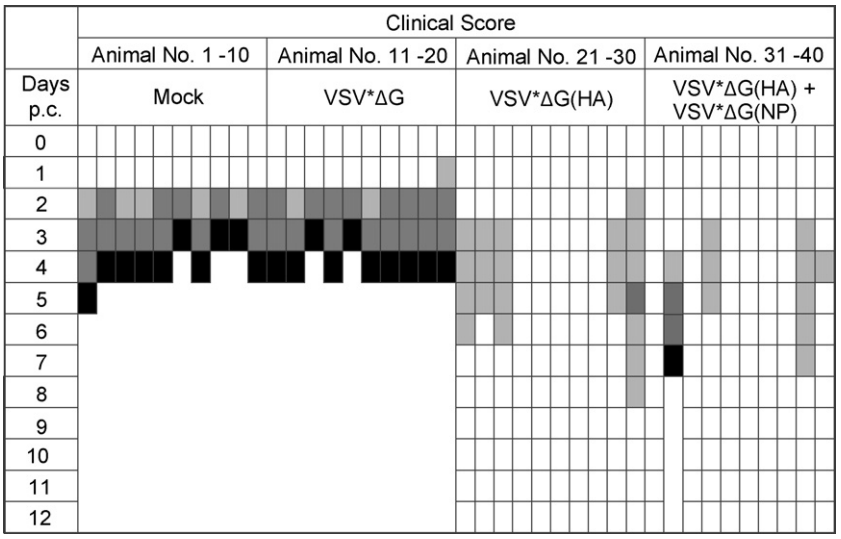

Fig. 5. Clinical monitoring of vaccinated chickens after AIV infection. SPF chickens vaccinated with either vehicle, VSV*ΔG, VSV*ΔG(HA), or a mixture of VSV*ΔG(HA) and VSV*ΔG(NP) were challenged with A/chicken/Italy/445/99 (H7N1) and surveyed daily for clinical symptoms which were scored as follows: healthy (white arrays), peri-ocular inflammation (light grey arrays), severely ill (dark grey arrays), and dead (black arrays).

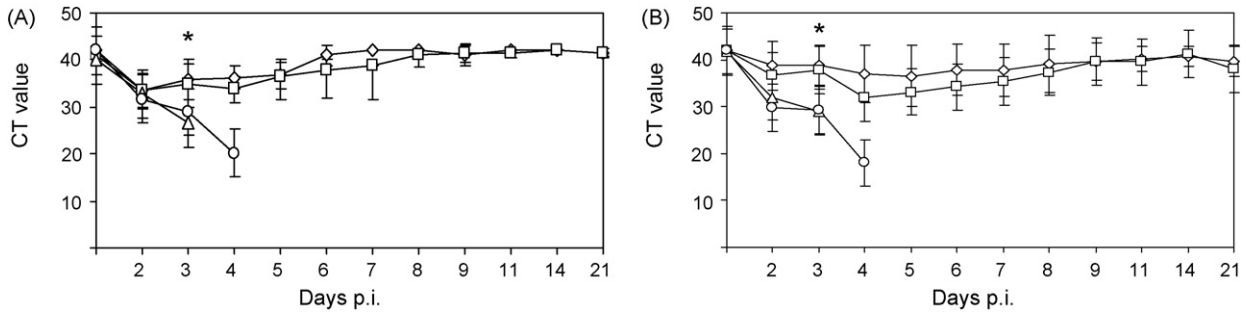

Fig. 6. Shedding of A/chicken/Italy/445/99 (H7N1) challenge virus. (A) RNA was extracted from oropharyngeal and (B) cloacal swab samples collected from SPF chickens at the indicated times post challenge with A/chicken/Italy/445/99 (H7N1). Real-time RT-PCR was performed for detection of viral RNA encoding the M gene. Mean Ct values and standard deviations from ten animals per group are given. Mock-vaccinated, VSV*ΔG-vaccinated, VSV*ΔG(HA)-vaccinated, and chickens that received both VSV*ΔG(HA) and VSV*ΔG(NP) are represented by circles, triangles, rhombs, and squares, respectively. Infectious virus could not be isolated in embryonated chicken eggs if samples showed Ct values higher than 38. Significant differences ($p < 0.01$) compared to mock-vaccinated or VSV*ΔG-vaccinated control birds are indicated by asterisks. At day 4, only one bird in the mock-vaccinated group was still alive.

18 at day 4. This indicates that significant amounts of virus were shed before the animals died. Oropharyngeal swabs collected from VSV*ΔG(HA)-vaccinated animals on days 2–4 p.c. showed mean Ct values ranging from 34 to 36. The analysis of cloacal swabs revealed Ct values of 37 at days 4 and 5 p.c. After day 6, only Ct values equal or higher than 38 were detected in all samples from chickens vaccinated with either VSV*ΔG(HA) or VSV*ΔG(HA) and VSV*ΔG(NP). These findings suggest that some virus was transiently shed from VSV*ΔG(HA)-vaccinated chickens but to a significantly reduced extent as compared to control birds. Chickens immunized with both VSV*ΔG(NP) and VSV*ΔG(HA) secreted virus to a similar extent than chickens that received only VSV*ΔG(HA). Thus, simultaneous vaccination of chickens with VSV*ΔG(NP) and VSV*ΔG(HA) did not result in further reduction of virus shedding.

3.6. Differentiation of infected from vaccinated animals

Since the VSVΔG replicon vaccines expressed HA and NP but not other AIV antigens, they were expected to meet the DIVA principle. To test this hypothesis, we used a competitive ELISA for detection of

Table 2
Reisolation of challenge virus in embryonated chicken eggs.

Egg passage	PBS	H7N1[a]	Isolation of AIV[b] from swab samples with Ct values of											
			29	30	31	32	33	34	35	36	37	38	39	42
1°	–	+	+	+	+	+	+	+	–	+	–	+	–	–
2°	ND[c]	ND	ND	ND	ND	+	ND	ND	–	+	–	+	–	–

[a] Embryonated chicken eggs were inoculated with 10 EID_{50} of A/chicken/Italy/445/99 (H7N1).

[b] Oropharyngeal swabs collected after challenge of vaccinated and non-vaccinated birds were selected according to their Ct values as determined by quantitative RT-PCR analysis (Fig. 6A). Each sample was inoculated into three 10-day-old embryonated SPF chicken eggs and incubated for 5 days (1° egg passage). Virus isolation was considered positive (+) if the allantoic fluid from at least one egg demonstrated haemagglutinating activity (≥2 HAU). Some samples were passaged in embryonated eggs a second time (2° egg passage).

[c] Not determined.

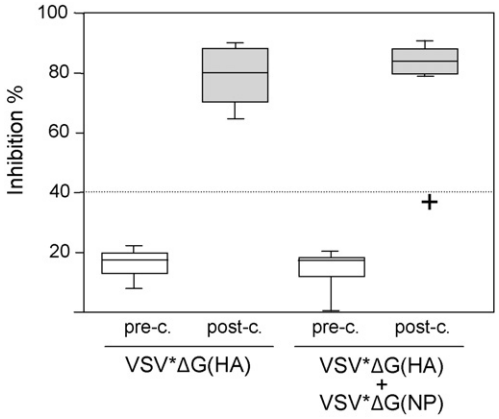

Fig. 7. Discrimination of infected from vaccinated animals by a competitive NA-ELISA. Sera were collected from SPF chickens 14 days after second immunization with the indicated VSVΔG replicons (pre-c.) and 21 days after challenge with A/chicken/Italy/445/99 (H7N1) (post-c.). Sera were tested for the presence of NA-antibodies by a competitive ELISA with anti-N1-peroxidase conjugate as tracer. The inhibition of tracer antibody binding to immobilized antigen is shown as box-whiskers plots. Percentages higher than 40 were considered positive. The "+" indicates a single bird of group 4 (HA + NP) which died on day 7.

antibodies that bind to the NA antigen of subtype N1 (Fig. 7). When serum was collected from vaccinated birds directly before challenge, NA-specific antibodies were not detected. However, when serum was sampled 3 weeks after challenge of the vaccinated birds, all birds had seroconverted and were tested positive for NA antibodies. Only one bird in the HA + NP group, which had died on day 7 (indicated by a cross in Fig. 7) did not produce NA-specific antibodies, probably because time was too short to allow seroconversion. These results indicate that vaccinated chickens can be easily discriminated from infected ones.

4. Discussion

HPAIV of subtypes H7 and H5 are highly contagious, rapidly disseminating pathogens causing fatal disease in poultry. They represent a permanent threat to poultry production, but also show zoonotic potential and may be transmitted to humans and other mammalian species [6]. Thus, vaccination of domestic poultry may not only reduce economic losses and secure the protein sources for millions of people but may also reduce the risk of transmission to humans. A high-quality vaccine against HPAIV is expected to be protective and to prevent unnoticed virus spread. To meet these requirements, a vaccine must reduce shedding of infectious virus as far as possible and must also allow differentiation of infected from vaccinated animals (DIVA). Conventional inactivated influenza vaccines do not comply with these requirements, and therefore their general use is not allowed in many countries.

We developed a non-transmissible VSV replicon vaccine for immunization of chickens with the HA and NP antigens of HPAIV (H7N1). We showed that VSV*ΔG(HA) protected chickens against lethal challenge with a heterologous H7N1 HPAIV, significantly reduced shedding of challenge virus, and complied with the DIVA principle. VSV*ΔG(HA) also satisfied high-safety criteria. Though HA has receptor-binding and fusion activities, and was previously found to be incorporated into VSV virions [32], VSV*ΔG(HA) did not produce any infectious progeny and can be regarded as a real single-cycle vector. The inability of HA to substitute VSV G functions can be explained by previous observations that showed that HA is inactive when expressed in the absence of either the receptor-destroying enzyme NA [33] or the ion channel protein M2 [34]. In addition, VSV replication/transcription takes place in the cytosol and does not include any cDNA intermediates that might recombine with or integrate into host chromosomal DNA. RNA recombination has been frequently observed with plus-strand RNA viruses [35,36], but appears to be a very rare event in the case of non-segmented negative-strand RNA virus [37,38].

Live virus vaccines (conventional or recombinant) normally are more efficient than inactivated vaccines. They stimulate both the humoral and cellular arms of the immune system, induce long lasting immune responses, and do not require adjuvants which may considerably add to the costs of conventional inactivated vaccines. However, a live virus vaccine has to be attenuated to rule out any residual virulence, and this may result in loss of immunogenicity. Often, it is difficult to find an adequate balance between virulence and immunogenicity. Non-transmissible vector vaccines might represent an attractive approach to overcome this problem.

The high efficacy of VSV vector vaccines might be explained by the high antigen expression levels achievable with this vector. The RNA replicon self-replicates in the cytosol whereby the genetic information is amplified. In addition, VSV-infected cells undergo rapid apoptosis [39]. Antigen-containing apoptotic bodies are generated and ingested by professional antigen-presenting cells thereby inducing a strong humoral immune response. Recently, it has been shown that the immunostimulatory properties of exosomal vaccines are enhanced by incorporation of fusion-competent VSV G protein [40]. This phenomenon may rely on the ability of VSV G to activate toll-like receptor-dependent pathways [41] and may also hold true for G-complemented VSV*ΔG vectors. Though the VSV*ΔG(HA) replicon vaccine was effective and protective, it did not show any adverse effects in chicken. Thus, further attenuation of the RNA replicon vaccine was not required.

Replication-competent, attenuated live virus vaccines are subject to mutation and selection, which may result in reversion to virulence although the probability to do so is rather low with most live virus vaccines. In this respect, VSV*ΔG(HA) can be regarded as completely safe as it does not produce any progeny. Thus, the VSV*ΔG replicon combines advantages of both classical types of viral vaccines in being as safe as inactivated virus but showing all the advantages of live virus vaccines. A clear booster effect was observed when the same vector was applied a second time two weeks after the primary immunization. As VSV G is not expressed in cells infected with VSV*ΔG, the immune response against the single VSV envelope glycoprotein appears to be too low to neutralize the vector when applied again [22]. This feature is important because quantity, affinity, and avidity of antibodies normally increase when the immune system contacts an antigen again.

The humoral immune response is believed to principally account for protection against influenza viruses. Viral vector-driven expression of HPAIV NP antigen alone failed to protect chickens against a lethal virus challenge [42,43]. However, it is believed that a cytotoxic T cell response directed against highly conserved T cell epitopes in the NP and M1 proteins may provide cross-protection against drift viruses or even viruses of other subtypes [44,45]. Since previous work showed that single-cycle VSV*ΔG replicons can trigger a cellular immune response in mice [46], we generated a VSV*ΔG(NP) replicon. Following infection of primary chicken fibroblasts with VSV*ΔG(NP), NP antigen was expressed primarily in the nucleus. However, no additional beneficial effect was observed when chickens were immunized with both VSV*ΔG(NP) and VSV*ΔG(HA). We may speculate that the VSV matrix protein may have interfered with the processing of NP protein, which may have suppressed the potential of VSV*ΔG(NP) to stimulate a cellular immune response. Previous studies showed that the VSV matrix protein inhibits cellular RNA polymerases in the nucleus and blocks nucleocytoplasmic RNA transport resulting in host shut-off and apoptosis [39]. This property of the VSV replicons may be advantageous for triggering a strong humoral, MHC-II restricted immune

response (see above). However, it may interfere with peptide presentation by MHC-I complexes so that cellular immunity is not adequately stimulated. It will be interesting to see whether M protein mutant VSV vectors [47] represent more promising vaccines in this respect.

The VSV*ΔG replicon vaccines were applied intramuscularly in this study. The serum antibodies induced in this way prevented systemic spread of challenge virus and protected immunized chickens against disease. However, localized mucosal replication of challenge virus probably was not completely prevented and this led to some virus secretion, local inflammation, and seroconversion. It is supposed that in order to achieve sterile immunity against influenza viruses, induction of mucosal immunity by applying the vaccine to mucosal surfaces would be advantageous. This application route would also favor mass application of the vaccine to chicken flocks for example by spray or drinking water. However, the mucosal surfaces of the respiratory and gastrointestinal tract are lined by epithelial cells that form polarized cell sheets. Previous studies showed that VSV does not efficiently infect polarized epithelial cells via the apical plasma membrane [48]. Using a replication-competent VSV vector this problem may be less important for vaccination. If VSV succeeds in infection of some – less polarized – cells in the epithelium, progeny will be released from the basolateral domain [48,49]. In this way infection is disseminated to subepithelial tissues and lymphoid organs. However, a VSV replicon is not able to spread and inefficient infection of epithelial cells might not be sufficient to trigger a mucosal immune response. Accordingly, previous work in mice showed that single-cycle VSV vector vaccines are less effective than replication-competent ones when applied nasally [50,51]. We have previously shown that VSV*ΔG pseudotyped with the influenza C virus glycoprotein HEF is able to infect polarized epithelial cells via the apical plasma membrane [23]. It will be interesting to see whether pseudotyping of VSV*ΔG(HA) with HEF or other appropriate viral glycoproteins will improve mucosal vaccination.

In conclusion, we showed for the first time that an RNA replicon based on VSV which is not a natural avian pathogen can be used as a marker vaccine for protection of chickens against highly pathogenic influenza virus. The vaccine is protective against clinical disease and limits virus shedding significantly. It complies with highest safety standards. Nevertheless, it still represents a prototype vaccine, which has to be improved and optimized in particular with respect to mucosal immunity. VSV*ΔG can be propagated to high titers on our packaging cell line, which ensures that it is available for mass vaccination used in modern poultry farming. As the VSV replicon system does not depend on inactivation and adjuvants, the costs for this vaccine are expected to be not higher than the costs for conventional live attenuated vaccines. Given the very broad host tropism of VSV, the vector might be also useful for vaccination of livestock other than poultry. Because any antigen can be expressed in principle by this vector system, it represents a promising platform for vaccination against a number of pathogens of veterinary importance. However, VSV replicon vaccines may be in particular valuable as emergency vaccines for protection against highly pathogenic and zoonotic agents for which other types of vaccines are not available.

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