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Chimeric avian paramyxovirus-based vector immunization against highly pathogenic avian influenza followed by conventional Newcastle disease vaccination eliminates lack of protection from virulent ND virus

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ABSTRACT

Recently, we described a chimeric, hemagglutinin of highly pathogenic avian influenza virus (HPAIV) H5 expressing Newcastle disease virus (NDV)-based vector vaccine (chNDVFHN$_{PMV8}$H5) in which NDV envelope glycoproteins were replaced by those of avian paramyxovirus-8 (APMV-8). This chimeric vaccine induced solid protection against lethal HPAIV H5N1 even in chickens with maternal antibodies against NDV (MDA+). However, due to the absence of the major NDV immunogens it failed to induce protection against Newcastle disease (ND). Here, we report on protection of MDA+ chickens against HPAI H5N1 and ND, by vaccination with chNDVFHN$_{PMV8}$H5 either on day 1 or day seven after hatch, and subsequent immunization with live attenuated NDV seven days later. Vaccination was well tolerated and three weeks after immunization, challenge infections with highly pathogenic NDV as well as HPAIV H5N1 were carried out. All animals remained healthy without exhibiting any clinical signs, whereas non-vaccinated animals showed morbidity and mortality. Therefore, vaccination with chNDVFHN$_{PMV8}$H5 can be followed by NDV vaccination to protect chickens from HPAIV as well as NDV, indicating that the antibody response against chNDVFHN$_{PMV8}$H5 does not interfere with live ND vaccination.

Introduction

Newcastle disease (ND) and avian influenza (AI) are the most important infectious diseases of poultry.

ND caused by Newcastle disease virus (NDV) [1,2] is a highly contagious disease of chickens listed by the world organization of animal health [3] and is responsible for high losses in poultry holdings. Depending on the pathogenicity of the virus strain, mortality can amount to 100% after infection with virulent (velogenic) strains. In contrast, lentogenic strains used for vaccination do not induce any clinical signs in adult chickens [4,5]. Highly pathogenic avian influenza virus (HPAIV) [6] is the cause of a highly lethal, systemic disease of poultry, with clinical signs similar to ND but with a more pronounced per-acute course of disease [7]. Currently circulating HPAIV H5N1 spread to 63 countries causing death of more than 400 million domestic poultry either by disease itself or disease control measures, e.g. culling [8]. 650 human infections leading to 386 fatalities have been confirmed until 24th of January in 2014, indicating a mortality rate of 60%. [9].

Thus, effective vaccination strategies to control ND and HPAI are important for the poultry industry and to protect human health.

NDV-based vector vaccines expressing HPAIV antigens are able to induce protection in chickens against HPAIV without the risk of reassortment [10]. Although, protective efficacy was high when used in specific pathogen free (SPF) chickens in experimental settings [10–13], their use in the field was frequently hampered by the presence of antibodies against NDV induced by prior vaccination with NDV vaccines. In young chickens, maternally-derived antibodies (MDA) against NDV presented a major obstacle [14]. To overcome this problem, a novel NDV-based vector virus was constructed in which F and HN were substituted by the homologous proteins of avian paramyxovirus 8 (APMV-8) [15]. However, the substitution of the NDV surface proteins by those of APMV-8 drastically decreased the protection against NDV infection. To induce protection against both, NDV and HPAIV infection, the vaccination protocol had to be optimized.

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Materials and methods

Cells and viruses

Quail muscle cells clone 9 (QM9) were used for immunofluorescence studies, while Leghorn hepatocellular epithelial cells (LMH) were used for virus titration of swabs. Chicken embryo fibroblasts (CEF) were prepared from 11-day-old SPF chicken embryos. Recombinant NDV (rNDVGu) and chimeric NDV (chNDVFHN$_{PMV8}$H5) have been described [15,16]. HPAIV A/duck/Vietnam/TG24-01/05(H5N1), kindly provided by P. Song Lien (National Centre for Veterinary Diagnosis, Dongda, Vietnam), and APMV-8 strain APMV 8/goose/Delaware/1053/76 were obtained from the National Reference Laboratories for Avian Influenza (T. Harder) and Newcastle disease. NDV strain Herts33/56 was kindly provided by MSD Animal Health.

Western Blot analyses

CEF were lysed 24 h after infection. Lysates were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membranes. Immunostaining and detection was done as described previously [15].

Preparation of monospecific anti-NP rabbit sera

The pET19b vector and Rosettagami *Escherichia coli* cells (both Novagen, Darmstadt, Germany) were used for expression of the C-terminal 81 amino acids of the nucleoprotein of APMV-8/Delaware/1053/1976. A hexa-histidin- and an Avi-tag (used as a biotin acceptor site) [17] were positioned in-frame and N-terminally of the APMV-8 NP$_{ct}$ fragment. DNA encoding the heptamerization domain of the human C4 binding protein (C4BP) [18,19] was inserted between the tags and the APMV-8 sequence with SGS-linker sequences placed between C4BP and the virus-specific sequences. Plasmid pBirCam (Avidity, Aurora, U.S.A) over-expressing the bacterial biotin ligase *BirA* was co-transformed into *E. coli* strain Rosettagami to ensure co-translational mono-biotinylation of the recombinant APMV protein fragments at their Avi-tag. Mono-biotinylated NP$_{ct}$ was purified from Rosettagami cultures induced with IPTG, refolded *in vitro*, emulsified in Freund's incomplete adjuvant and used to immunize rabbits (legal permission for animal immunization granted under LALLF M-V/TSD/7221.3-2.5-010/10).

Fluorescence staining and microscopic examination

For confocal microscopy, QM9 cells were seeded in 24-well plates on glass coverslips and infected at a multiplicity of infection (moi) of 5. After 1 h incubation on ice and 12 h incubation at 37 °C and 3% CO_2, cells were fixed with 3.7% formaldehyde in phosphate buffered saline (PBS), permeabilized with 0.1% Triton in PBS, and blocked with 5% BSA in PBS. Subsequently, cells were incubated with primary antibodies (mab-F$_{NDV}$, mab-HN$_{NDV}$, mab-NP$_{NDV}$ [20]) or sera (rabbit-F$_{APMV8}$, rabbit-HN$_{APMV8}$ [15], rabbit-NP$_{APMV8}$, chicken-HIS$_{APMV8}$, rabbit-HIS$_{NDV}$), followed by washes with 5% bovine serum albumin (BSA) in PBS. Subsequently, species specific secondary antibodies labeled with Alexa Fluor® 568 (α-rabbit) (Invitrogen) or Alexa Fluor® 488 (α-mouse) (Invitrogen) were added, and after three washing steps, cells were mounted in Mowiol (Roth, Karlsruhe, Germany). Images were collected on a Leica SP5 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) using a 63× oil immersion objective with a numerical aperture of 1.4. Fluorochromes were excited using a 488-nm laser and a 561-nm laser. For higher sensitivity, hybrid detectors were applied (HyD2, 488 nm; HyD4, 568 nm). Brightfield images were collected simultaneously with the fluorescence images using the transmitted light detector. Sequential *z*-sections of stained cells were acquired for maximum projection. Images were processed using ImageJ software and Adobe Photoshop CS5 (Adobe systems).

Animal experiments

Vaccination and challenge experiments were carried out in the BSL3 and BSL3+ experimental animal facilities of the Friedrich-Loeffler-Institut. All animal experiments were conducted following German animal welfare regulations (LALLF M-V/TSD/7221.3-1.1-053/10) and are given in Table SI2. Briefly, ten SPF chickens without maternally derived NDV antibodies (MDA-), hatched at the Friedrich-Loeffler-Institut, were vaccinated oculonasally at three weeks of age with 1e + 06 TCID$_{50}$/animal of chNDVFHN$_{PMV8}$H5. MDA+ chickens were derived from a flock of SPF-white leghorn chickens, which had been immunized with a commercially available inactivated NDV vaccine (Nobilis® Newcavac, MSD Animal Health). 10–11 MDA+ chickens each were immunized oculonasally with 1e + 06 TCID$_{50}$/animal either on day 1 after hatch with chNDVFHN$_{PMV8}$H5 and on day seven with rNDVGu (group A), or on day seven with chNDVFHN$_{PMV8}$H5 and on day 14 with rNDVGu (group B). Four to six non-vaccinated control chickens were kept as control animals for each group.

Oropharyngeal and cloacal swabs of MDA- chickens were taken on two, four, six and 12 days post vaccination (dpv), whereas MDA+ chickens were sampled by combined oropharyngeal and cloacal swabs taken four dpv.

Three weeks after vaccination, MDA− animals immunized with chNDVFHN$_{PMV8}$H5 and non-immunized control chickens were challenged by infection with velogenic NDV strain Herts 33/56. Furthermore, MDA+ chickens immunized with chNDVFHN$_{PMV8}$H5 followed by ND vaccination seven days later as well as non-immunized control chickens were infected with HPAIV A/duck/Vietnam/TG24-01/05 (group A$_{AIV}$ and B$_{AIV}$) or velogenic NDV strain Herts 33/56 (group A$_{NDV}$ and B$_{NDV}$). Three weeks after the respective immunization, animals were infected via the oculonasal route with 1e + 06 TCID$_{50}$/animal. Clinical signs were evaluated over a period of eight days and scored as described previously [15]. Combined oropharyngeal and cloacal swabs were taken on two, four, six and 10 days after challenge (dpch). Heparinised blood samples of all animals were investigated before vaccination, before challenge infection, and of all surviving birds at the end of the 14-day-observation period by testing for AIV-, NDV- and APMV-8 specific antibodies using the hemagglutination inhibition (HI) assay.

Serology

HI assay was performed according to standard (92/66/EEC).

Detection of virus and viral genome

Swabs were titrated in triplicate. RNA was isolated, detected by RT-qPCR and transformed to genome equivalents (GEQ) as described previously [15].

Statistical data analysis

Differences between groups were statistically tested by appropriate Wilcoxon-tests for paired or unpaired data, respectively. Bonferroni correction was applied in case of multiple testing. The global significance level was 0.05. All calculations were performed using R software [21], Version 2.13.0 (2011-04-13).