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

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Infectious laryngotracheitis virus (ILTV), a member of the Alphaherpesvirinae, possesses several unique genes. One of them, UL0, encodes an abundantly expressed protein that accumulates in the nuclei of ILTV-infected cells. This study demonstrates that this protein is dispensable for in vitro virus replication and that UL0 deletion mutants exhibit only minor growth defects in cultured cells. The UL0 gene locus of ILTV was also used for insertion of foreign DNA sequences encoding enhanced GFP or haemagglutinin (HA), subtype H7, of a highly pathogenic avian influenza virus under the control of the human cytomegalovirus immediate–early gene promoter. Expression of foreign proteins was shown by (immuno)fluorescence tests and Western blot analyses. After experimental infection of chickens, UL0 deletion mutants proved to be attenuated when compared to both parental wild-type ILTV and an UL0 rescue mutant. Nevertheless, all animals immunized with UL0-negative ILTV were protected from clinical disease after subsequent infection with virulent ILTV. Furthermore, all animals immunized with HA-expressing ILTV survived a lethal challenge with H7 subtype avian influenza virus with minimal clinical signs. Thus, an UL0-negative and HA-expressing ILTV recombinant may be used as a bivalent live virus vaccine against ILT and fowl plague. Unlike inactivated influenza virus vaccines, HA-expressing ILTV recombinants should be suitable for mass application and would also permit serological discrimination between vaccinated and virus-infected animals in the field.

INTRODUCTION

Infectious laryngotracheitis (ILT), a serious respiratory disease of chickens that occurs worldwide, affects growth and egg production and may lead to the death of the animals (Bagust & Guy, 1997). The causative agent, ILT virus (ILTV or gallid herpesvirus-1) is a member of the subfamily Alphaherpesvirinae, family Herpesviridae, and represents the hitherto only species in the genus Infectious Laryngotracheitis-like Viruses (Minson et al., 2000). Like other alphaherpesvirus genomes, the ca. 150 kbp DNA genome of ILTV consists of a long (UL) and a short (US) unique region and of inverted repeat sequences (IRs and TRs) that flank the US region (Fig. 1) (Johnson et al., 1991; Leib et al., 1987). DNA sequencing of major parts of the ILTV genome revealed that gene content and arrangement are, overall, similar to that found in other alphaherpesviruses (reviewed by Fuchs et al., 2000). Therefore, most of the gene designations introduced originally for herpes simplex virus type 1 (HSV-1) (McGeoch et al., 1988) were also adopted for ILTV.

However, the low degree of sequence conservation between ILTV gene products and their homologues indicated a considerable phylogenetic distance to other mammalian or avian alphaherpesviruses (Johnson & Tyack, 1995; McGeoch et al., 2000). This was confirmed by several unique features of the ILTV genome, including the absence of an UL16 homologue (Fuchs & Mettenleiter, 1999), the translocation of the UL47 gene from the UL1 to the US region (Wild et al., 1996; Ziemann et al., 1998a) and an internal inversion within the UL3 region that encompasses the UL22 to UL44 genes (Ziemann et al., 1998a). Furthermore, the ILTV...
genome contains several ORFs that possess no homologies to any other known herpesvirus genes (Fuchs & Mettenleiter, 1996; Johnson et al., 1997; Wild et al., 1996; Ziemann et al., 1998a, b). Recently, we demonstrated that in infected chicken cell cultures, seven of these ILTV-specific genes are indeed transcribed and translated into proteins (Veits et al., 2003b; Ziemann et al., 1998b). The protein products of a cluster consisting of five ORFs (A–E) located downstream of UL22 were detected predominantly in the host cell cytoplasm (Veits et al., 2003b). In contrast, the products of the ILTV-specific UL0 and UL[−1] genes are expressed from spliced mRNAs. Transfer plasmids were generated for deletion (pΔUL0) or rescue (pILT-E43BX) of the UL0 gene and for insertion of foreign genes (pΔUL0-G and pΔUL0-H7). The insertion of pΔUL0-G consists of the coding sequence of EGFP flanked by the human cytomegalovirus immediately–early gene promoter (P_{HCMV-IE}) and a polyadenylation signal of simian virus 40 [SV40 poly(A)], whereas pΔUL0-H7 contains the H7 subtype HA gene of AIV. Virus recombinants (bold italics) were isolated after co-transfection of chicken cells with transfer plasmids and genomic ILTV DNA. The EGFP-expressing mutant ILTV ΔUL0-G was derived from ILTV A489 and served as parental virus for all other recombinants.

In the present study, UL0 deletion mutants of ILTV were isolated and characterized in vitro and in vivo to evaluate their suitability as live virus vaccines. To date, the only ILT vaccines in use were generated by serial egg or cell culture passages of field virus isolates and these vaccines have not been genetically characterized (Bagust & Guy, 1997). These ILTV strains are suitable for mass application via eye drop, aerosol or drinking water, but, in numerous cases, turned out to be insufficiently attenuated or reverted to a virulent phenotype (Bagust & Guy, 1997). ILTV recombinants with defined gene deletions might be safer vaccines, but, to date, only a few virus mutants of this type have been tested in vivo. Whereas deletion of the viral thymidine kinase gene UL23 led to attenuation of ILTV in chickens (Okamura et al., 1994; Schnitzlein et al., 1995), ILTV mutants lacking
the UL50 gene, which encodes another enzyme of nucleotide metabolism, dUTPase, remained virulent (Fuchs et al., 2000; Lüsschow et al., 2001).

Considering the easy mass application of ILTV live virus vaccines, ILTV recombinants might be useful vectors for expression of immunogenic proteins of other chicken pathogens. As demonstrated by recent epidemics in Hong Kong, Italy and The Netherlands (Abbott, 2003; Capua et al., 1999; Capua & Marangon, 2000; Shortridge et al., 1998), fowl plague is one of the economically most important infectious diseases of chickens, with mortality rates of up to 100%. Fowl plague is caused by highly pathogenic avian influenza A viruses (AIV) carrying the antigenically distinct haemagglutinin (HA) subtypes H5 or H7 (Alexander, 2000). The HA of influenza viruses is an envelope glycoprotein that is required for attachment and penetration of host cells and it represents a major target of the host immune response (Easterday et al., 1997; Lamb & Krug, 2001). Thus, not only inactivated AIV (Allan et al., 1971; Swayne et al., 1999, 2001) but also subunit vaccines containing vacinia virus- or baculovirus-expressed HA proteins (Chambers et al., 1988; Crawford et al., 1999), DNA vaccines containing the HA gene (Fynan et al., 1993; Kodihalli et al., 2000) or fowlpox virus recombinants expressing HA (Swayne et al., 2000; Taylor et al., 1988) have been shown to protect domestic fowl against infections with highly pathogenic AIV strains possessing the corresponding HA subtypes. HA subtype H5 has already been expressed in an UL50-negative ILTV recombinant and chickens vaccinated with this virus were protected against lethal AIV challenge (Lüsschow et al., 2001).

In the present study, the UL0 gene locus of ILTV was used for insertion of a H7 subtype HA gene, which had been reverse-transcribed and cloned from an AIV field isolate of the recent fowl plague epidemic in Italy (Capua & Marangon, 2000). The ILTV recombinant obtained was tested for HA expression by immunofluorescence and Western blot analyses. In vitro growth properties of the HA-expressing ILTV mutant, an UL0 deletion mutant without foreign gene insertion and an UL0 rescue mutant were also analysed. Furthermore, animal experiments were performed to determine whether the UL0 deletion mutants are attenuated and whether they confer protective immunity against ILT and fowl plague.

**METHODS**

**Viruses and cells.** Virus recombinants were derived from the pathogenic ILTV strain A489 and propagated in primary chicken embryo kidney (CEK) cells (Fuchs & Mettenleiter, 1996). For transfection experiments and plaque assays, a chicken hepatoma cell line (LMH) (Kawaguchi et al., 1987; Schnitzlein et al., 1994) was used. Cells were grown in MEM with 10% FCS (Invitrogen). The highly pathogenic AIV isolate A/chicken/Italy/445/99 (H7N1) was propagated in 10-day-old embryonated chickens' eggs (Lohmann Tierzucht).

**Construction of UL0 deletion plasmids and ILTV recombinants.** Plasmid pILT-E43, which contains an 11-kbp EcoRI fragment of the ILTV genome (Ziemann et al., 1998b), was shortened by consecutive HindIII/NotI and EcoRI/XhoI double digestions and re-ligation. As in subsequent experiments, non-compatible, single-stranded overhangs of digested DNA were polished by treatment with Klenow polymerase prior to ligation. The 3997 bp insert of the resulting plasmid pILT-E43BX included the entire UL0 gene of ILTV (Fig. 1). To obtain pUL0, 1137 bp of the UL0 ORF were removed by double digestion of pILT-E43BX with BstHI/Xbal and subsequent re-ligation (Fig. 1). A second deletion plasmid, pUL0-G (Fig. 1), was generated by substitution of a 984 bp ClaI–BsrGI fragment by a 1615 bp ClaI–AflII fragment of pB-gFP (Fuchs & Mettenleiter, 1999), which contains an expression cassette for EGFP under the control of the major immediate-early promoter of human cytomegalovirus (pHCMV-IE). Plasmid pUL0-G and genomic DNA of ILTV A489 were used for calcium phosphate-mediated co-transfection of LMH cells (Fuchs et al., 2000). Eukaryotic expression constructs encoding the trans-activating ICP4 and UL48 proteins of ILTV were also added to the transfection mixture, as they have been shown to enhance virus replication (Fuchs et al., 2000). EGFP-expressing virus recombinants were identified by fluorescence microscopy and isolated by limiting dilutions of the transfection progeny on CEK cells grown in microtitre plates. Genomic DNA of the isolated virus recombinant ILTV ΔUL0-G (Fig. 1) was prepared (Fuchs & Mettenleiter, 1996) and used for subsequent co-transfection experiments with plasmids pILT-E43BX and pUL0. By screening transfection progeny for non-fluorescent plaques, the UL0 rescue mutant ILTV UL0R and the deletion mutant ILTV ΔUL0, which contain no reporter gene insertion, were isolated.

**Cloning and expression of influenza virus HA.** Influenza virus particles were sedimented from allantoic fluid harvested 4 days after inoculation of embryonated chickens' eggs with AIV A/chicken/Italy/445/99 by centrifugation at 50,000 g for 30 min, and genomic viral RNA was prepared (Chomczynski & Sacchi, 1987). Consensus primers for amplification of genome segment 4 were deduced from published nucleotide sequences of H7 subtype HA genes of other influenza viruses. The custom-made (Invitrogen) primers AI-H7F (5’-GGGATACAAAAATGAACT-3’) and AI-H7R (5’-CCAACTTATACAAATGTC-3’) were used for reverse transcription and subsequent PCR amplification, as described (Lüsschow et al., 2001). The 1711 bp amplification products were inserted into the Smal-digested plasmid vector pUC19. Using 23rd-labelled vector- and insert-specific primers, the cloned products of two independent PCR reactions were sequenced (Fuchs & Mettenleiter, 1999). The resulting insert DNA sequence (GenBank accession no. AF580535) of the two pUC-H7 plasmids was identical and encoded a HA protein of 564 aa. For HA expression, pHCMV-IE was excised from pcDNA3 (Invitrogen) as a 685 bp NruI–HindIII fragment and inserted upstream of the cloned HA ORF after digestion of pUC-H7 with Xbal/Sall. From the plasmid obtained, the HA expression cassette was re-cloned as a 2431 bp HindIII–KpnI fragment into pUL0-G, from which the GFP insertion had been removed by digestion with HindIII/XbaI. The resulting plasmid, pUL0-H7 (Fig. 1), together with genomic DNA of ILTV ΔUL0-G, was used for co-transfection of LMH cells. Recombinant ILTV UL0-H7 was isolated from non-fluorescent progeny virus plaques.

**Western blot analyses.** CEK cells were inoculated with AIV A/chicken/Italy/445/99 (H7N1), wild-type ILTV A489 or ILTV mutants at an m.o.i. of 2 and incubated at 37°C for 24 h. Lysates of ca. 10⁶ infected or uninfected cells per lane were separated by SDS-PAGE in 10% polyacrylamide gels and transferred to nitrocellulose filters, as described (Fuchs & Mettenleiter, 1999). Blots were incubated with an ILTV UL0-specific rabbit antiseraum at a dilution of 1:10,000 (Ziemann et al., 1998b), an ILTV glycoprotein.
C-specific monoclonal antibody (mAb) at a dilution of 1:500 (Veits et al., 2003a) or with chicken antiserum, which was obtained after repeated immunization with the inactivated influenza virus isolate A/FPV/Dutch/27 (H7N7) and subsequent booster infection with the same virus (dilution 1:1000). Antibody binding was detected by luminescence reactions (SuperSignal System, Pierce) of peroxidase-conjugated secondary antibodies (Dianova) and recorded on X-ray films (Hyperfilm MP, Amersham).

**Indirect immunofluorescence (IIF) assays.** CEK or LMH cells were fixed with a 1:1 mixture of methanol and acetone at 1–3 days after ILTV- or AIV-infection at low m.o.i. (≤ 0:001) and incubated with an AIV-specific antiserum (see above), with chicken sera from the animal trials (see below) or with an ILTV glycoprotein J-specific mAb (Veits et al., 2003a) at dilutions of 1:100, as described (Veits et al., 2003a). Binding of fluorescein-conjugated secondary antibodies (Dako and Dianova) was analysed by fluorescence microscopy (Diaphot 300, Nikon) after chromatin counterstaining with propidium iodide.

**Plaque assays and one-step growth kinetics.** For determination of plaque sizes, LMH cell monolayers were infected in parallel with the respective ILTV mutants at low m.o.i. After 2 h, the inoculum was replaced with medium containing 6 g methylcellulose 1 l−1 and incubation was continued for 3 days at 37 °C. The diameters of 50 plaques per virus mutant were determined by fluorescence microscopy either directly (ILTV ΔUL0-G) or after IF with a glycoprotein J-specific mAb. Average diameters and SD values were calculated and the statistical significance of differences was analysed using Student’s t-test. One-step growth analyses of ILTV were performed essentially as described previously (Fuchs et al., 2000). At different times post-infection (p.i.) (1, 6, 12, 18, 24 and 48 h) at an m.o.i. of 4, CEK cells were scraped into the medium, lysed by freeze–thaw incubation and progeny virus titres were determined by plaque assays on LMH cells. The average results of two independent experiments were plotted.

**Animal experiments.** White Leghorn chickens were bred from specific-pathogen-free eggs (Lohmann Tierzucht). At 10 weeks of age, the animals were separated into four groups, and infected with either wild-type ILTV A489 or recombinants ILTV AUL0, ΔUL0-H7 and UL0R. The viruses were diluted in a blue-coloured solvent (Intervet) and ca. 5 × 103 p.f.u. of virus per animal were administered via eye drop. Chickens were observed daily for clinical symptoms for a 2 week period and tracheal swabs were taken at 3, 4 and 5 days after infection (p.i.) for re-isolation of ILTV. Sera collected before infection and at day 15 p.i. were tested for ILTV- and AIV-specific antibodies by IF or HAI tests (Lüschow et al., 2001), respectively. At day 25 p.i., six animals from each group, as well as eight non-immunized chickens, were infected intratracheally with 2 × 103 p.f.u. of the virulent ILTV strain A489. Furthermore, 14 chickens that had been immunized with ILTV ΔUL0-H7, and three chickens that had been immunized with ILTV ΔUL0, were challenged by oculonasal inoculation with 0·2 ml allantoic fluid containing 6 × 107 mean embryo infectious doses (EID50) of the highly pathogenic AIV isolate A/chicken/Italy/445/99 (H7N1). Clinical signs were monitored and tracheal and cloacal swabs were taken to re-isolate AIV or ILTV. For re-isolation of ILTV, tracheal swabs taken at 3, 4 and 5 days after immunization or challenge infection were analysed by plaque assay on LMH cells, whereas AIV was identified in tracheal and cloacal swabs taken at 3, 6 and 10 days after infection by passage in embryonated chickens’ eggs and subsequent testing of the allantoic fluids for haemagglutinating activity (Lüschow et al., 2001). At 2 weeks after challenge infection, all surviving animals were necropsied and investigated for pathologica alterations. Clinical scores were determined over the 2 week period after immunization as well as after challenge infection. To this end, the chickens were classified on a daily basis as healthy (0), ill (1), severely ill (2) or dead (3). For all animals of each group, mean values were calculated for each day (daily scores) and for the period of days 2–12 after ILTV, or days 1–10 after AIV, infection, respectively (total scores). To determine total scores, dead animals were considered until the end of the monitoring period but were no longer included in the daily scores after the day of death.

**RESULTS AND DISCUSSION**

**Isolation of virus mutants**

The virulent ILTV strain A489 was mutated by cotransfection of cultured chickens’ cells with genomic viral DNA and transfer plasmids containing deletions and/or foreign gene insertions at the UL0 locus (Fig. 1). To facilitate isolation of virus mutants, codons 16–318 of the UL0 ORF were initially replaced by an expression cassette encoding EGFP. Recombinant ILTV ΔUL0-G could be isolated easily from transfection progeny since it formed green fluorescent plaques. After repeated plaque purification, ILTV ΔUL0-G appeared homogeneous and was used as parental virus for the generation of further ILTV recombinants. Since previous studies demonstrated that EGFP reporter gene insertions may affect *in vitro* and *in vivo* replication of ILTV, independent of the viral genes deleted (Fuchs et al., 2000; Veits et al., 2003b), a second UL0 deletion mutant without any foreign DNA sequences was generated from ILTV ΔUL0-G and isolated by selection of non-fluorescent virus plaques. This mutant, ILTV UL0 (Fig. 1), exhibited a deletion of codons 1–352 of UL0. The 3’-terminal part of the 507 codon UL0 ORF was not removed, since it overlaps the conserved UL1 gene encoding the presumably essential glycoprotein gl. of ILTV (Fuchs & Mettenleiter, 1996). In similar approaches, two other virus recombinants were derived from ILTV ΔUL0-G: the UL0 rescue mutant ILTV UL0R and the influenza virus HA gene insertion mutant ILTV ΔUL0-H7 (Fig. 1). All virus recombinants were characterized by restriction analyses and Southern blot hybridization of genomic DNA, as well as by PCR amplification and sequencing of the mutated region of the genome (results not shown).

Protein synthesis of wild-type ILTV and UL0 mutants was compared by Western blot analyses of infected CEK cells (Fig. 2). Whereas similar amounts of glycoprotein C were detectable with a specific mAb (Veits et al., 2003a) in cells infected with either virus, the reactions of an UL0-specific antiserum revealed that the 63 kDa gene product (Ziemann et al., 1998b) was only expressed by ILTV A489 and ILTV ΔUL0-G and isolated by selection of non-fluorescent virus plaques. This mutant, ILTV ΔUL0 (Fig. 1), exhibited a deletion of codons 1–352 of UL0. The 3’-terminal part of the 507 codon UL0 ORF was not removed, since it overlaps the conserved UL1 gene encoding the presumably essential glycoprotein gl. of ILTV (Fuchs & Mettenleiter, 1996). In similar approaches, two other virus recombinants were derived from ILTV ΔUL0-G: the UL0 rescue mutant ILTV UL0R and the influenza virus HA gene insertion mutant ILTV ΔUL0-H7 (Fig. 1). All virus recombinants were characterized by restriction analyses and Southern blot hybridization of genomic DNA, as well as by PCR amplification and sequencing of the mutated region of the genome (results not shown).

**The UL0 protein is dispensable for *in vitro* replication of ILTV**

Successful isolation of UL0-negative ILTV mutants from non-complementing chickens’ cells already indicated that
this ILTV-specific gene is not essential for virus replication. For detection of sub-lethal defects, one-step growth kinetics and plaque sizes of the different UL0 deletion mutants were compared to those of parental wild-type ILTV and an UL0 rescue virus (Fig. 3). Growth curves were determined in CEK cells, in which ILTV replicates more efficiently than in other chickens' cells (Bagust & Guy, 1997; Veits et al., 2003b). These studies demonstrated that the final titres of wild-type and mutant viruses were very similar (Fig. 3A). Whereas the growth curves of parental ILTV A489 and of the rescue mutant ILTV UL0R were indistinguishable, replication of all UL0 deletion mutants appeared to be delayed slightly (Fig. 3A). This moderate delay in virus growth might indicate a beneficial function of UL0 during early steps of virus replication. The UL0 protein was detected predominantly within the nucleus of ILTV-infected cells (Ziemann et al., 1998b), implicating a possible role in regulation of viral gene expression, DNA synthesis or encapsidation. Remarkably, the UL0 protein of ILTV is encoded at a genome position corresponding to that of the structurally and functionally conserved transactivator protein ICP0 of HSV-1 and other alphaherpesviruses (Everett et al., 1993). Unlike its positional homologues, the UL0 gene product of ILTV is not an immediate–early protein and also lacks their typical RING finger domain (Ziemann et al., 1998b). However, a related function cannot be ruled out.

Plaque formation of ILTV UL0 mutants was analysed in the chicken hepatoma cell line LMH (Fig. 3B) and in primary CEK cells (results not shown). In both cell types, the plaques of the EGFP-expressing deletion mutant ILTV ΔUL0-G were significantly smaller than those of wild-type ILTV A489. As expected, wild-type plaque sizes were found after repair of the UL0 gene in ILTV UL0R (Fig. 3B). Remarkably, the defect of ILTV ΔUL0-G was corrected almost completely by removal of the EGFP expression cassette in ILTV ΔUL0 (Fig. 3B). Thus, cell-to-cell spread of ILTV ΔUL0-G was not affected by the deletion of UL0 but...
by insertion or expression of the foreign gene. Similar effects were also observed after insertion of the EGFP reporter gene into the dUTPase gene UL50 of ILTV; possible toxic effects of the EGFP protein were discussed as a reason for this phenomenon (Fuchs et al., 2000; Lüscho et al., 2001). However, insertions at other loci, such as the ILTV-specific ORF B gene (Veits et al., 2003b) did not affect plaque formation. Furthermore, the present study indicates that not only expression of EGFP but also expression of influenza virus HA affects plaque formation of the corresponding virus mutant ILTV DUL0-H7 (Fig. 3B). Therefore, the reason for the small plaque phenotypes observed might be a de-regulation of adjacent virus genes by PHCMV-IE, which is present in both foreign gene insertions (Fig. 1), or a deple-

tion of metabolites, such as ribonucleotides and amino acids, due to the strong activity of PHCMV-IE in infected cells.

### Table 1. Summary of animal experiments

Chickens were immunized via eye drop with ILTV recombinants (DUL0-H7, DUL0 or UL0R) or wild-type ILTV A489 and challenged after 25 days either intratracheally with ILTV A489 or oculonasally with AIV A/chicken/Italy/445/99 (H7N1). For the indicated time-periods after immunization (p.i.) and after challenge infection, animals were examined daily and total clinical scores were determined. At 2 weeks p.i., the sera of the animals were analysed for ILTV-specific antibodies (Ab) by IIF assay and for HA-specific antibodies by HAI tests. Shedding of ILTV was detected by plaque assays of tracheal swabs on LMH cells and AIV was re-isolated from tracheal and cloacal swabs by cultivation in embryonated chickens' eggs. Some groups were not tested (NT) for individual parameters and some tests were prevented by the death (D) of the animals.

(a) Immunization: each animal was immunized with 10^5-7 p.f.u. of the virus indicated per animal.

<table>
<thead>
<tr>
<th>Time (days p.i.)</th>
<th>Group 1 ILTV DUL0-H7 (n = 20)</th>
<th>Group 2 ILTV DUL0 (n = 9)</th>
<th>Group 3 ILTV UL0R (n = 6)</th>
<th>Group 4 ILTV A489 (n = 6)</th>
<th>Group 5 Control (n = 8)</th>
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<tbody>
<tr>
<td>Mortality</td>
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<td>0/9</td>
<td>0/6</td>
<td>0/6</td>
<td>0/8</td>
</tr>
<tr>
<td>Morbidity</td>
<td>2–12</td>
<td>2/20</td>
<td>1/9</td>
<td>2/6</td>
<td>4/6</td>
</tr>
<tr>
<td>Clinical score</td>
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<td>0–04</td>
<td>0–18</td>
<td>0–27</td>
<td></td>
</tr>
<tr>
<td>ILTV shedding</td>
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<td>7/20</td>
<td>2/9</td>
<td>5/6</td>
<td>NT</td>
</tr>
<tr>
<td>ILTV-specific Ab</td>
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<td>14/20</td>
<td>7/9</td>
<td>6/6</td>
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<td>AIV-specific Ab</td>
<td>15</td>
<td>20/20</td>
<td>0/9</td>
<td>NT</td>
<td>NT</td>
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<tr>
<td>HAI titre</td>
<td>2^{+6}</td>
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(b) Challenge: each animal was challenged with 10^5-3 p.f.u. ILTV A489 per animal at day 25 p.i.

<table>
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<tr>
<th>Time (days post-challenge)</th>
<th>Group 1A ILTV A489 (n = 14)</th>
<th>Group 2A ILTV A489 (n = 3)</th>
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<td>0/14</td>
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<td>Morbidity</td>
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<td>14/14</td>
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<tr>
<td>Clinical score</td>
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<td>0–64</td>
</tr>
<tr>
<td>AIV shedding</td>
<td>3</td>
<td>9/14</td>
</tr>
</tbody>
</table>

(c) Challenge: each animal was challenged with 10^7-8 EID_{50} AIV A/chicken/Italy/445/99 (H7N1) per animal at day 25 p.i.

<table>
<thead>
<tr>
<th>Time (days post-challenge)</th>
<th>Group 1B ILTV A489 (n = 14)</th>
<th>Group 2B ILTV A489 (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mortality</td>
<td>0/14</td>
<td>3/3</td>
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<tr>
<td>Morbidity</td>
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<td>3/3</td>
</tr>
<tr>
<td>Clinical score</td>
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<td>2–57</td>
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<tr>
<td>AIV shedding</td>
<td>9/14</td>
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Apart from these effects of foreign gene insertions, our studies demonstrated that the UL0 gene plays only a minor role during in vitro replication of ILTV. Possibly, this can be explained by the presence of the adjacent Ul(-1) gene (Fig. 1), which encodes a protein that shares significant sequence homology with the Ul0 gene product (Ziemann et al., 1998b) and therefore might possess related functions.

UL0 deletion mutants are attenuated in vivo and protect against ILTV challenge infection

Although UL0 is not required for ILTV replication in cultured chickens’ cells, the situation in the animal host might be different. To test this possibility, 10-week-old chickens were infected by ocular administration of 5 × 10^3 p.f.u. of wild-type ILTV A489, ILTV Ul0R or deletion mutants ILTV ΔUl0 and ΔUl0-H7. From days 2 to 12 after immunization, clinical signs of ILT, such as respiratory disorder and conjunctivitis, were monitored and scored (Table 1 and Fig. 4A). Only 3 of 29 chickens infected with the Ul0 deletion mutants ILTV ΔUl0 or ΔUl0-H7 developed slight respiratory symptoms for a few days, whereas the other animals remained completely healthy (Table 1). Morbidity rates and clinical scores were significantly higher in animals infected with wild-type ILTV A489 or with the rescue mutant virus ILTV Ul0R (Table 1 and Fig. 4A). Tracheal swabs were taken from all animals at days 3, 4 and 5 after infection to detect virus shedding. Whereas ILTV A489 and Ul0R were isolated from most animals of the respective groups, ILTV ΔUl0 was re-isolated from only 2 of 9 animals (Table 1). This finding indicates a correlation between the efficiency of in vivo replication and virulence. Attenuation of Ul0 deletion mutants of ILTV has also been confirmed by safety tests performed by intratracheal administration of 10^5 p.f.u. of virus to 10-day-old chickens. In these studies, all animals tested survived infection with ILTV ΔUl0, whereas in chickens infected with virulent ILTV A489 the mortality rate was 49% (J. Claessens & W. Fuchs, European patent no. EP1241177).

Although most of the animals immunized with UL0-negative ILTV exhibited neither clinical signs nor virus shedding, many of these chickens produced ILTV-specific antibodies at levels detectable by IIF assay (Table 1). To test whether the immune response was sufficient to confer protection against subsequent wild-type virus infection, vaccinated chickens and non-vaccinated control animals were challenged intratracheally with high doses (2 × 10^5 p.f.u. per animal) of ILTV A489 at day 25 after immunization. As expected, all non-vaccinated chickens developed severe symptoms of ILT, shed high titres of virus and 2 of 8 animals died within 10 days after infection (Table 1 and Fig. 4B). In contrast, the chickens that had been immunized previously with virulent ILTV A489 or Ul0R showed no clinical signs and no challenge virus shedding could be detected. Most of the animals vaccinated with the Ul0 deletion mutants ILTV ΔUl0 or ΔUl0-H7 were also completely protected (Table 1 and Fig. 4B). Only 1 of 6 animals of each of the two groups shed low titres of challenge virus and exhibited mild respiratory symptoms. Thus, Ul0 deletion mutants, like thymidine kinase-negative ILTV (Schnitzlein et al., 1995), could be used as attenuated live virus vaccines against ILT.

Immunization with an ILTV UL0 deletion mutant expressing influenza virus HA (H7) protects chickens from fowl plague

To generate a vector vaccine against one group of fowl plague viruses, we have previously used the Ul50 gene locus of ILTV for insertion and expression of an AIV HA gene, subtype H5 (Lüschoh et al., 2001). Since fowl plague is also caused by influenza viruses possessing HA genes of subtype H7, a second ILTV recombinant containing the respective ORF of the highly pathogenic AIV isolate A/chicken/Italy/...
445/99 (H7N1) was constructed. However, considering the insufficient attenuation of ILTV UL50 mutants (Fuchs et al., 2000; Lüscho et al., 2001), the foreign gene was inserted at the UL0 gene locus. In ILTV ΔUL0-H7 (Fig. 1), the HA gene is preceded by a strong heterologous promoter (PHCMV-IE), and the endogenous polyadenylation signal of the co-terminally transcribed UL0, UL1 and UL2 genes (Fig. 1) (Fuchs & Mettenleiter, 1996) permits processing of the 3' end of the HA mRNA. Abundant HA expression was shown by IIF and Western blot analyses of infected CEK cells (Figs 5 and 6). After incubation with an AIV (H7N7)-specific chicken antiseraum (α-AIV) or a mAb against glycoprotein J of ILTV (α-gJ) was detected with fluorescein-conjugated secondary antibodies. Cell nuclei were counterstained with propidium iodide.

Fig. 5. For indirect immunofluorescence tests, CEK cells were fixed 24 h after infection with ILTV A489, ILTV ΔUL0-H7 or AIV A/Chicken/Italy/445/99 (H7N1). Binding of an AIV-specific chicken antiserum (α-AIV) or a mAb against glycoprotein J of ILTV (α-gJ) was detected with fluorescein-conjugated secondary antibodies. Cell nuclei were counterstained with propidium iodide.

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Abundant HA expression was shown by IIF and Western blot analyses of infected CEK cells (Figs 5 and 6). After incubation with an AIV (H7N7)-specific chicken antiserum, a pronounced cytoplasmic fluorescence was detectable in cells infected with either AIV A/chicken/Italy/445/99 (H7N1) or ILTV ΔUL0-H7 but not in cells infected with wild-type ILTV A489 (Fig. 5, left panels). As expected, a mAb against glycoprotein J of ILTV (Veits et al., 2003a) reacted with cells infected with ILTV A489 or ΔUL0-H7 but not with AIV-infected cells (Fig. 5, right panels). In Western blot analyses of infected cell lysates, the AIV-specific chicken serum detected two proteins (ca. 48 and 26 kDa) of ILTV ΔUL0-H7, which were not expressed by ILTV A489 but which correspond to proteins found in AIV-infected cells (Fig. 6A). Presumably, these proteins represent HA1 and HA2, which are generated by protease cleavage during the processing of influenza virus HA (Lamb & Krug, 2001). Similar to the situation that occurs in other highly pathogenic AIV strains, the predicted cleavage site at position 343 of the protein investigated is preceded by multiple basic amino acids (KGSRVRR/GLF), which permit efficient processing by ubiquitous cellular proteases (Alexander, 2000). Identical cleavage site sequences were also determined for other H7N1 influenza virus isolates of the fowl plague outbreak in Italy during 1999 and 2000 (Capua & Marangon, 2000). Efficient cleavage might explain that no HA proteins exhibiting the apparent mass of the primary translation product (62-7 kDa) or larger glycosylated precursor proteins were detectable in cells infected with ILTV ΔUL0-H7. A third AIV-specific protein of ILTV ΔUL0-H7 (ca. 42 kDa) (Fig. 6A) does not resemble any of the authentic AIV gene products and might result from either incomplete glycosylation or degradation of HA1. A control blot was incubated with an ILTV-specific mAb (Veits et al., 2003a) that detected comparable amounts of
glycoprotein C in cells infected with ILTV ΔUL0-H7 or A489 (Fig. 6B).

To analyse the immunogenicity of the influenza virus HA protein expressed by ILTV ΔUL0-H7, the sera of immunized chickens were analysed by HAI tests. These tests revealed that after 2 weeks, HA-specific serum antibodies were present at moderate titres in all animals that had been infected with ILTV ΔUL0-H7, whereas the sera of chickens infected with ILTV ΔUL0 remained negative (Table 1). After 25 days, immunized animals of these two groups were challenged with a lethal dose of the highly pathogenic AIV isolate A/chicken/Italy/445/99 (H7N1), which had been the donor of the HA gene expressed in ILTV ΔUL0-H7. As expected, the chickens immunized with ILTV ΔUL0 were not protected, developed signs of fowl plague (depression and diarrhoea) and died within 5 days (Fig. 4C and Table 1). After dissection, oedema of the head, multifocal cutaneous cyanosis, congestion in spleen and kidneys, as well as subepicardial haemorrhage, non-suppurative myocarditis and necrotizing pancreatitis were observed (results not shown). In contrast, all chickens that had been immunized with ILTV ΔUL0-H7 survived the AIV challenge and did not develop serious disease, and no significant lesions were found at necropsy in four animals investigated at days 3 and 6 after challenge (Table 1). However, since all animals immunized with ILTV ΔUL0-H7 were somewhat apathetic for several days after challenge (Fig. 4C), and since a few of the animals exhibited moderate respiratory symptoms, a total clinical score of 0·64 was determined (Table 1). At 3 days after challenge, shed AIV could be re-isolated from tracheal and/or cloacal swabs of 75% of the animals (Table 1) but at later time-points the virus was apparently eliminated.

Although it remains to be tested to what extent ILTV ΔUL0-H7 is efficacious against heterologous fowl plague viruses of the H7 subtype, like that of the recent outbreak in The Netherlands (Abbott, 2003), our studies demonstrate that a single live virus vaccination with this recombinant ILTV protected chickens from a lethal infection with homologous AIV and from severe symptoms of fowl plague. Virus shedding and the minor clinical signs observed after challenge infection might be reduced further by repeated immunization or by administration of higher doses of ILTV ΔUL0-H7. On the other hand, it should be mentioned that inactivated AIV, as well as other vector, subunit and DNA vaccines, did not completely prevent shedding of highly pathogenic AIV strains of the H5 or H7 subtypes (Crawford et al., 1999; Kodihalli et al., 2000; Swayne et al., 1999, 2000, 2001). Apparently, the efficacy of inactivated AIV and of recombinant vaccines containing only the HA gene or protein is comparable (Easterday et al., 1997). However, the use of recombinant HA vaccines would facilitate serological discrimination between immunized and field virus-infected animals by diagnostic tests for antibodies against other AIV proteins and might permit a relaxation of the present restrictions on vaccination against fowl plague. When compared to inactivated AIV, as well as to subunit, DNA or fowlpox vector vaccines, ILTV live virus vaccines have the additional advantage that they are suitable for mass application, not only by eye drop but also by aerosol or drinking water (Bagust & Guy, 1997). The only disadvantage of the vector vaccine presented might be that the narrow host range of ILTV permits efficient replication only in chickens but not in turkeys and ducks, which are also infected frequently with AIV (Bagust & Guy, 1997; Alexander, 2000).

However, since chickens are among the most economically important domestic animals, we started to generate further ILTV recombinants containing foreign gene insertions at the UL0 gene locus, which apparently confers a better attenuation than the UL50 gene locus used previously (Fuchs et al., 2000; Lüschof et al., 2001). These insertions include not only the HA gene of highly pathogenic AIV of the H5 subtype but also genes encoding immunogenic proteins of other chicken pathogens, such as Newcastle disease virus and Marek’s disease virus.

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REFERENCES


