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Protection of chickens from lethal avian influenza A virus infection by live-virus vaccination with infectious laryngotracheitis virus recombinants expressing the hemagglutinin (*H5*) gene

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Abstract

The *H5* hemagglutinin (HA) gene of a highly pathogenic avian influenza virus (AIV) isolate (A/chicken/Italy/8/98) was cloned and sequenced, and inserted at the non-essential *UL50* (dUTPase) gene locus of a virulent strain of infectious laryngotracheitis virus (ILTV). Northern and Western blot analyses of the obtained ILTV recombinants demonstrated stable expression of the HA gene under control of the human cytomegalovirus immediate-early gene promoter. In vitro replication of the HA-expressing ILTV mutants was not affected, and infection of chickens revealed a reduced but still considerable virulence, similar to that of a *UL50* gene deletion mutant without foreign gene insertion. The immunized animals produced specific antibodies against ILTV and AIV HA, and were protected against challenge infections with either virulent ILTV, or two different highly pathogenic AIV strains (A/chicken/Italy/8/98, A/chicken/Scotland/59). After challenge, no ILTV could be reisolated from protected animals, and shedding of AIV was considerably reduced. Thus, although attenuation remains to be improved, genetically engineered ILTV live-virus vaccines might be used as vectors to protect chickens also against other pathogens.

Keywords: Infectious laryngotracheitis; Avian influenza; Recombinant live-virus vaccines

1. Introduction

Avian influenza (AI), and infectious laryngotracheitis (ILT) are worldwide occurring, and economically important chicken diseases. ILTV (gallid herpesvirus 1) causes serious respiratory problems which invariably affect growth and egg production, but may also entail death of the animals [1]. Moderate avian influenza A virus infections are also primarily characterized by respiratory symptoms, whereas highly pathogenic virus strains cause 'fowl plague', a generalized disease with mortality rates close to 100% [2,3]. All reported outbreaks of highly pathogenic avian influenza, including the recent episodes in Hong Kong and Italy were caused by viruses expressing hemagglutinin (HA) subtypes *H5* or *H7*, whereas viruses possessing any other of the 15 known HA serotypes, and also many *H5* and *H7* isolates, exhibit low or moderate pathogenicity [3]. The hemagglutinin of orthomyxoviruses is required for attachment to and penetration of host cells, and represents a major target of the host immune response [2,4]. Therefore, not only inactivated whole virus preparations [5], but also recombinant vaccines containing the HA gene or protein are promising candidates for protection of poultry from fatal influenza A virus infections. In experimental studies, vaccinia virus- or baculovirus-expressed HA proteins [6,7], DNA vaccines encoding HA [8–10], as well as recombinant HA-expressing live fowl pox viruses [11–13] have been shown to protect chickens and other domestic fowl against challenge infections with highly pathogenic influenza virus strains.

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In contrast to fowl pox virus, ILTV has hitherto not been described as a suitable vector for the presentation of antigens of other chicken pathogens. Although classically attenuated live-virus vaccines of ILTV which can easily be applied to huge flocks by aerosol or drinking water have been in use for many years, molecular characterization and gene manipulation of ILTV were hampered for a long time by the restriction of in vitro virus propagation to embryonated chicken eggs and primary cell cultures [1]. Meanwhile, a chicken hepatoma cell line has been established, which permits productive ILTV replication and could be successfully transfected with infectious virus DNA and plasmids [14–16]. Furthermore, the 155 kbp DNA genome of ILTV was almost completely sequenced during the last decade ([14,17–21] and others). Since these studies revealed, that genome structure and gene arrangement of ILTV are widely similar to those found in mammalian alphaherpesviruses such as herpes simplex virus (HSV-1) [22] or pseudorabies virus (PrV) [23], the knowledge about gene functions of these viruses might be utilized for the development of genetically engineered ILTV vaccines. In several alphaherpesviruses viral enzymes participating in nucleotide metabolism such as thymidine kinase (UL23), or dUTPase (*UL50*) were demonstrated to be dispensable for in vitro virus replication but relevant for in vivo virulence [24–26]. Thus, the first described ILTV recombinants were deletion mutants of the *UL23* and *UL50* genes [14,27]. As expected, thymidine kinase negative ILTV mutants were shown to be significantly attenuated in chickens [27]. Also, substitution of the *UL50* gene of a pathogenic strain by an expression cassette encoding the enhanced green fluorescent protein (EGFP) led to viable but completely apathogenic virus recombinants, whereas, remarkably, simple deletion mutants of the *ILTV* dUTPase gene were less attenuated in vivo [14].

To test the suitability of ILTV as a vector, we inserted the open reading frame (ORF) encoding the hemagglutinin (*H5*) of a recently isolated, highly pathogenic avian influenza virus (A/chicken/Italy/8/98) [28] at the *UL50* gene locus. Since herpesviruses, in contrast to most RNA viruses and poxviruses, replicate in the nucleus of infected cells [22], the transcripts of inserted RNA virus genes are frequently unstable, artificially spliced, or inefficiently translocated to the cytoplasm. In the case of an influenza virus gene such problems were improbable, because transcription of orthomyxovirus mRNAs also takes place in the host cell nucleus [4]. Furthermore, HA is an immunodominant antigen of an important chicken pathogen which, similar to ILTV, initially replicates in the respiratory tract [1,2]. The cloned HA gene was expressed under control of the human cytomegalovirus immediate-early gene promoter ($P_{HCMV\text{-}IE}$) which was shown to permit efficient EGFP expression in previously described ILTV mutants [14,18]. The novel ILTV recombinants were tested for hemagglutinin expression, and used for animal trials to investigate, whether live vaccination is able to confer protective immunity against virulent ILTV, and against different highly pathogenic *H5* influenza A viruses.

2. Materials and methods

2.1. Viruses and cells

The influenza virus isolate A/chicken/Italy/8/98 (H5N2; kindly provided by I. Capua, Legnaro, Italy), and the virus strain A/chicken/Scotland/59 (H5N1) [29] were propagated in 10-day-old embryonated chicken eggs (purchased from Lohmann Tierzucht, Cuxhaven, Germany). All infectious laryngotracheitis virus recombinants used in this study were derived from the virulent strain ILTV A489 (obtained from D. Lütticken, Boxmeer, The Netherlands). The dUTPase gene deletion mutants ILTV ΔUL50 and ILTV ΔUL50G were described previously [14]. ILTV was generally propagated in primary chicken embryo kidney (CEK) cells, but for transfection experiments and plaque-assays the chicken hepatoma cell line LMH [15] was used. Cells were cultivated in minimum essential medium (MEM, Life Technologies) which was supplemented with 5–10% fetal calf serum (FCS, Life Technologies).

2.2. Cloning and sequencing of the hemagglutinin (H5) gene

Influenza virus (A/chicken/Italy/8/98) particles were sedimented from allantoic fluid by centrifugation at $50000 \times g$ for 30 min, and RNA was isolated by guanidinium thiocyanate–phenol–chloroform extraction [30]. For reverse transcription and amplification of the complete hemagglutinin ORF from genomic RNA segment 4 the consensus primers AI H5F (AGC AAA AGC AGG GGT $^{A}/_{C}T^{A}/_{G}$ AT), and AI H5R (TTT TAA ATG CAA ATT CTG CAC TGC AAT GA) were deduced from available GenBank sequences of other *H5* influenza virus isolates. Virus RNA (ca. 1 μg) was hybridized with 2.5 pmol of AI H5F and incubated for 1 h at 42°C with 200 U reverse transcriptase (Superscript II, Life Technologies). After digestion of template RNA with RNases H and T1, aliquots of the cDNA were PCR-amplified with 50 pmoles of AI H5F and AI H5R, and 1 U Pfx DNA polymerase (Life Technologies) by 30 cycles of 94°C for 15 s, 55°C for 30 s, 68°C for 120 s, and a final elongation step for 10 min at 68°C (Primus 96 Thermocycler, MWG Biotech). The purified 1.7 kbp PCR products were treated with Klenow polymerase and T7 polynucleotide kinase, and cloned into SmaI-digested plasmid pUC18. Two independently obtained plasmids, designated pUC-HA5a and pUC-

HA5b (Fig. 1) were subcloned by digestion with either *Xba*I, *Sph*I, *Hin*dIII, or *Sma*I and *Bam*HI followed by religation, and sequenced with vector specific primers as described previously [18]. DNA sequences were assembled and analyzed with the GCG software package in UNIX version 9.1 [31].

2.3. Construction and DNA analysis of the ILTV recombinants

The influenza virus HA gene was first recloned as a 1930 bp *Bam*HI/*Pvu*II fragment of pUC-HA5a into *Bam*HI and *Eco*RV doubly digested plasmid pcDNA-3 (Invitrogen). The resulting plasmid pCD-HA5a (Fig. 1) was used for in vitro transcription and translation studies. For insertion of the influenza virus HA gene at the UL50 locus of ILTV we utilized previously described transfer plasmids [14], which carry a EGFP expression cassette either in parallel (pILT-CSGa, not shown) or in opposite (pILT-CSGb, Fig. 1) orientation to the deleted UL50 ORF. First, an undesired *Bam*HI site of these constructs was removed by double digestion with *Bst*XI and *Sph*I, Klenow treatment, and religation. Then, the EGFP ORF was released by digestion with *Bam*HI and *Not*I, and replaced by a 1947 bp fragment of the similarly cleaved plasmid pCD-HA5a. The obtained plasmids pILT-CSHAa (not shown) and pILT-CSHAb (Fig. 1), together with viral DNA of ILTV ΔUL50-GB, and the *trans*-activating plasmid pRc-UL48 were used for calcium–phosphate mediated

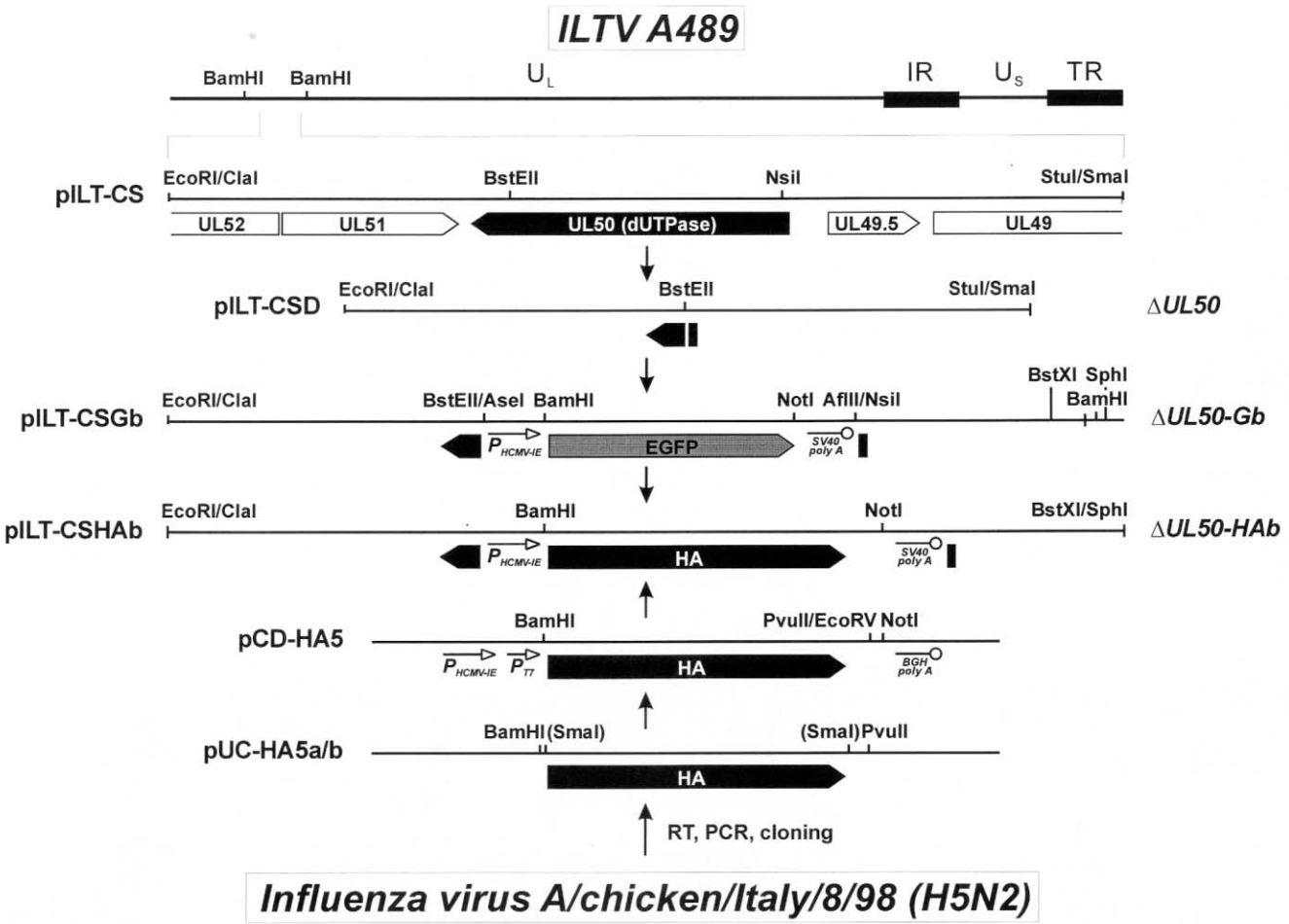

Fig. 1. Plasmid cloning and construction of ILTV recombinants. A schematic map shows the localization of the *UL50* gene region within a 10.6 kbp *Bam*HI fragment of the ILTV genome which consists of a long (U_L) and a short (U_S) unique region, and of inverted repeat sequences (IR, TR). The plasmid-cloned *UL50* gene (pILT-CS) was either deleted (pILT-CSD), or substituted by an enhanced GFP (EGFP) expression cassette (pILT-CSGb), which includes a herpesvirus promoter ($P_{HCMV\text{-}IE}$) and a polyadenylation signal (SV40 poly A). The influenza virus HA gene was reverse transcribed (RT), amplified (PCR), and cloned in pUC18 (pUC-HA5a/b). Recloning in pcDNA3 (pCD-HA) permits expression in eucaryotic cells utilizing $P_{HCMV\text{-}IE}$ and the bovine growth hormone polyadenylation signal (BGH poly A), as well as in vitro transcription and translation from the bacteriophage T7 promoter. Finally, the HA gene was inserted at the *UL50* gene locus of ILTV (pILT-CSHAb). Relevant restriction sites are indicated (see text). Designations of parental virus strains and ILTV recombinants obtained after cotransfection of plasmids and viral DNA are given in italics. Several plasmids and ILTV recombinants were described previously [14].

cotransfection of LMH cells [14]. Transfection progenies were analyzed by plaque-assays, non-fluorescent plaques were picked by aspiration, and purified to homogeneity by limiting dilutions on CEK cells grown in microtitre plates [18]. Single virus isolates (ILTV ΔUL50-HAa, ILTV ΔUL50-HAb) obtained from cotransfections with either transfer plasmid were further propagated, and characterized by restriction analyses and Southern blot hybridization of virus DNA, which were performed as described [17,18].

2.4. Western blot analyses

CEK cells were infected with ILTV or AIV at a multiplicity of five plaque forming units (pfu) or mean embryo infectious doses (EID_{50}) per cell, and incubated for 24 h at 37°C. Lysates of ca. 10^5 cells/lane were separated on discontinuous SDS-10% polyacrylamide gels, and electrotransferred to nitrocellulose membranes. The blots were incubated for 1 h each with 5% low-fat milk, primary antibodies, and peroxidase-conjugated secondary antibodies (Dianova). All reagents were diluted in TBS-T (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.25% Tween 20) which was also used for repeated washing after each step. Antibody binding was detected by luminescence (ECL Western blot detection system, Amersham-Pharmacia), and visualized on X-ray films.

2.5. Animal experiments

White leghorn chickens were bred from specific pathogen-free eggs (purchased from Lohmann Tierzucht, Cuxhaven, Germany). At the age of 18 weeks, three groups of 12 animals each were separated and infected intratracheally with 10^5 pfu of either ILTV ΔUL50-HAa, ILTV ΔUL50-HAb, or the previously characterized mutant ILTV ΔUL50 [14]. The chickens were observed daily for clinical symptoms, and tracheal swabs were taken 2, 3, and 4 days after immunization (p.i.) for titration of shed ILTV. Before infection, and 14, 21, and 28 days p.i., sera were collected and tested for ILTV and AIV specific antibodies by indirect immunofluorescence or hemagglutination inhibition (HI) tests, respectively. At day 28 p.i., four animals of each group, as well as four non-immunized chickens were intratracheally infected with 10^5 pfu of the virulent ILTV strain A489. The other animals were challenged by intranasal inoculation with 0.5 ml allantoic fluid containing ca. 10^8 EID_{50} of the influenza virus isolates A/chicken/Italy/8/98, or A/chicken/Scotland/59. Clinical signs were monitored for 2 weeks, and tracheal and cloacal swabs were taken to reisolate shed influenza A or ILT viruses. After 3 weeks all surviving animals were killed, and investigated for pathological alterations.

2.6. Plaque tests for titration of ILTV

For reisolation of ILTV tracheal swabs were taken 2, 3, and 4 days after challenge infection. To release the virus, swabs were incubated for 2 h at room temperature in 1 ml MEM, sonicated, and stored at −70°C until use. The serially diluted samples were incubated for 2 h with monolayers of LMH cells, and then replaced by MEM containing 5% FCS, 0.8% methyl cellulose, and 50 μg/ml enrofloxacin (Baytril, Bayer). After 4–5 days at 37°C the virus plaques were visualized by fixation of the cells with 2% formaldehyde (1 h), and subsequent staining with 1% crystal violet in 50% ethanol (15 min).

2.7. Indirect immunofluorescence tests

For detection of ILTV-specific antibodies CEK cells were grown in 24 well tissue culture plates and infected with wild type ILTV (ca. 100 pfu/well). After 24 h at 37°C the cells were fixed for 15 min with methanol–acetone (1:1), and subsequently incubated for 30 min each with diluted chicken sera and fluorescein-conjugated secondary antibodies (Dianova). After each step, the cells were washed three times for 5 min with phosphate buffered saline (PBS), and finally overlaid with 90% glycerol containing 25 mg/ml 1, 4 diazabicyclo[2.2.2.]octane and 1 μg/ml propidium iodide. Serum reactions with ILTV-infected and similarly treated non-infected CEK cells were analyzed by fluorescence microscopy.

2.8. Reisolation of AIV

For reisolation of AIV tracheal and cloacal swabs were taken 2, 3, 4, 7, and 10 days after challenge infection, placed in 1.7 ml MEM containing 10% FCS, 1 mg/ml enrofloxacin, 100 μg/ml lincomycin (Heinrich Fromme, Marburg, Germany), and 50 μg/ml gentamycin (Life Technologies), and stored at −70°C until use. According to standard protocols [32], three 10-day-old embryonated chicken eggs were inoculated with 0.2 ml of each sample via the allantoic cavities and further incubated at 37°C. Allantoic fluid was harvested after death of the embryos, and from surviving embryos which had been killed 5 days after infection. After examination for hemagglutinating activity, an additional egg-passage of negative probes from dead embryos was performed and tested.

2.9. Hemagglutination inhibition tests

Chicken sera collected 14, 21, and 28 days after immunization, and 21 days after challenge infection were

examined for HA-specific antibodies by HI tests [32] using the influenza virus isolates A/chicken/Italy/8/98, or A/chicken/Scotland/59 as antigens. In U-bottomed microtiter plates serial two-fold serum dilutions in PBS were subsequently mixed with equal volumes (25 μl) containing four hemagglutinating units of virus, and 1% chicken red blood cells, and incubated for 40 min at room temperature each. The HI titers were determined as reciprocals of the highest serum dilutions in which inhibition of hemgglutination was observed.

3. Results

3.1. Characterization of the cloned HA gene

The hemagglutinin gene of the highly pathogenic AIV isolate A/Chicken/Italy/8/98 (H5N2) was reverse transcribed from genomic RNA segment 4, amplified by PCR, and cloned in two independent experiments. To facilitate sequencing of the obtained plasmids pUC-HA5a and b (Fig. 1) the inserts were subcloned by utilization of internal restriction sites. The assembled cDNA sequence of the HA gene coding region (GenBank accession number AJ305306) comprises 1731 bp in and proved to be identical in both clones, except that an 'A' at position 628 of pUC-HA5a was replaced by 'T' in pUC-HA5b. This base substitution, however, has no effect on the deduced sequence of the HA gene product, which is 566 amino acids in size. The calculated molecular mass of the protein is 63.9 kDa, whereas electrophoretic mobility of the in vitro translation product of pCD-HA5a (Fig. 1) revealed an apparent mass of only 58 kDa (not shown). The protein contains hydrophobic signal and membrane anchor sequences close to the N- and C-termini, and seven putative *N*-glycosylation sites at positions 26, 27, 39, 181, 302, 498, and 557. The predicted protease cleavage site at position 344 is preceded by a cluster of basic amino acids (..PQRRRKKR/GLF..) as is typical for the HA proteins of highly pathogenic AIV strains [3]. Comparison of the amino acid and nucleotide sequences with that of previously described *H5* hemagglutinin genes showed greatest homologies to the recently published sequence of another AIV isolate from Italy [33], which differs from our sequence only in three amino acids. In general, the investigated HA gene is more closely related to that of European, Asian, and African virus isolates (93–99% identical amino acids), than to that of American viruses (89–92% identity). The *H5* hemagglutinin of strain A/Chicken/Scotland/59 [29], which was used for challenge infections in the present study (see below) exhibits an identity of 94% to the HA gene product expressed in recombinant ILTV.

3.2. Generation of hemagglutinin-expressing ILTV recombinants

In a recent study, we have shown that the dUTPase gene (UL50) of ILTV is non-essential for virus replication, and that this locus can be used for foreign gene insertions [14]. For expression of the influenza virus HA gene, we modified transfer plasmids which were previously used for *UL50* gene deletion and reporter gene insertion (Fig. 1). To utilize the HCMV-IE promoter and the SV40 polyadenylation signal of the expression cassette, only the EGFP ORF was replaced by the cloned cDNA encoding hemagglutin. Besides pILT-CSHAb (Fig. 1), a second plasmid (pILT-CSHAa, not shown) was generated which contains the same deletion, and the same HA expression cassette, but in parallel orientation with the UL50 ORF. Both transfer plasmids were used for calcium–phosphate mediated cotransfection of LMH cells. The expression construct pRc-UL48 encoding the ILTV homologue of the herpesviral *trans*-activator VP16 or αTIF [21,22] was added to transfection mixtures, because it reproducibly increases the infectivity of naked ILTV DNA [14]. Viral DNA was prepared from the described mutant ILTV ΔUL50-Gb (Fig. 1) which permitted detection of the desired novel recombinants by the absence of EGFP expression. Purification of recombinants was further facilitated because the parental virus, like most GFP-expressing ILTV-mutants, forms very small plaques [14], which were restored to wild type-like sizes in the new mutants ILTV ΔUL50-HAa and -HAb. Their maximum titers on CEK-cells were higher than 10^6 pfu/ml, and indistinguishable from that of wild type ILTV (A498), or an *UL50* gene deletion mutant without any foreign gene insertion (ILTV ΔUL50, Fig. 1).

To verify correct insertion of the HA gene, *Bam*HI digested DNA of the ILTV recombinants was analyzed by Southern blotting (Fig. 2). Hybridization with the labelled plasmid pILT-CS (Fig. 1) showed, that the *UL50* gene is localized within a 10612 bp *Bam*HI fragment of the wild type *ILTV* genome (Fig. 2B). In the genomes of ILTV ΔUL50-Gb, -HAa, and -HAb this fragment is replaced by two subfragments, since both the EGFP and the HA expression cassettes contain an additional *Bam*HI site (Fig. 1). Sizes of the detected subfragments were as expected for each of the virus recombinants (Fig. 2). Further hybridizations with EGFP- (Fig. 2C), and HA-specific (Fig. 2D) probes confirmed the presence of these genes in the genomes of the respective ILTV mutants.

After Northern blot hybridization of total RNA isolated 8 h after infection of CEK cells at a multiplicity of 5 pfu/cell with either ILTV ΔUL50-HAa, or ILTV ΔUL50-HAb an abundant HA gene-specific transcript of 2.3 kb was detectable, which matches the expected size of a polyadenylated full-length mRNA (not

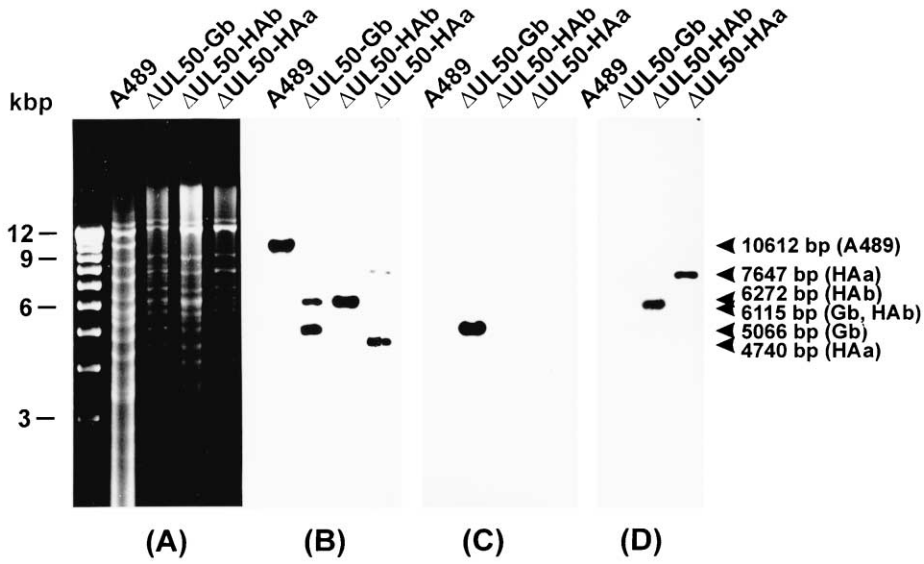

Fig. 2. Analysis of *Bam*HI digested DNA of wild-type ILTV (A489) and ILTV recombinants (ΔUL50-Gb, ΔUL50-HAb, ΔUL50-HAa). The ethidium-bromide-stained gel is shown in (A). Southern bots were hybridized with ^{32}P-labeled pILT-CS (B), the EGFP (C), or influenza virus HA gene (D). DNA markers and expected restriction fragment sizes of the individual ILTV mutants are indicated on the left and right, respectively.

shown). In Western blot analyzes of CEK cell lysates prepared 24 h after ILTV infection, proteins of similar sizes of ILTV ΔUL50-HAa, and ILTV ΔUL50-HAb were detected by three different *H5* influenza A virus-specific chicken antisera. As an example, the reactions of a serum raised against a heterologous H5N1 virus strain are shown (Fig. 3, upper panel). The strong band at 52 kDa is presumably formed by the N-terminal cleavage product of HA (HA1), whereas fainter signals at 75 and 26 kDa (the latter band is hardly visible) might represent a glycosylated precursor (HA0), and the C-terminal protein fragment (HA2), respectively. Proteins of similar sizes were found in cells infected with AIV A/chicken/Italy/8/98, but were absent from non-infected, or wild type ILTV infected cells. As a control, a monoclonal antibody detected glycoprotein C in all ILTV-infected cells (Fig. 3, lower panel). Stable expression of the HA protein by ILTV ΔUL50-HAa, and ILTV ΔUL50-HAb was further confirmed by indirect immunofluorescence reactions of all tested *H5* influenza virus specific chicken antisera (not shown).

3.3. Protection of chickens by immunization with HA expressing ILTV recombinants

To analyze the in vivo growth properties and immunogenicity of the generated ILTV recombinants, two groups of 12 chickens were intratracheally infected with either ILTV ΔUL50-HAa, or ΔUL50-HAb. A control group of the same number was immunized with the previously characterized mutant ILTV ΔUL50 [14], which exhibits a similar deletion of the dUTPase gene, but no foreign gene insertion (Fig. 1). From 2 to 10 days p.i. all animals of the three groups suffered from ILT-specific clinical signs, such as respiratory disorder and apathy, and one of the chickens infected with ILTV ΔUL50-HAa died after 2 weeks, possibly as a consequence of a secondary infection (Table 1). Shed ILTV could be reisolated from all animals by tracheal swabs taken 2–4 days p.i., but titers were much lower than those observed after challenge infection of non-immunized chickens with wild-type ILTV (Table 1). After 2 weeks, ILTV-specific antibodies were detectable in sera of almost all chickens by indirect immunofluorescence tests with infected CEK cells (Table 1). The sera of four animals showed strong cross-reactions with cellular proteins which prevented evaluation of the tests. In sera of all chickens immunized with either ILTV ΔUL50-HAa, or -HAb, the presence of influenza virus HA-specific antibodies could be demonstrated by HI tests (Table 1). As expected, the HI-titers achieved using the heterologous AIV strain A/chicken/Scotland/59 (H5N1) were slightly lower than those obtained with the strain A/chicken/Italy/8/98 (H5N2). Since the HI-titers remained nearly constant at low levels from 2 to 4 weeks p.i. (not shown), the challenge infections were performed at day 28 after immunization.

For that purpose, each group of immunized chickens was divided into three subgroups. Of each group, four animals (three in case of the ILTV ΔUL50-HAa group), as well as four non-vaccinated chickens were infected with the pathogenic ILTV strain A489. Whereas the non-vaccinated animals developed severe disease, and shed high amounts of ILTV, all immunized chickens remained healthy, and no challenge virus could be re-isolated (Table 1). A second set of four animals from

each group was challenged with the highly pathogenic AIV strain A/chicken/Italy/8/98. As expected, the ILTV ΔUL50-vaccinated chickens were not protected against avian influenza, and three of them died within 4 days. In contrast, no clinical signs were detectable in eight animals previously vaccinated with either ILTV ΔUL50-HAa, or ILTV ΔUL50-HAb (Table 1). Animals of these two groups were also significantly protected against challenge with the heterologous influenza virus strain A/chicken/Scotland/59. Six out of eight chickens remained healthy over 3 weeks, whereas two animals appeared sick and showed mild diarrhea for several days (Table 1). In contrast, animals immunized with ILTV ΔUL50 were not protected against challenge with the AIV strain A/chicken/Scotland/59. One chicken died after 3 days, and the others exhibited clinical signs of severe disease as huddling, edema of the head, lacrimation, diarrhea, and blood in tracheal, as well as in cloacal swabs (Table 1).

Using these swabs, shedding of influenza virus by protected and non-protected animals was monitored from 2 to 10 days after challenge infection (Table 2). From all animals immunized with ILTV ΔUL50 which contains no HA gene sequences challenge virus could be reisolated from both tracheal and cloacal swabs until day 7 after challenge, or until death. After 10 days infectious influenza virus was still detectable in all surviving animals, but only in cloacal swabs. In chickens immunized with the HA expressing ILTV recombinants ΔUL50-HAa or ΔUL50-HAb the challenge virus was eliminated much faster, since no influenza virus could be detected later than 4 days after challenge (Table 2). Moreover, the HA gene donor strain A/chicken/Italy/8/98 could be reisolated only from four out of eight protected animals. In two chickens the challenge virus was detected only in the intestinal tract, and in the remaining two animals AIV was restricted to the respiratory tract. Replication of the heterologous AIV strain A/chicken/Scotland/59 was inhibited somewhat less efficiently, since it was shed by seven out of eight vaccinated chickens, and in three of them it was found in tracheal and cloacal swabs as well. Only one animal never shed influenza virus A/chicken/Scotland/59 during investigation.

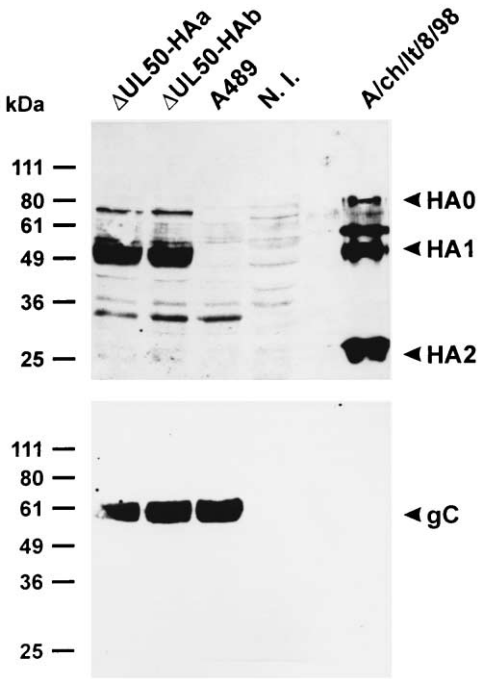

Fig. 3. Western blot analysis of ILTV-infected (ΔUL50-HAa, ΔUL50-HAb, A489), AIV-infected (A/chicken/Italy/8/98), and non-infected (N.I.) CEK cell lysates. Proteins were separated in SDS-10% polyacrylamide gels, transferred to nitrocellulose membranes, and incubated with an influenza A virus (H5N1) specific chicken antiserum (upper panel), or a monoclonal antibody directed against glycoprotein C of ILTV (lower panel). Antibody binding was visualized by chemiluminescense reactions of peroxidase-conjugated secondary antibodies. Molecular weight markers and calculated masses of the detected proteins are indicated.

At the end of the trial, 3 weeks after the AIV challenge, all surviving animals were necropsied and dissected. In convalescent as well as in protected chickens the titers of HA specific serum antibodies were significantly higher than after immunization with the ILTV recombinants (Table 1), but no conspicuous pathological alterations were found. In contrast, all animals which died within a few days after challenge infection showed edema, hyperemia, and hemorrhages in many tissues, including the myocardium, liver, kidney, and skin (not shown).

In summary, our experiments have shown that a single live-virus vaccination of chickens with ILT virus recombinants expressing the HA (subtype *H5*) gene of an avian influenza A virus confers protective immunity against ILTV, and against different highly pathogenic AIV isolates of the *H5* subtype. Immunization not only prevents disease, but also reduces shedding of challenge virus.

4. Discussion

To our knowlegde, this is the first report which describes the use of ILTV as a vector for an antigenic protein of another chicken pathogen, and it is also the first approach to utilize a recombinant herpesvirus for prevention of avian influenza. Other animal herpesviruses, as for example PrV or herpesvirus of turkey, have already been shown to be suitable vectors for antigens of hog cholera virus, or Newcastle disease virus, respectively [34,35]. Since the 155 kbp DNA genome of ILTV also contains non-essential regions, which can be deleted and replaced by reporter gene expression constructs encoding β-galactosidase or GFP [14,18,27], we started to substitute the reporter genes by

Table 1
Animal experiments with UL50 negative, and influenza virus HA expressing ILTV recombinants

	Time scale[a]	Group 1	Group 2	Group 3	Group 4
Immunization[b]	0	ILTV ΔUL50	ILTV ΔUL50-HAa	ILTV ΔUL50-HAb	None
Morbidity[c]	2–10 days p.i.	12/12	12/12	12/12	
Clinical signs		+	++	++	
Mortality	14 days p.i.	0/12	1/12	0/12	
ILTV-specific antibodies[d]	14 days p.i.	12/12	9/11	10/12	
HA-specific antibodies[e]	14 days p.i.	0/12	11/11	12/12	
A/chicken/Italy/8/98		≤2	34.3	55.7	
A/chicken/Scotland/59		≤2	22.6	26	
ILTV shedding[f]	2–4 days p.i.	12/12	11/11	12/12	
Pfu/ml		6.8×10^1	2.5×10^2	4.4×10^2	
Challenge[b]	28 days p.i.	ILTV A489			
Morbidity[c]	1–10 days p.c.	0/4	0/3	0/4	4/4
Clinical signs		−	−	−	+++
Mortality		0/4	0/4	0/4	0/4
ILTV shedding[f]	2–4 days p.c.	0/4	0/4	0/4	4/4
Pfu/ml		0	0	0	1.5×10^4
Challenge[g]	28 days p.i.	Influenza virus A/chicken/Italy/8/98 (H5N2)			
Morbidity[c]	1–7 days p.c.	4/4	0/4	0/4	
Clinical signs		+++	−	−	
Mortality	4 days p.c.	3/4	0/4	0/4	
HA specific antibodies[e]	21 days p.c.	1/1	4/4	4/4	
A/chicken/Italy/8/98		1024	512	512	
Challenge[g]	28 days p.i.	Influenza virus A/chicken/Scotland/59 (H5N1)			
Morbidity[c]	1–10 days p.c.	4/4	2/4	0/4	
Clinical signs		+++	+	−	
Mortality	2 days p.c.	1/4	0/4	0/4	
HA specific antibodies[e]	21 days p.c.	3/3	4/4	4/4	
A/chicken/IScotland/59		1024	512	512	

[a] The time scale of the experiments discriminates between days after immunization (days p.i.), and days after challenge infection (days p.c.).
[b] Immunization of chickens, as well as ILTV challenge infections, were performed by intratracheal application of ca. 10^5 pfu of the indicated ILT virus per animal.
[c] Clinical symptoms were valued from low (+) to severe (+++, for details see text).
[d] ILTV-specific antibodies were detected by indirect immunofluorescence tests on infected CEK cells.
[e] HA-specific antibodies were quantified by hemagglutinin inhibition tests using two different influenza virus isolates. The given titers are the geometric means of the reciprocals of the maximum positive serum dilutions.
[f] The mean titers of shed ILTV in tracheal swabs were determined by plaque assays on LMH cells.
[g] AIV-challenge was performed by intranasal administration of 10^8 EID_{50} of the indicated virus isolates.

genes of other chicken viruses at the same loci. In the present study, the HA gene of an *H5* avian influenza virus was inserted into the non-essential UL50 (dUTPase) locus of ILTV. A recently described [14] GFP-expressing mutant (ILTV ΔUL50-Gb) was chosen as parental virus for DNA cotransfection experiments to permit selection of the desired novel recombinants which should no longer express the easily detectable reporter protein. Previous investigations have shown that ILTV ΔUL50-Gb and some other GFP-expressing ILTV mutants exhibit a small plaque phenotype in vitro, which is not a consequence of the viral gene deletion, since the growth defect could be repaired by simple removal of the reporter gene cassette consisting of the HCMV-IE promoter and of the ORF encoding EGFP ([14], and unpublished results). In the present study, we discovered that replacement of the GFP ORF by that encoding influenza virus HA has the same

effect, since parental virus was rapidly outgrown by the new recombinants ILTV ΔUL50-HAa, or -HAb. This demonstrates that neither simple insertion of foreign DNA, nor the activity of the heterologous HCMV-IE promoter, which is also present in the genomes of the HA expressing ILTV mutants, impairs virus replication in vitro. Although the molecular basis for the inhibitory effect of GFP on ILTV replication remains unclear, it might facilitate substitution by almost any other foreign gene, which would be advantageous for further use of ILTV as a vector.

Apparently, the in vitro growth deficiency of the GFP-expressing *UL50* gene deletion mutant of ILTV correlates with a complete loss of in vivo virulence [14]. Conversely, either removal of the *GFP* gene or substitution by the influenza virus HA gene increases pathogenicity for chickens, leading to an only slightly attenuated phenotype when compared to that of the original wild-type strain ILTV A489. Thus, deletion of the viral dUTPase gene might not be sufficient for construction of recombinant ILTV live-virus vaccines with or without foreign antigens. However, the use of lower doses of the investigated *UL50* gene mutants, as well as their administration by spray or drinking water remain to be tested, since it was shown that efficacy and remaining virulence of classically attenuated ILTV vaccine strains significantly depend on dose and application route [1]. As potential alternatives to *UL50*, several other non-essential genes of ILTV could be deleted, and two of these deletions, affecting thymidine kinase (UL23), or glycoprotein G (US4) have already been shown to confer reporter gene-independent attenuation in vivo ([18,27], and unpublished results).

Despite their incomplete attenuation, the generated virus recombinants ILTV ΔUL50-HAa, and -HAb demonstrate, that construction of efficient bivalent vaccines against both ILTV and avian influenza viruses should be possible. In vitro studies showed, that independent of orientation within the ILTV genome, the inserted HA gene was abundantly expressed under control of the HCMV-IE promoter in both virus mutants. In all chickens immunized with these ILTV recombinants hemagglutinin-specific antibodies were detectable, but their titers remained significantly lower than in animals which survived infection with the highly pathogenic AIV isolates A/chicken/Italy/8/98, or A/chicken/Scottland/59. A possible explanation for the different immune responses might be the fact, that the latter viruses cause generalized infections, whereas replication of ILTV remains almost restricted to the upper respiratory tract [1,2]. However, the HI titers achieved after immunization of chickens with inactivated virus, DNA vaccines, or recombinant fowl pox viruses are also rather low [9,10,13], but nevertheless, these vaccines, including our ILTV recombinants, confer protective immunity to chickens. In all these cases, the steep increase of HI titers after challenge infection indicated that HA-specific antibodies produced by activated memory B cells are most likely responsible for influenza virus neutralization [9,13].

Although hemagglutinin is an immunodominant antigen, development of influenza A virus vaccines is hampered by the extreme variability of this protein which encompasses 15 subtypes up to now [2]. Even within the *H5* subtype, which is present in many highly pathogenic avian influenza virus isolates, variations of more than 10% of the amino acid residues could be observed. Our studies revealed that after immunization with ILTV recombinants expressing the *H5* type hemagglutinin of influenza virus A/chicken/Italy/8/98 the animals were well protected against challenge with the parental AIV isolate. Protection against challenge with another *H5* type influenza virus isolate (A/chicken/Scotland/59) [29], whose HA is 94% identical

Table 2
Reisolation of AIV after challenge of chickens vaccinated with ILTV recombinants

Immunization[a]	Group 1b	Group 2b	Group 3b	Group 1c	Group 2c	Group 4c
	ILTV ΔUL50	ILTV ΔUL50HAa	ILTV ΔUL50HAb	ILTV ΔUL50	ILTV ΔUL50HAa	ILTV ΔUL50HAb
Challenge[b] (28 days p.i.)	Influenza virus A/chicken/Italy/8/98			Influenza virus A/chicken/Scotland/59		
2 days p.c.[c]	4/4	1/4	1/4	4/4	2/4	3/4
3 days p.c.	4/4	1/4	3/4	3/3	3/4	2/4
4 days p.c.	1/1	1/4	0/4	3/3	3/4	2/4
7 days p.c.	1/1	0/4	0/4	3/3	0/4	0/4
10 days p.c.	1/1	0/4	0/4	3/3	0/4	0/4

[a] Chickens were immunized by intratracheal application of ca. 10^5 pfu/animal of the indicated ILTV recombinant.

[b] Challenge infections were performed 28 days after immunization by intranasal administration of ca 10^8 EID_{50} of the indicated AIV isolate per animal.

[c] At different times after challenge infection (days p.c.) tracheal and cloacal swabs were taken from all animals and tested by inoculation of embryonated chicken eggs. The number of chickens, from which AIV could be reisolated from one or both swabs, was compared to the total number of surviving animals at that particular time point.

to the HA protein used for immunization, was also sufficient to prevent severe disease, but moderate clinical signs were observed in several animals. As observed with all other types of inactivated or recombinant influenza virus vaccines, shedding of challenge virus was reduced, but not completely abolished in our experiments. Correlating with the clinical observations, shedding of the HA gene donor strain A/chicken/Italy/8/98 was reduced to a greater extent than that of the heterologous strain A/chicken/Scotland/59. In agreement with our results, a recent in vivo study performed with a fowl pox virus recombinant also demonstrated that the degree of sequence diversity between the HA proteins of vaccine and challenge virus at least partly correlates with the rate of local challenge virus replication and shedding [11].

Apparently it is difficult to generate reliable vaccines against a broader range of avian influenza A viruses since the neuraminidase (NA) as the second surface protein is as variable as HA, and use of the higher conserved nucleoprotein (NP) in recombinant virus- or DNA vaccines conferred only partial protection even against challenge with homologous virus strains [2,10,13]. Thus, to achieve protection against different influenza A viruses multivalent vaccines containing or expressing different HA subtypes or variants of the same subtype should be used. If utilizing ILTV as a vector, insertions of multiple foreign genes at different non-essential sites of the virus genome would be possible. Furthermore, the easy selection of foreign antigen containing ILTV recombinants by substitution of the *GFP* reporter gene also permits the fast generation of emergency vaccines against influenza epidemic strains.

When compared to inactivated influenza virus, our ILTV mutants and other recombinant vaccines containing only HA have the advantage to permit serological discrimination between immunized and field-virus infected animals by the absence or presence of antibodies against NA and NP, which are detectable by standard diagnostic tests [2]. A particular advantage of ILTV live-virus vaccines is their applicability to huge flocks by spray or drinking water [1]. In contrast, inactivated virus and DNA vaccines require individual administration to each animal, and also with fowl pox live-virus vaccines mass application techniques were found to be less efficient than the commonly used wing-web method [36]. One restriction for the use of ILTV based vector vaccines is the very narrow host range of ILTV which replicates efficiently only in chickens and pheasants, but not in turkeys and ducks which are frequently infected by avian influenza viruses [1,3]. However, chickens are the economically most important host of highly pathogenic AIV and other viruses causing Newcastle disease, infectious bronchitis, infectious bursitis, or Marek's disease, and we are currently preparing ILTV recombinants encoding immunogenic proteins of these viruses. For these approaches non-essential gene loci other than UL50 are chosen as insertion sites, which should ensure that the resulting virus mutants are sufficiently attenuated.

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