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Protection of chickens against H5N1 highly pathogenic avian influenza virus infection by live vaccination with infectious laryngotracheitis virus recombinants expressing H5 hemagglutinin and N1 neuraminidase

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ABSTRACT

Attenuated vaccine strains of the alphaherpesvirus causing infectious laryngotracheitis of chickens (ILTV, gallid herpesvirus 1) can be used for mass application. Previously, we showed that live virus vaccination with recombinant ILTV expressing hemagglutinin of highly pathogenic avian influenza viruses (HPAIV) protected chickens against ILT and fowl plague caused by HPAIV carrying the corresponding hemagglutinin subtypes [Lüschow D, Werner O, Mettenleiter TC, Fuchs W. Protection of chickens from lethal avian influenza A virus infection by live-virus vaccination with infectious laryngotracheitis virus recombinants expressing the hemagglutinin (H5) gene. Vaccine 2001;19(30):4249–59; Veits J, Lüschow D, Kindermann K, Werner O, Teifke JP, Mettenleiter TC, et al. Deletion of the non-essential UL0 gene of infectious laryngotracheitis (ILT) virus leads to attenuation in chickens, and UL0 mutants expressing influenza virus haemagglutinin (H7) protect against ILT and fowl plague. J Gen Virol 2003;84(12):3343–52]. However, protection against H5N1 HPAIV was not satisfactory. Therefore, a newly designed dUTPase-negative ILTV vector was used for rapid insertion of the H5-hemagglutinin, or N1-neuraminidase genes of a recent H5N1 HPAIV isolate. Compared to our previous constructs, protein expression was considerably enhanced by insertion of synthetic introns downstream of the human cytomegalovirus immediate-early promoter within the 5′-nontranslated region of the transgenes. Deletion of the viral dUTPase gene did not affect in vitro replication of the ILTV recombinants, but led to sufficient attenuation in vivo. After a single ocular immunization, all chickens developed H5- or N1-specific serum antibodies. Nevertheless, animals immunized with N1-ILTV died after subsequent H5N1 HPAIV challenge, although survival times were prolonged compared to non-vaccinated controls. In contrast, all chickens vaccinated with either H5-ILTV alone, or H5- and N1-ILTV simultaneously, survived without showing any clinical signs. Real-time RT-PCR indicated limited challenge virus replication after vaccination with H5-ILTV only, which was completely blocked after coimmunization with N1-ILTV. Thus, chickens can be protected from H5N1 HPAIV-induced disease by live vaccination with an attenuated hemagglutinin-expressing ILTV recombinant, and efficacy can be further increased by coadministration of an ILTV mutant expressing neuraminidase. Furthermore, chickens vaccinated with ILTV vectors can be easily differentiated from influenza virus-infected animals by the absence of serum antibodies against the AIV nucleoprotein.

1. Introduction

Avian influenza (AI) and infectious laryngotracheitis (ILT) are worldwide occurring infectious diseases of poultry causing significant economic losses. Both infections primarily affect the respiratory tract. However, whereas highly pathogenic avian influenza A viruses (HPAIV) lead to generalized infections in chickens with mortality rates close to 100%, lytic replication of the alphaherpesvirus causing ILT (ILTV, gallid herpesvirus 1) remains largely restricted to larynx, trachea and, frequently, the conjunctiva, and mortality rates are usually moderate [1,2]. ILTV also establishes livelong asymptomatic latent infections of sensory neurons, and reactivated virus can be shed [3]. Since acute ILTV infections of chickens result in significantly reduced weight gain or egg production, live virus vaccines which can be easily administered to large numbers of animals by eye-drop, aerosol, or drinking water, are in use for prevention of disease [1]. The conventionally attenuated

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ILTV vaccine strains applied up to now are genetically uncharacterized and sometimes revert to more virulent phenotypes after animal passage [4–6]. However, several genetically engineered ILTV recombinants have been generated, which are attenuated by irreversible deletions of defined virus genes (reviewed in Ref. [7]). Such ILTV recombinants might also be suitable as vectors for the expression of immunogenic proteins of other chicken pathogens like HPAIV [8,9].

During the last decade extensive outbreaks of fowl plague caused by HPAIV of hemagglutinin (HA) subtypes H5 or H7 combined with various neuraminidase (NA) subtypes occurred in most parts of the world [10,11]. In particular, the current H5N1 HPAI viruses which spread from Asia to Europe and Africa are responsible for the death of several hundred millions of birds, and, due to their increased zoonotic potential, they also account for more than 240 fatal cases in humans. Therefore, preventive vaccination of poultry is an option at least in the most endangered regions where the virus has become endemic. Since vaccination with inactivated AIV is usually efficacious, but requires time-consuming individual administration and interferes with serological and syndrome surveillance of natural AIV infections [2], numerous attempts have been made to develop subunit, DNA or vectored vaccines which give good protection and permit reliable differentiation of infected from vaccinated animals (DIVA strategy) [12]. Most of these approaches focus on the two highly variable influenza virus envelope glycoproteins HA and NA, since the more conserved inner virion proteins are apparently not sufficiently antigenic to induce solid protective immunity. In contrast, subtype-specific protection against lethal HPAIV infections of chickens could be achieved by immunization of chickens with retrovirus- or baculovirus-expressed HA [13,14], with plasmid DNA or defective adenoviruses containing HA gene expression cassettes [15,16], and with vectored HA-expressing live virus vaccines based on fowlpox virus (FPV) [17] or Newcastle disease virus (NDV) [18,19]. A FPV-based vectored vaccine coexpressing H5 and N1 proteins was shown to protect chickens not only against H5N1 but also against H7N1 HPAIV, indicating that NA can significantly contribute to the immune response [20]. In line with this observation, vaccination of mice with NA expression plasmids or baculovirus-expressed NA has also been shown to induce pronounced immune responses, and to protect against lethal infections with homologous influenza viruses [21,22].

Most of the recombinant vaccines developed so far could support the DIVA strategy since they fail to induce antibodies against conserved AIV proteins like the nucleoprotein (NP), which are formed during AIV infection and are detectable by enzyme-linked immunosorbent assays (ELISA) [23,24]. However, with the exception of modified live vaccines, most of the recombinant vaccines are not suitable for mass application, but require repeated individual administration of high amounts of DNA or antigen. On the other hand, the AIV-specific efficacy of vectored NDV and FPV vaccines is significantly reduced in animals, which had been previously immunized with conventional vaccines against ND or FP (J. Veits et al., unpublished results) [25]. Thus, although FPV- and NDV-based AIV vaccines are already used in practice, alternative vectors are required to produce emergency vaccines for animals which are resistant against these viruses. Whereas in many countries chickens are vaccinated against ND within the first days of life [26], vaccination against ILT is less common, and, if at all, performed later [1]. Therefore, we modified ILTV mutants, which had been attenuated by deletion of nonessential genes encoding dUTPase (UL50), or the ILTV-specific UL0 protein, to express the HA of Italian H5N2 or H7N1 HPAIV isolates from the late 1990s [8,9]. Live vaccination with these ILTV recombinants protected chickens against disease after challenge with pathogenic ILTV, or lethal doses of the respective parental HPAIV isolates.

However, recent studies revealed that an NDV recombinant expressing the same H5 type HA like our described ILTV mutant was efficacious against Italian H5N2 HPAIV, but conferred only partial protection against Asian H5N1 HPAIV [19,27]. In contrast, solid protection against the latter challenge virus could be achieved with an NDV recombinant expressing the matching H5 subtype HA [27,28]. Thus, either the ca. 6% amino acid substitutions, or different posttranslational modifications of the two H5 proteins might prevent sufficient cross-immunity. To test, whether this also applies to ILTV, chickens immunized with the described H5 expressing mutant [8] were challenged with a recent H5N1 HPAIV isolate from Vietnam. As suspected, the results (see below) were similar to those obtained with NDV recombinants, resulting in the need for improvement of the ILTV-based vector vaccine.

Due to the antigenic variability of influenza viruses, fast and frequent substitutions of the expressed AIV proteins might be required to ensure the efficacy of vectored vaccines. Since directed mutagenesis of herpesvirus genomes in transfected eukaryotic cells is greatly facilitated by cleavage of the virus DNA at unique restriction sites [29], we devised a novel ILTV vector which provides such a site at the UL50 gene locus, together with an expression cassette for enhanced green fluorescent protein (EGFP) for convenient selection. This vector was used for insertion of the HA or NA genes of Asian H5N1 HPAIV. Although these genes were under control of the strong immediate-early promoter-enhancer complex of human cytomegalovirus ($P_{HCMV\text{-}IE}$), protein analyses with potent H5- or N1-specific rabbit sera revealed very low expression levels. Since mRNA splicing can enhance transcription efficiency, stability and nuclear export of transcripts, and translation rates in higher eukaryotes [30], artificial introns were inserted upstream from the cloned open reading frames (ORFs). The resulting ILTV recombinants expressed high levels of HA and NA and were used separately, and in combination, for live virus vaccination of chickens, and tested for their protective efficacy against subsequent H5N1 HPAIV infection.

2. Materials and methods

2.1. Viruses and cells

The highly pathogenic avian influenza viruses (HPAIV) A/chicken/Vietnam/P41/2005 (H5N1), A/duck/Vietnam/TG24-01/2005 (H5N1), and A/chicken/Italy/8/98 (H5N2) (obtained from T. Harder, Insel Riems, Germany) were propagated in 10-day-old embryonated chicken eggs (Lohmann Tierzucht, Cuxhaven, Germany). All ILTV recombinants were derived from the pathogenic ILTV strain A489 (obtained from D. Lütticken, Boxmeer, The Netherlands), and propagated in primary chicken embryo kidney (CEK) cells as described previously [31]. The described ILTV mutant ILTV-ΔUL50HAa [8], which contains an expression cassette for the HA gene of HPAIV A/chicken/Italy/8/98 (H5N2) at the deleted UL50 gene locus, was used for comparison. Cotransfections with genomic ILTV DNA were performed in the chicken hepatoma cell line LMH [32], and rabbit kidney cells (RK13) were used for transfection with eukaryotic expression plasmids. Vaccinia virus recombinants derived from strain Copenhagen were propagated in the monkey cell line CV-1. Cells were grown to monolayers in minimum essential medium (MEM, Invitrogen) supplemented with 10% fetal calf serum (FCS, Invitrogen) at 37 °C, and maintained at the same temperature in MEM containing 2–5% FCS after infection or transfection. For plaque assays, the virus inoculum was removed 2 h after infection, and cells were overlaid with medium containing 6 g/l methylcellulose.

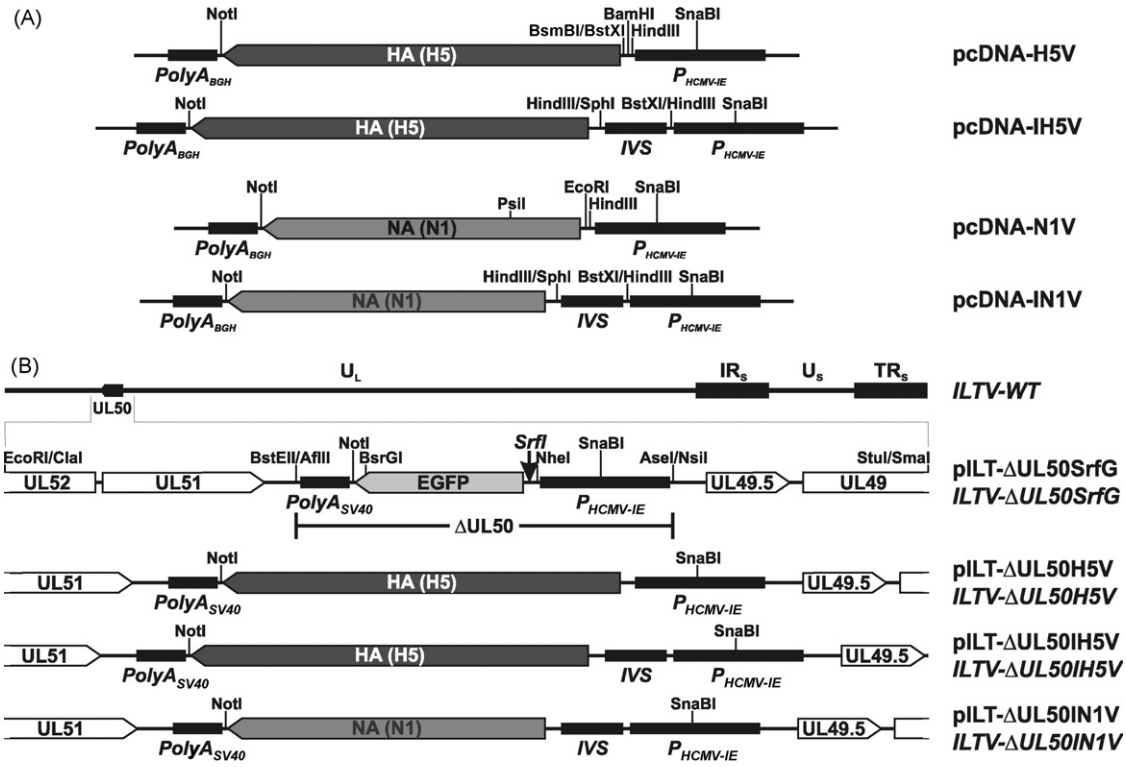

Fig. 1. Construction of expression plasmids and ILTV recombinants. (A) Plasmids pcDNA-H5V and pcDNA-N1V contain the HA and NA ORFs of HPAIV A/chicken/Vietnam/P41/2005 (H5N1) flanked by the HCMV immediate-early promoter/enhancer complex ($P_{HCMV-IE}$) and the polyadenylation signal of the bovine growth hormone gene ($PolyA_{BGH}$). In pcDNA-IH5V and pcDNA-IN1V synthetic intron sequences (IVS) were inserted. (B) The transfer plasmid pILT-ΔUL50SrfG contains an expression cassette consisting of $P_{HCMV-IE}$, the coding sequence of enhanced green fluorescent protein (EGFP), and the SV40 polyadenylation signal ($PolyA_{SV40}$), as well as an artificial SrfI restriction site inserted at the UL50 gene locus of ILTV. In pILT-ΔUL50H5V, pILT-ΔUL50IH5V, and pILT-ΔUL50IN1V the EGFP ORF was replaced by the HA or NA ORFs with or without the upstream IVS. After cotransfection of chicken cells with these plasmids and genomic ILTV DNA virus recombinants (designations printed in italics) were isolated. Also indicated are relevant restriction sites, open reading frames (pointed rectangles), and a diagram of the ILTV genome, which consists of long and short unique regions (U_L, U_S), and of inverted repeat sequences (IR_S, TR_S) flanking the U_S region.

2.2. Construction of expression plasmids and vaccinia virus recombinants

The plasmid-cloned HA gene of HPAIV A/chicken/Vietnam/P41/2005 (H5N1) [28] was isolated as a 1784 bp BsmBI/NotI-fragment, and inserted into eukaryotic expression vector pcDNA3 (Invitrogen), which had been digested with BstXI and NotI. The NA gene of the same HPAIV isolate was amplified from genomic RNA with SuperScript II reverse transcriptase and Platinum Pfx DNA polymerase (Invitrogen), using the primers N1V-F (5′-CAGAATTCCACCATGAATCCAAATCAGAAGATAATAAC-3′) and N1V-R (5′-CAGCGGCCGCAAACTACTTGTCAATGGTGAATG-3′). Artificial EcoRI and NotI restriction sites (underlined) permitted direct cloning of a 1364 bp fragment of the amplification product into the accordingly cleaved vector pcDNA3. Since the obtained plasmids pcDNA-H5V and pcDNA-N1V showed insufficient gene expression in transfected cells, a synthetic intron sequence (IVS), which had been isolated as a 301 bp BstXI/SphI-fragment from pIRES1neo (Clontech), was inserted at the unique HindIII sites upstream of the ORFs, resulting in plasmids pcDNA-IH5V and pcDNA-IN1V (Fig. 1A).

For generation of recombinant vaccinia viruses a 1809 bp BamHI/NotI fragment of pcDNA-H5V, or the EcoRI/NotI insert of pcDNA-N1V were cloned into the SmaI-digested transfer vector pCS43 [33], which permits protein expression under control of the vaccinia virus p7.5K gene promoter. Virus recombinants were generated as described [34,35], and tested for HA or NA expression by indirect immunofluorescence (IIF) reactions of infected cells with AIV-specific chicken sera.

For prokaryotic expression of NA the EcoRI/NotI-insert of pcDNA-N1V (Fig. 1A) was recloned into the similarly digested expression vector pGEX-4T-3 (GE Healthcare). Subsequently, a 295 bp fragment encoding the N-terminal 97 amino acids of NA was deleted by double digestion with EcoRI and PsiI, followed by religation. After transformation of bacteria with the resulting plasmid a 64 kDa fusion protein of NA with glutathione-S-transferase (GST) was isolated as described previously [36].

In all cloning experiments, non-compatible fragment ends were blunt-ended with Klenow polymerase prior to ligation, and correct insertions of all generated plasmids were verified by DNA sequencing.

2.3. Generation of ILTV recombinants

To facilitate substitution of transgenes, a novel ILTV vector was constructed containing an expression cassette for EGFP, as well as a unique SrfI restriction site (Fig. 1B). To this end, plasmid pEGFP-N1 (Clontech) was digested with BamHI, and one nucleotide of the resulting single-stranded 5′-overhang was filled by treatment with Klenow polymerase in the presence of only dGTP. The remaining three unpaired nucleotides were removed with mung bean nuclease, and the plasmid was religated. As a consequence of this treatment the BamHI site was deleted from the multiple cloning site of pEGFP-N1, and the adjacent SmaI site was expanded to a SrfI site (5′-GCCCGGGC-3′). Subsequently, a 792 bp NheI/BsrGI-fragment which included the modified sequence, was isolated and used to substitute the corresponding fragment of recombination plasmid pILT-CSGa [8,37], which contained an EGFP expression cas-

sette instead of the UL50 gene of ILTV. After cotransfection of LMH cells with the resulting plasmid pILT-ΔUL50SrfG (Fig. 1B), transactivator plasmid pRc-UL48, and genomic DNA of ILTV A489 (FuGene HD reagent, Roche) the novel mutant ILTV-ΔUL50SrfG (Fig. 1B) was isolated and virus DNA was prepared as described previously [31,37].

For substitution of the EGFP gene by the cloned AIV genes, pILT-ΔUL50SrfG was digested with SnaBI and NotI, and the released 1060 bp fragment was replaced by corresponding 2126 bp, 2431 bp or 2017 bp fragments of pcDNA-H5V, pcDNA-IH5V or pcDNA-IN1V, respectively (Fig. 1B). After recloning of the HA and NA genes the artificial intron was preserved and $P_{HCMV\text{-}IE}$ was restored, whereas the downstream polyadenylation signal of the bovine growth hormone gene ($PolyA_{BGH}$) was replaced by that of simian virus 40 ($PolyA_{SV40}$). Plasmids pILT-ΔUL50H5V, pILT-ΔUL50IH5V or pILT-ΔUL50IN1V, and pRc-UL48 were used for cotransfection with genomic DNA of ILTV-ΔUL50SrfG, which had been digested with SrfI. Nonfluorescent progeny virus plaques were picked by aspiration, and the isolated mutants ILTV-ΔUL50H5V, ILTV-ΔUL50IH5V and ILTV-ΔUL50IN1V (Fig. 1B) were characterized by restriction analyses and Southern blot hybridization of genomic DNA, as well as by PCR-amplification and DNA sequencing of the modified genome part. Furthermore, expression of the transgenes was tested by IIF tests and Western blot analyses with monospecific antisera (see below).

2.4. Preparation of monospecific anti-HA (H5) and anti-NA (N1) rabbit sera

Two adult rabbits were infected with 5×10^8 plaque forming units (PFU) of the HA- or NA-expressing vaccinia virus recombinants twice at a 2-week-interval. A third rabbit was immunized by intramuscular application of 100 μg of the purified 64 kDa GST-NA fusion protein emulsified in mineral oil four times at 3-week-intervals. Sera collected before and 2 or 3 weeks after the last immunization were analyzed.

2.5. Virion preparation and Western blot analyses

For purification of ILT virions infected CEK cell lysates were cleared by centrifugation at $4500 \times g$ for 15 min. Virus particles were sedimented from the supernatants by centrifugation through a cushion of 40% sucrose in phosphate buffered saline (PBS), and purified in a continuous 20–50% sucrose gradient at 20,000 rpm and 4 °C for 1 h (Beckman SW28 rotor). The virions were aspirated from the gradient, sedimented again, and resuspended in PBS. Allantoic fluid of embryonated chicken eggs was harvested 48 h after infection with HPAIV. Total CEK cell lysates were prepared 24 h after infection with ILTV at a multiplicity (MOI) of 5 PFU per cell. 2 μg of virion proteins, 2 μl of allantoic fluid, or lysates of ca. 10^4 cells per lane were denatured in sample buffer [38] containing 10% β-mercaptoethanol for 5 min at 95 °C, separated by discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes (Mini-Protean III and Trans-Blot SD cells, Bio Rad). The blots were blocked for 1 h in TBS-T (10 mM Tris–HCl pH 8, 150 mM NaCl, 0.25% Tween 20) containing 5% low fat milk, and then incubated with rabbit antisera against influenza virus H5 hemagglutinin (dilution 1:10,000), GST-N1 neuraminidase (dilution 1:1,000,000), or ILTV glycoprotein G (gG) [39] (dilution 1:1,000,000), or a monoclonal antibody against ILTV gC [40] (dilution 1:100) in TBS-T containing 0.5% milk. After repeated washing with TBS-T, antibody binding was detected with horseradish peroxidase-conjugated secondary antibodies (Dianova), and visualized by chemiluminescence (SuperSignal, Pierce) recorded on X-ray films.

2.6. Indirect immunofluorescence tests

CEK cells were infected with serial dilutions of ILTV or HPAIV and incubated under semi-solid medium. RK13 cells were transfected (FuGene HD reagent, Roche) with ca. 1 μg of pcDNA3 expression plasmids per 10^5 cells. After 48 h the cells were washed with PBS and fixed with a 1:1 mixture of methanol and acetone for 30 min at −20 °C. To prevent unspecific antibody binding monolayers were blocked with PBS containing 10% FCS, and then incubated with the rabbit antisera against vaccinia virus-expressed HA or NA (dilutions 1:200), chicken sera from the animal experiments (dilutions 1:50), or an ILTV gJ-specific monoclonal antibody [40] (dilution 1:50) for 1 h each. Bound antibodies were visualized by incubation with species-specific Alexa Fluor 488- or 594-conjugated secondary antibodies (Invitrogen) diluted in PBS for 30 min. After each step cells were repeatedly washed with PBS, and, prior to fluorescence microscopy (Olympus IX51), preserved with a 9:1 mixture of glycerol and PBS, containing 25 mg/ml 1,4-diazabicyclooctane and, optionally, 1 μg/ml propidium iodide for chromatin staining.

2.7. Animal experiments

White Leghorn chickens were bred from specific-pathogen-free eggs (Lohmann Tierzucht), and groups of 10 animals each were vaccinated by ocular application of 10^5 PFU of the tested ILTV recombinants. In a first trial, 6-week-old chickens were immunized with the described mutant ILTV-ΔUL50HAa [8], and in a subsequent study ILTV-ΔUL50IH5V, ILTV-ΔUL50IN1V or both new recombinants were applied to 12-week-old animals. During the following 10 days, the chickens were checked daily for clinical symptoms and classified as healthy (0), ill (1), severely ill (2) or dead (3). The average clinical scores of each group were calculated for each day, and for the entire monitoring period. In the second trial tracheal swabs were taken for virus reisolation on days 3, 4 and 5 after infection (p.i.) and incubated in MEM with 5% FCS, 1 mg/ml enrofloxacin, 1 mg/ml lincomycin and 0.05 mg/ml gentamycin for 2 h at room temperature. After freeze-thawing ILTV titers were determined by plaque assays on CEK cells. Before immunization, and on day 20 p.i sera were collected from all animals, and tested for the presence of specific antibodies by IIF tests on CEK cells infected with ILTV A489, and on RK13 cells transfected with pcDNA-IH5V or pcDNA-IN1V, respectively. HA-specific antibodies were also detected by hemagglutinin inhibition (HI) tests using HPAIV A/chicken/Italy/8/98 (H5N2), which were performed as described [8].

After 3 weeks all immunized chickens as well as naive control animals were challenged by oculonasal application of 10^8 egg infectious doses (EID_{50}) of HPAIV A/chicken/Italy/8/98 (H5N2), or 10^6 EID_{50} of HPAIV A/duck/Vietnam/TG24-01/05 (H5N1) per animal. During the following 10 days, the chickens were again monitored for clinical signs and scores were determined as described above. Oropharyngeal and cloacal swabs were taken on days 2, 3, 4, 5, 6, 7, 9, 11 and 14 after challenge (p.c.), and their content of AIV RNA was quantified by real-time reverse transcription and polymerase chain reaction (RT-PCR). Amplification of a 101 nucleotide fragment of the matrix protein (M1) gene was performed as described [19,41]. Three weeks p.c. all surviving chickens were necropsied and sera were prepared for IIF and HI tests. Furthermore, the sera collected before and after challenge were tested for influenza A virus nucleoprotein-specific antibodies by a competitive ELISA (ID-Vet, Montpellier, France) as recommended by the manufacturer.

2.8. Statistical analyses

Evaluation of data sets was performed essentially as described recently [28]. Maximum clinical scores and survival times after

challenge infection, as well as numbers of AIV-shedding animals in individual groups were pairwise compared using two-sided Fisher's exact tests. Differences were considered as significant if probabilities of the null hypothesis were less than 5% ($p < 0.05$).

3. Results

3.1. Isolation and in vitro replication of ILTV recombinants

ILTV mutants can be generated by homologous recombination between virion DNA and transfer plasmids in transfected chicken cells [7]. To facilitate identification and isolation of the desired mutants, expression cassettes for reporter proteins like EGFP have been frequently inserted into the genome, which subsequently were removed or substituted by other transgenes [8,37]. However, detection of few non-fluorescent virus plaques in transfection progenies was difficult, and several consecutive rounds of plaque-purification were usually required to obtain homogeneous virus populations. Previous studies had shown that cleavage of herpesvirus DNA at a unique restriction site prior to cotransfection with transfer plasmids containing homologous sequences spanning this site increased the proportion of recombinant progeny viruses [29]. Since no SrfI site is present in the published sequence of the ILTV genome [42], we introduced an artificial unique SrfI restriction site together with an EGFP expression cassette at the nonessential UL50 gene locus. Except for this site, the novel mutant ILTV-ΔUL50SrfG (Fig. 1B) was identical to a previously described ILTV vector [8,37].

SrfI-digested DNA of ILTV-ΔUL50SrfG was used for cotransfection of LMH cells together with plasmids containing expression cassettes for HA or NA of the recent HPAIV isolate A/chicken/Vietnam/P41/2005 (H5N1) at the UL50 gene locus (Fig. 1B). In addition, an expression plasmid for pUL48 of ILTV was included, which, like its homologs in other alphaherpesviruses, enhances viral immediate-early gene expression and thereby increases the infectivity of naked virion DNA [37,39]. This was of particular importance in the present cotransfection experiments, since, as expected, progeny virus titers obtained after SrfI cleavage were significantly lower than with similar amounts of undigested ILTV DNA. However, the proportions of recombinant viruses expressing the novel transgenes instead of the EGFP were increased from less than 1% to nearly 100% (data not shown). Thus, unlike earlier mutants, the new recombinants ILTV-ΔUL50H5V, -ΔUL50IH5V and -ΔUL50IN1V (Fig. 1B) could be directly isolated from single nonfluorescent virus plaques of the transfection progenies without repeated plaque purification.

One-step growth analyses and plaque assays of the novel ILTV recombinants in CEK cells revealed that deletion of the viral dUTPase gene UL50, and insertion of the HPAIV genes did not significantly affect in vitro replication (not shown), which was in agreement with the results of earlier studies [8,37]. Thus, efficient in vitro production of the putative ILTV vector vaccines should be possible.

3.2. Expression and specific detection of the AIV HA (H5) and NA (N1) proteins

As a prerequisite for expression of influenza virus proteins in recombinant herpesviruses the respective genes had to be flanked by an RNA polymerase II-dependent promoter, and by a suitable polyadenylation signal. Furthermore, potent monospecific antisera were required for sensitive detection and quantitation of the expressed proteins.

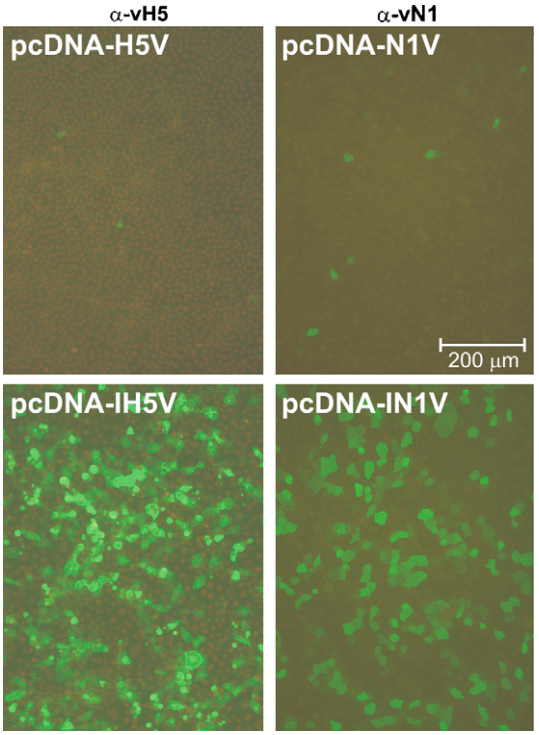

Fig. 2. Presence of a synthetic intron enhances expression of HPAIV HA and NA. RK13 cells were fixed 2 days after transfection with intron-less (pcDNA-H5V, pcDNA-N1V) or intron-containing (pcDNA-IH5V, pcDNA-IN1V) expression plasmids, and analyzed by IIF tests with monospecific rabbit antisera against vaccinia virus-expressed HPAIV proteins (α-vH5, α-vN1). Antibody binding was detected with Alexa Fluor 488-conjugated secondary antibodies (green fluorescence), and chromatin was counterstained with propidium iodide (orange fluorescence). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Therefore, rabbits were infected with recombinant vaccinia viruses expressing the HA (568 codons) and NA (450 codons) ORFs of HPAIV A/chicken/Vietnam/P41/2005 (H5N1). The obtained sera showed specific reactions in IIF tests of cells transfected with HA- or NA-expression plasmids (Fig. 2), or AIV-infected cells (Fig. 3). However, only the anti-HA-serum was also suitable for Western blot analyses (Fig. 4; α-vH5), whereas the NA-specific serum did not react in this assay (not shown). Therefore, a major part of NA (amino acids 98–449) was expressed in *E. coli* as a GST-fusion protein, which was then used for immunization of another rabbit. In Western blot analyses the resulting antiserum specifically detected an AIV protein with an apparent molecular mass of ca. 60 kDa, which could be clearly differentiated from a cross-reacting 68 kDa chicken cell protein (Fig. 4B, α-bN1).

For eukaryotic expression the HA and NA genes were cloned behind the immediate-early promoter–enhancer complex of HCMV (Fig. 1A). However, after transfection with the originally obtained plasmids pcDNA-H5V or -N1V, only few faintly positive RK13 cells were detected in IIF tests with the monospecific antisera (Fig. 2, upper panels), and no proteins were specifically recognized in Western blot analyses (not shown). Since Northern blot analyses indicated that this was presumably due to inefficient transcription or unstable mRNAs, synthetic introns were inserted into their 5′-untranslated regions to enhance expression (Fig. 1A). These modifications resulted in unambiguous detection of HA as well as NA in Western blot (not shown) and IIF analyses of transfected cells (Fig. 2, lower panels).

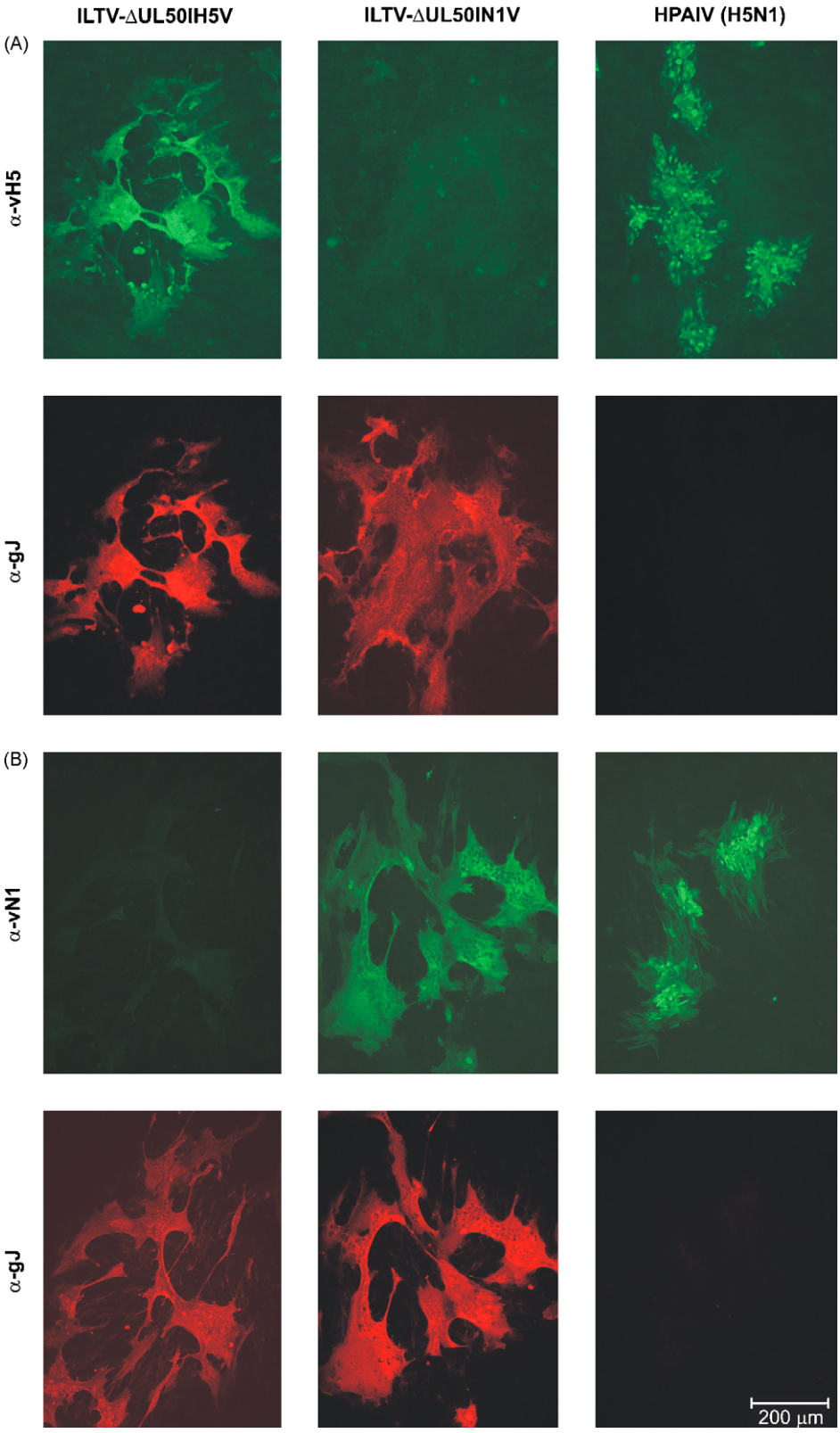

Fig. 3. IIF analyses of protein expression by ILTV recombinants and HPAIV. CEK cells were fixed 2 days after infection with ILTV-ΔUL50IH5V, ILTV-ΔUL50IN1V, or HPAIV A/duck/Vietnam/TG24-01/2005 (H5N1). Binding of HA-specific (A, α-vH5) or NA-specific (B, α-vN1) antisera, and of an ILTV gJ-specific monoclonal antibody (α-gJ) was visualized using Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibodies. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

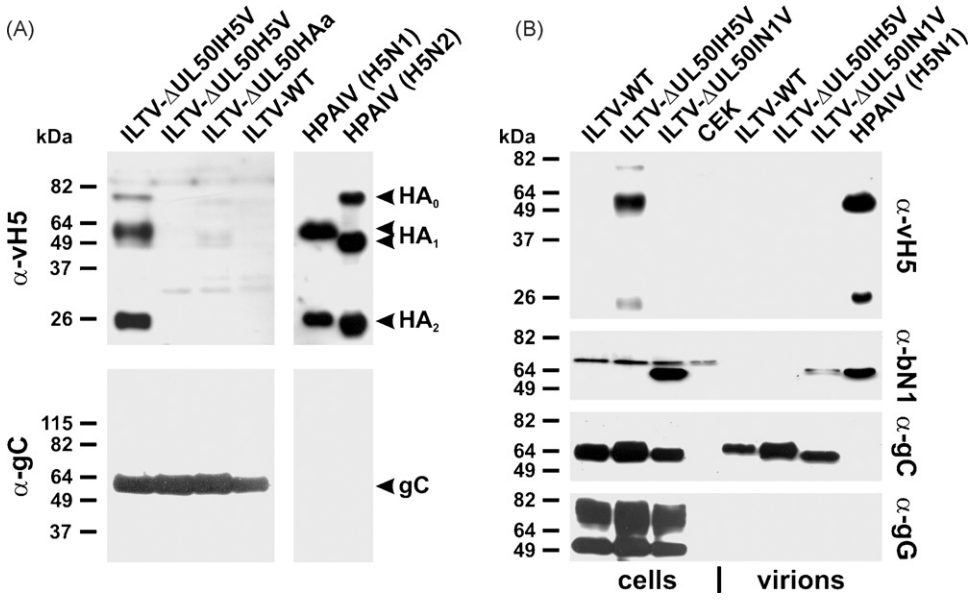

Fig. 4. Western blot analyses of infected cells and ILT virions. CEK cells were harvested 24 h after infection with the indicated ILTV recombinants at an MOI of 5. ILT virions were isolated from continuous sucrose gradients, and HPAIV was prepared from the allantoic fluid of embryonated chicken eggs. After separation in discontinuous SDS–10% polyacrylamide gels the proteins were blotted and incubated with monospecific antibodies against influenza virus HA (α-vH5), NA (α-bN1), or ILTV glycoproteins (α-gC, α-gG). Antibody binding was detected with peroxidase-conjugated secondary antibodies and visualized by chemiluminescence. Parallel blots are shown in the different panels of (A) and (B), respectively. Molecular masses of marker proteins and processing products of HA (HA_0, HA_1, HA_2) are indicated.

3.3. Protein expression of the ILTV recombinants

To ascertain whether the synthetic intron sequences also increased expression of the investigated AIV proteins in the genetic context of ILTV, the expression cassettes of pcDNA-H5V or pcDNA-IH5V (Fig. 1A) were inserted at the UL50 locus of ILTV (Fig. 1B). In Western blot analyses of CEK cells infected with ILTV-ΔUL50IH5V abundant proteins possessing apparent masses of ca. 75, 52, and 23 kDa were specifically detected by the anti-HA serum (Fig. 4A, α-vH5), which represent the hemagglutinin precursor (HA_0), and the mature fragments (HA_1, HA_2) resulting from proteolytic cleavage [43]. The latter proteins exhibited similar sizes to those detected in the allantoic fluid of chicken embryos infected with HPAIV A/duck/Vietnam/TG24-01/2005 (H5N1). IIF tests confirmed, that in CEK cells infected with ILTV-ΔUL50IH5V HA was expressed at levels comparable to those in HPAIV-infected cells (Fig. 3A, green fluorescence), and showed a similar cytoplasmic distribution of AIV HA and ILTV gJ (Fig. 3A, red fluorescence) within the virus-induced syncytia. In contrast, cells infected with intron-less ILTV-ΔUL50H5V showed only very weak HA-specific IIF reactions (not shown), and HA was barely detectable by Western blot (Fig. 4A, α-vH5). Since the amounts of ILTV gC on a control blot indicated similar infection rates (Fig. 4A, α-gC), HA-expression was obviously greatly enhanced in the presence of the synthetic intron.

In cells infected with the previously described intron-less mutant ILTV-ΔUL50HAa [8], HA-expression appeared more pronounced than that of ILTV-ΔUL50H5V, although it was still significantly lower than that of ILTV-ΔUL50IH5V (Fig. 4A, α-vH5). Since in all compared ILTV mutants the foreign genes were under control of $P_{HCMV\text{-}IE}$, expression rates are most likely also influenced by differences between the cDNA sequences of the HA genes of HPAIV A/chicken/Vietnam/P41/2005 (H5N1) and A/chicken/Italy/8/98 (H5N2), which amount to ca. 7.5% [8,28]. The 6% differences between the deduced amino acid sequences include formation of an additional, eighth N-glycosylation consensus sequence [44] at HA position 170 of the recent Asian influenza viruses. Apparently, glycosylation at this site indeed occurs in AIV-infected cells, as well as in cells infected with ILTV-ΔUL50IH5V, since the N-terminal HA1 fragment of the Vietnamese virus appears significantly larger than that of the Italian virus (Fig. 4A, α-vH5), in spite of almost identical calculated masses of the primary translation products.

Because of the results obtained with HA, only an intron-containing NA-expressing ILTV recombinant was generated (Fig. 1B). In Western blot analyses of CEK cells infected with ILTV-ΔUL50IN1V, an abundant 60 kDa protein was detected by the GST-NA-specific antiserum (Fig. 4B, α-bN1), which was not found in cells infected with wild-type ILTV or other mutants, but was similar in size to the corresponding gene product of HPAIV A/duck/Vietnam/TG24-01/2005 (H5N1). Like HA, the ILTV-expressed NA showed a cytoplasmic localization (Fig. 3B, green fluorescence), which was comparable to that of ILTV gJ (Fig. 3B, red fluorescence).

Western blot analyses further revealed that NA, despite its abundant expression by ILTV-ΔUL50IN1V, was present in purified virions in only very low amounts (Fig. 4B, α-bN1), and that the HA expressed by ILTV-ΔUL50IH5V was not detectable at all in virus particles (Fig. 4B, α-vH5). The quality of the investigated virion preparations was verified by control blots demonstrating the presence of envelope glycoprotein gC, and the absence of gG (Fig. 4B, lower two panels), which has been identified as a nonstructural glycoprotein of ILTV [39,45].

3.4. Protective efficacy of ILTV mutants expressing AIV HA or NA

To investigate whether the described ILTV recombinant expressing H5 hemagglutinin of HPAIV A/chicken/Italy/8/98 (H5N2) [8] is protective not only against homologous HPAIV challenge, but also against recent H5N1 HPAIV isolates, 6-week-old chickens were immunized by ocular administration of 1×10^5 PFU of ILTV-ΔUL50HAa. Whereas after intratracheal application in earlier experiments this mutant had shown considerable residual pathogenicity [8], it was avirulent after

Table 1
Summary of animal experiments.

Animals		1st trial				2nd trial			
		10 White Leghorn chickens each (6 weeks old)				10 White Leghorn chickens each (12 weeks old)			
Immunization 10^5 *PFU/animal ocular*	0 d p.i.	ILTV-ΔUL50HAa	Controls	ILTV-ΔUL50HAa	Controls	ILTV-ΔUL50IH5V	ILTV-ΔUL50IN1V	ILTV-ΔUL50IH5V ΔUL50IN1V	Controls
Mortality		0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Morbidity	1–10 d p.i.	0/10	0/10	0/10	0/10	4/10	7/10	7/10	0/10
Clinical score[a]						*0.1*	*0.16*	*0.15*	
ILTV shedding[b]	4–5 d p.i.	NT[c]	NT	NT	NT	5/10	1/10	1/10	NT
ILTV-specific Ab[d]	20 d p.i.	NT	NT	NT	NT	10/10	10/10	10/10	0/10
HA-specific Ab[e]	20 d p.i.	6/10	0/10	9/10	0/10	10/10	0/10	10/10	NT
HI titer		$2^{3.8}$		$2^{4.6}$		$2^{4.2}$		$2^{4.1}$	
NA-specific Ab[f]	20 d p.i.	NT	NT	NT	NT	0/10	10/10	10/10	NT
NP-specific Ab[g]	20 d p.i.	0/10	NT	0/10	NT	0/10	0/10	0/10	NT
Challenge	21 d p.i.	HPAIV A/chicken/Italy/8/98 (H5N2)		HPAIV A/duck/Vietnam/TG24-01/05 (H5N1)					
Dose		10^8 *EID_{50}/animal oculonasal*		10^6 *EID_{50}/animal oculonasal*					
Mortaility	1–8 d p.c.	0/10	9/10	5/10	10/10	0/10	10/10	0/10	10/10
Morbidity	1–10 d p.c.	0/10	10/10	7/10	10/10	0/10	10/10	0/10	10/10
Clinical score			*2.38*	*1.27*	*2.75*		*2.46*		*2.77*
AIV shedding[h]	2–14 d p.c.	10/10	10/10	9/9	†[i]	7/10	10/10	1/10	†
Oropharyngeal swabs		*≤10*	*≤10*	*≤9*		*≤7*	*≤10*	*≤1*	
Cloacal swabs		*≤10*	*≤10*	*≤9*		*0*	*≤10*	*0*	
HA-specific Ab	21 d p.c.	10/10	1/1	5/5	†	10/10	†	10/10	†
HI titer		$2^{6.8}$	2^{12}	$2^{8.4}$		$2^{5.3}$		$2^{3.7}$	
NP-specific Ab	21 d p.c.	10/10	1/1	5/5	†	5/10	†	0/10	†

[a] Mean clinical scores (0: healthy, 1: ill, 2: severely ill and 3: dead) of all animals of the group over 10 days.
[b] ILTV shedding was determined by virus reisolation from tracheal swabs.
[c] Not tested.
[d] ILTV-specific antibodies were detected by IIF tests on ILTV-infected cells.
[e] HA-specific antibodies were detected by IIF tests on cells transfected with pcDNA-IH5V, and HI tests.
[f] NA-specific antibodies were detected by IIF tests on cells transfected with pcDNA-IN1V.
[g] NP-specific antibodies were detected by a competitive ELISA.
[h] AIV shedding was quantified by real-time RT-PCR.
[i] Not tested because of death of the animals.

eye-drop administration (Table 1). Nevertheless, 3 weeks p.i. all animals were protected against a lethal challenge with 10^8 EID_{50} of HPAIV A/chicken/Italy/8/98 (H5N2) without exhibiting any clinical signs (Table 1). In contrast, protection against HPAIV A/duck/Vietnam/TG24-01/2005 (H5N1) was significantly ($p = 0.003$) less pronounced, and 50% (5 of 10) of the chickens died after challenge infection with 10^6 EID_{50} of this virus (Table 1). However, the five surviving animals developed only mild symptoms of AIV infection. The incomplete protection might be explained by the different amino acid sequences and modifications of the HA proteins expressed by the ILTV vector and the challenge virus (see above), and/or by the increased virulence of the more recent HPAIV isolate, which is reflected by higher clinical scores in non-immunized chickens (Table 1).

Therefore, in a second animal trial three groups of 12-week-old chickens were immunized by ocular administration of 10^5 PFU of the novel recombinants ILTV-ΔUL50IH5V, ILTV-ΔUL50IN1V, or both. Unlike in the previous experiment, many of the animals developed slight to moderate conjunctivitis between days 3 and 7 p.i., leading to total clinical scores of 0.1–0.16 (Table 1). This might be due to the higher susceptibility of older chickens for ILTV [1]. However, all animals recovered completely, and the typical respiratory symptoms of infectious laryngotracheitis were not observed. Nevertheless, replication of the ILTV mutants in the respiratory tract was demonstrated by the presence of low amounts of infectious virus in tracheal swabs from 7 out of 30 immunized chickens taken at days 4 and 5 p.i. (Table 1). All animals developed ILTV-specific antibodies which were detectable in sera collected at day 20 p.i. by IIF tests on ILTV-infected cells. Moreover, IIF tests on cells transfected with pcDNA-IH5V or pcDNA-IN1V (Fig. 1A) revealed that all sera contained antibodies against the AIV protein(s) expressed by the ILTV recombinant(s) used for immunization (Table 1). All sera of animals infected with ILTV-ΔUL50IH5V or both recombinants were also positive in HI tests, but, despite increased HA expression (Fig. 4A), titers of HA-specific antibodies appeared to be in the same range as after immunization with ILTV-ΔUL50HAa (Table 1). However, these results might be biased, since the donor virus of the HA gene expressed in ILTV-ΔUL50HAa, HPAIV A/chicken/Italy/8/98 (H5N2), was used as antigen in all HI tests.

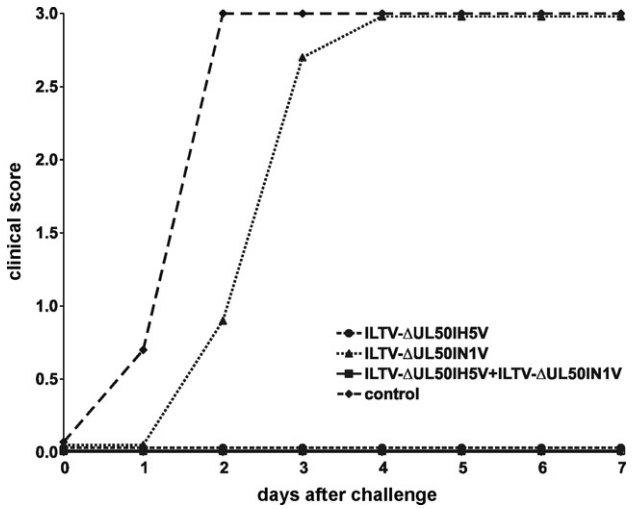

Fig. 5. Clinical scores after HPAIV challenge. At day 21 after vaccination with ILTV-ΔUL50IH5V, ILTV-ΔUL50IN1V or both, immunized chickens and naive control animals were challenged with 10^6 EID_{50} HPAIV A/duck/Vietnam/TG24-01/2005 (H5N1), and daily classified as healthy (0), moderately ill (1), severely ill (2), or dead (3). The average scores of all 10 animals of each group are indicated.

At day 21 p.i. the immunized chickens and naive control animals were challenged with HPAIV A/duck/Vietnam/TG24-01/05 (H5N1). As expected, all control animals died within 48 h, most of them without visible clinical signs (Table 1, Fig. 5). All animals immunized with ILTV-ΔUL50IN1V developed severe symptoms of avian influenza like depression, diarrhea, edema of the head and necroses at comb, wattles and legs, and died between 72 and 96 h p.c. (Table 1, Fig. 5). Thus, vaccination with NA-expressing ILTV significantly ($p < 0.001$) delayed, but did not prevent fatal HPAIV infection. In contrast, solid protection was achieved by immunization with

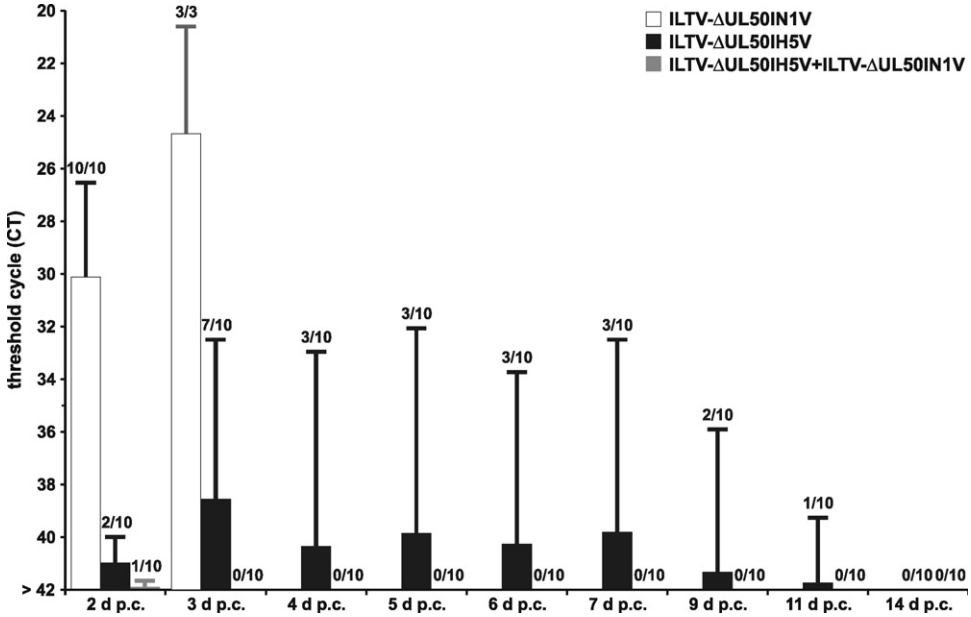

Fig. 6. Detection of HPAIV RNA in oropharyngeal swabs. Samples were taken from all surviving animals immunized with ILTV-ΔUL50IH5V, ILTV-ΔUL50IN1V or both at different times after challenge infection (d p.c.) with HPAIV A/duck/Vietnam/TG24-01/2005, and analyzed for presence of the AIV M1 gene sequences by real-time RT-PCR. Mean (bars) and minimum (vertical lines) threshold cycle numbers (CT) are indicated for each group and day. Total (n) and positive (x) animal numbers are noted above the bars (x/n).

ILTV-ΔUL50IH5V (Table 1, Fig. 5). Thus, the novel HA-expressing ILTV vector vaccine proved to prevent disease and death caused by recent H5N1 HPAIV significantly ($p = 0.003$) more efficient than the previously described mutant ILTV-ΔUL50HAa.

Although all animals vaccinated with ILTV-ΔUL50IH5V, or with ILTV-ΔUL50IH5V and ILTV-ΔUL50IN1V survived challenge infection without any clinical signs (Table 1, Fig. 5), quantitative real-time RT-PCR analyses indicated different amounts of challenge virus in oropharyngeal swabs taken after 2–14 days (Table 1, Fig. 6). Whereas AIV RNA could be detected for several days in oropharyngeal swabs of up to 7 of 10 chickens immunized with ILTV-ΔUL50IH5V, only one of the animals vaccinated with ILTV-ΔUL50IH5V and ILTV-ΔUL50IN1V was slightly positive at day 2 p.c., possibly due to residual input virus. This difference was statistically significant ($p = 0.02$). Thus, in this experiment double-vaccination with HA- and NA-expressing ILTV conferred almost sterile immunity against infection with homologous HPAIV. However, even vaccination with ILTV-ΔUL50IH5V alone inhibited replication and spread of the challenge virus efficiently, since it was never detected in cloacal swabs (Table 1). In contrast, after immunization with ILTV-ΔUL50IN1V or ILTV-ΔUL50HAa [8], lower threshold cycle numbers indicated the presence of higher amounts of H5N1 HPAIV in oropharyngeal as well as in cloacal swabs (Table 1, Fig. 6). Similar quantitative investigations of HPAIV replication in non-vaccinated control animals were impossible because of their early death.

The different rates of H5N1 challenge virus replication were also reflected by serological tests. Whereas in animals immunized with ILTV-ΔUL50HAa, HI tests revealed much higher titers of HA-specific antibodies at day 21 p.c. than at day 20 p.i., titers increased only moderately in chickens immunized with ILTV-ΔUL50IH5V, and even decreased in the doubly vaccinated animals (Table 1). Antibodies against the nucleoprotein of influenza A viruses (NP) were detected by commercial ELISA tests in all post-challenge sera taken from surviving chickens which had been immunized with ILTV-ΔUL50HAa, but only in 50% of the animals vaccinated with ILTV-ΔUL50IH5V (Table 1). The positive animals included those chickens, which shed AIV for several days. No NP-specific antibodies were found at day 21 p.c. in sera of animals previously immunized with ILTV-ΔUL50IH5V and ILTV-ΔUL50IN1V (Table 1), which confirmed the inhibition of AIV replication after vaccination with HA- and NA-expressing vectors. As expected, NP-specific antibodies were also not detectable in sera of immunized chickens prior to challenge (Table 1), demonstrating the suitability of ILTV recombinants as AIV marker vaccines in eradication programs based on the DIVA strategy [12].

Three weeks after HPAIV challenge infections all surviving animals were necropsied. In most cases no significant pathological changes were detectable, whereas control animals, and chickens which died after administration of ILTV-ΔUL50IN1V or ILTV-ΔUL50HAa, showed numerous lesions including petechiae in pericardium, haemorrhages in larynx and proventriculus, as well as pancreas necrosis.

4. Discussion

In the present study we show that a single live vaccination with a novel attenuated ILTV recombinant expressing influenza virus HA (subtype H5) is able to protect chickens reliably against a lethal challenge with homologous Asian H5N1 HPAIV, and that double-vaccination with HA and NA (subtype N1) expressing ILTV mutants can confer sterile immunity against homologous HPAIV challenge. These findings extend earlier investigations on the use of the avian herpesvirus ILTV as a vector for expression of AIV HA subtypes H5 or H7 [8,9].

Although the previously described recombinant ILTV-ΔUL50HAa expressing the HA gene of an Italian H5N2 HPAIV isolate from 1998 was efficacious against homologous challenge [8], it did not confer solid protection against recent H5N1 HPAIV (this study). Similar results have been obtained with a matching NDV recombinant [27]. In both cases, the incomplete protection might be partly explained by antigenic differences between the HA proteins of vaccine and challenge virus caused by ca. 6% amino acid substitutions and distinct glycosylation patterns. However, evaluation of conventional inactivated AIV vaccines revealed sufficient cross-protection even if the H5 subtype HA proteins exhibited less homology, indicating that high antigen masses, adjuvants, and other AIV proteins can compensate for the reduced HA specificity of the immune response [28,46].

To overcome the shortcomings of the ILTV vector system, several improvements have been introduced: (1) the vector was modified to permit rapid substitution of transgenes, (2) expression levels of the foreign proteins were significantly increased and (3) besides HA, NA was expressed as a second immunogenic AIV protein.

Rapid mutagenesis of the ILTV genome is still hampered by the lack of infectious full-length clones [7], which are available for several other herpesviruses [47], or for NDV [48,49]. Whereas the plasmid cloned virus genomes can be directly manipulated by well-established techniques in *E. coli*, generation of ILTV recombinants still requires homologous recombination between cloned genome fragments and genomic virus DNA in transfected eukaryotic cells, followed by usually time-consuming identification and isolation of the desired mutants. Similar to previous approaches with other herpesviruses [29], the construction of new ILTV recombinants could be greatly facilitated by introduction of an artificial unique SrfI restriction site together with an EGFP reporter gene cassette at the UL50 gene locus. If genomic ILTV DNA is cleaved at this unique site prior to transfection, infectious genomes are only reconstituted by recombination with homologous sequences of cotransfected transfer plasmids containing the desired transgenes. Therefore, repeated plaque purification becomes dispensable, and virus mutants can be isolated even faster than after mutagenesis of cloned herpesvirus genomes in bacteria, which requires subsequent removal of plasmid sequences containing critical antibiotic resistance genes. Thus, it is now possible to generate ILTV-vectored vaccines expressing antigens matching the circulating HPAIV strains or other chicken pathogens within a few weeks.

To overcome the low expression levels of the cloned HA and NA genes of several recent H5N1 HPAIV isolates from eukaryotic expression vectors utilizing the strong immediate-early promoters of human or murine cytomegalovirus and mammalian polyadenylation signals, synthetic introns were introduced into the 5′-nontranslated regions of the AIV genes. Splicing is known to significantly influence transcription rates, polyadenylation, stability and nuclear export of mRNAs, as well as translation efficiency [30], and, in the present case, substantially enhanced the expression levels of HA and NA from expression plasmids, and ILTV recombinants. These findings might be irrelevant for development of AIV-specific vectored vaccines based on viruses which replicate exclusively in the cytoplasm, like FPV and NDV [26,50]. However, the efficacies of DNA vaccines and vectored vaccines based on other herpesviruses or adenoviruses might be improved by the addition of intron sequences to the cloned AIV genes.

Despite abundant expression of HA and NA after intron insertion, NA was only inefficiently, and HA was not detectably incorporated into virions of the ILTV recombinants. In contrast, virions of HA-expressing NDV recombinants contained large amounts of the foreign protein [19,27]. This may be due to the different egress pathways of the viruses. Influenza viruses, like NDV, are enveloped at the plasma membrane to which the viral surface gly-

coproteins are targeted [43,51]. In contrast, herpesviruses including ILTV acquire their final, glycoprotein-containing envelopes by budding into vesicles of the trans-golgi network [52]. Remarkably, animal experiments revealed that presence or absence of HA in virions does not significantly influence the immune response after live virus vaccination. ILTV-ΔUL50IH5V (this study) and an NDV recombinant expressing the same transgene [27,28] induced comparable levels of HA-specific antibodies and conferred similar protection against HPAIV challenge. Thus, expression and presentation of HA in infected tissues seems to be sufficient for induction of solid immunity. Moreover, HA mediates host cell penetration by influenza viruses, and the furin-cleavable HA of HPAIV permits infection of almost any cell [2,43]. Since it cannot be excluded that HA is also utilized by the vector to alter its cell tropism, host range or virulence, absence of HA from virions might indeed increase the safety of vectored HPAIV vaccines.

Compared to immunization with the previously described recombinant ILTV-ΔUL50HAa [8] vaccination with the novel mutant ILTV-ΔUL50IH5V showed substantially improved protective efficacy against H5N1 HPAIV challenge. It remains to be elucidated, whether this was exclusively caused by the matching amino acid sequence and modification of HA, or also promoted by the increased HA expression level. However, it could be ruled out that the different ages of the chickens used for the compared trials played a critical role, since in current studies ILTV-ΔUL50IH5V proved to confer solid protection against H5N1 HPAIV not only to 12-week-old animals, but also to 6-week-old chickens (S. Pavlova et al., unpublished results). It also appears unlikely that efficacy was influenced by different in vivo replication rates of the used live vaccines, since both ILTV vectors exhibited the same deletion of the viral dUTPase gene UL50.

With respect to safety, the residual virulence of UL50-negative ILTV was also reevaluated. It has been shown that UL50-deleted ILTV recombinants are still pathogenic for chickens after intratracheal application of high virus doses [37]. Nevertheless, UL50-negative ILTV mutants were chosen as vectors since this deletion does not detectably affect in vitro virus replication and economic production of the vaccines [8,37]. Furthermore, replication of vaccine virus in chickens is required for successful mass application. We demonstrate here that UL50-negative, AIV HA-expressing ILTV is efficacious after eye-drop administration, and also sufficiently attenuated since it did not cause serious clinical signs in immunized SPF chickens. Deletions of other ILTV genes, encoding thymidine kinase, gG, gJ, pUL0 or pUL47, resulted in a more pronounced attenuation without affecting in vitro replication severely [9,53–56], and might be introduced in ILTV vectors designed for individual administration.

HA is the most variable protein of influenza viruses but, at least in avian viruses, also the most relevant antigen for induction of protective immunity [2]. This was confirmed by the present study, in which ILTV-recombinants expressing the AIV envelope glycoproteins HA or NA of subtypes H5 or N1 were analyzed in parallel. Although both virus mutants induced antibodies against the respective AIV proteins in all immunized chickens, only the HA-expressing mutant ILTV-ΔUL50IH5V conferred solid protection against H5N1 HPAIV challenge. However, although none of the animals vaccinated with NA-expressing ILTV-ΔUL50IN1V survived challenge infection, their survival times were prolonged by 1–2 days compared to non-vaccinated controls. Vaccination of chickens with different DNA and subunit vaccines providing N2 subtype NA also conferred partial protection against H5N2 HPAIV challenge [57] which parallels our results. In the mouse model, however, HA or NA vaccines were similarly efficacious against challenge infections with human influenza viruses, and, as in our studies, the best results were achieved by combined administration of both antigens [21,22].

After coimmunization of chickens with ILTV-ΔUL50IH5V and ILTV-ΔUL50IN1V protection from clinical disease was identical to that of single immunization with ILTV-ΔUL50IH5V, which already prevented any symptoms after H5N1 HPAIV challenge. However, whereas immunization with HA-expressing ILTV alone did not prevent limited replication of the challenge virus in the respiratory tract of most animals, no virus was detectable in oropharyngeal swabs from doubly vaccinated animals taken 2–14 days after challenge. These results were obtained by real time RT-PCR analyses of the samples, which proved to be more sensitive than conventional AIV reisolation on embryonated chicken eggs [19]. Rapid elimination of the challenge virus was further confirmed by serological tests. Unlike in animals immunized with HA-expressing ILTV recombinants alone, HPAIV challenge did not lead to increased HI titers or development of NP-specific antibodies in chickens coimmunized with ILTV-ΔUL50IH5V and ILTV-ΔUL50IN1V. The additive effects of HA- and NA-specific immunity might be explained by the different functions of the two influenza virus envelope proteins. Whereas HA is essential for attachment and penetration, NA activity permits efficient virus release [43], and simultaneous inhibition of both steps of the viral replication cycle should accelerate virus elimination. Since NA exhibits lower mutation rates than HA [2,43], the additional use of NA as a component of recombinant vaccines could also expand the efficacy to a broader range of influenza virus strains of the matching NA subtype. This was confirmed by evaluation of an FPV recombinant co-expressing H5 and N1 proteins, which conferred protection not only against experimental challenge with homologous HPAIV, but also against an H7N1 isolate [20].

Whereas HA-expressing vector vaccines based on FPV and NDV are already in use [58], corresponding ILTV recombinants have been tested in laboratory experiments only [8,9]. Nevertheless, ILTV-based vector vaccines against HPAIV show significant promise, since they confer reliable protection and support the DIVA strategy [12]. The ILTV genome contains dispensable regions and exhibits considerable variability in size [7], and, therefore, should be suitable for concomitant insertion and expression of several heterologous genes like HA and NA or different HA subtypes. Like NDV vaccines, ILTV vaccines are suitable for mass application by aerosol or drinking water [1,26], whereas FPV vaccines require individual wing-web administration for optimal efficacy [50]. Compared to NDV, shortcomings of ILTV might be the lower susceptibility of very young chickens, and its very narrow host range [1]. However, the narrow host range of ILTV can be advantageous since it prevents uncontrolled spread of the genetically engineered viruses into other species. This possibility cannot be ruled out for vaccines based on NDV, which exhibits a wide host range [26]. Furthermore, it has been shown that vectored FPV or NDV vaccines failed to induce solid immunity against AIV infection in chickens which had been already immunized with conventional vaccines against ND or FP, respectively (J. Veits et al., unpublished results) [25]. It remains to be tested, whether efficacy of ILTV-based AIV vaccines is also reduced in vaccinated animals. However, irrespective of the result, chickens are not generally immunized against ILT, and, if at all, much later than against ND [1,26]. Thus, whereas in countries with endemic HPAI general immunization of 1-day-old chickens with bivalent NDV-based vector vaccines might be adequate, other vectors are required to provide emergency vaccines for endangered older animals in case of emerging HPAI outbreaks in previously HPAI-free regions. Therefore, ILTV recombinants expressing AIV HA and NA singly or in combination could be valuable additional tools for containment of the present epidemic caused by H5N1 HPAIV, and future outbreaks of fowl plague.

Acknowledgments

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