In vitro and in vivo characterization of glycoprotein C-deleted infectious laryngotracheitis virus

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Infectious laryngotracheitis is an important respiratory disease of chickens that is caused by an alphaherpesvirus [infectious laryngotracheitis virus (ILTV); Gallid herpesvirus 1]. As herpesvirus envelope glycoproteins are main targets of the humoral host immune response, they are of particular interest for development of vaccines, as well as of diagnostic tools. The conserved, N-glycosylated envelope protein gC has been identified as a major surface antigen of ILTV. To study the function of gC, we now isolated a gC-deleted ILTV recombinant as well as a gC rescuant after co-transfection of permissive chicken cells with virion DNA and transfer plasmids containing engineered subgenomic fragments. Like other alphaherpesvirus homologues, ILTV gC proved to be non-essential for replication. ILTV-ΔgC exhibited delayed penetration kinetics and slightly reduced plaque sizes in cultured chicken cells, whereas virus titres were not reduced significantly compared with wild-type or gC-rescued virus. In vivo studies revealed that ILTV-ΔgC is attenuated in chickens. However, infection with high doses of ILTV-ΔgC was still fatal for approximately 20% of the animals, whereas wild-type or gC-rescued ILTV led to 50% mortality. Interestingly, innate and specific immune responses against ILTV-ΔgC were not reduced but enhanced, and surviving chickens were protected completely against challenge infection. Furthermore, ILTV-ΔgC might serve as a basis for marker vaccines permitting differentiation between vaccinated and field-virus-infected animals, as gC-specific antibodies could be detected easily in sera of animals infected with wild-type ILTV.

INTRODUCTION

Infectious laryngotracheitis (ILT) is a worldwide-occurring and economically important respiratory disease of chickens caused by infectious laryngotracheitis virus (ILTV), also designated Gallid herpesvirus 1. After primary infection, an incubation period of 3–12 days is followed by an acute phase lasting 1–2 weeks. During this time, the virus replicates mainly in trachea, larynx and conjunctiva, leading to respiratory disorders such as gasping, coughing, expectoration of bloody mucus and, less frequently, conjunctivitis. ILTV infection further results in reduced weight gain and egg production, and induces mortality rates between 0 and 80% depending on strain virulence (reviewed by Guy & Garcia, 2008). ILTV establishes lifelong latency in sensory neurons of surviving animals, and the virus can be reactivated by various stress factors with subsequent virus shedding (Williams et al., 1992). For prevention of ILT, conventionally attenuated live-virus vaccines are in use, which are suitable for mass application, but mostly possess significant residual virulence, which might increase after animal passage (Andreasen et al., 1990; Guy et al., 1991; Guy & Garcia, 2008; Kotiw et al., 1995). To overcome this problem, stably attenuated ILTV recombinants have been generated by targeted deletion of non-essential genes (reviewed by Fuchs et al., 2007).

Gallid herpesvirus 1 (ILTV) has been classified as the prototype member of the genus Iltovirus of the subfamily Alphaherpesvirinae of the family Herpesviridae (Davison et al., 2009). Gene content and arrangement within the approximately 150 kbp ILTV genome are similar to those in other alphaherpesvirus genomes, and designations of many open reading frames (ORFs) and several proteins have been adopted from herpes simplex virus type 1 (HSV-1) (McGeoch et al., 1988; Roizman et al., 2007; Thureen & Keeler, 2006). However, ILTV and the related parrot pathogen psittacid herpesvirus 1 (Thureen & Keeler, 2006) exhibit a considerable phylogenetic distance from other
avian and mammalian alphaherpesviruses (Johnson & Tyack, 1995; McGeoch et al., 2000), which correlates with the presence of several itlovirus-specific proteins, the translocation of the conserved UL47 gene from the U5 region to the U3 region and, compared with most other alphaherpesvirus genomes, the inversion of a conserved gene cluster within the U5 region ranging from UL22 to UL44 (Veits et al., 2003a; Wild et al., 1996; Ziemann et al., 1998a, b).

ILTV is predicted to encode 12 glycoproteins conserved within alphaherpesviruses (Fig. 1a), of which up to now only gB (Poulsen & Keeler, 1997), gC (Kingsley et al., 1994), gG (Kongsuwan et al., 1993), gI (Fuchs et al., 2005), gM and gN (Fuchs & Mettenleiter, 2005) homologues have been identified at the protein level. gG, gI, gM and gN were shown to be non-essential for virus replication in cell culture, and gG- as well as gI-deleted ILTV were shown to be attenuated in chickens (Devlin et al., 2006; Fuchs & Mettenleiter, 2005; Fuchs et al., 2005).

The gC homologues of several alphaherpesviruses, including HSV-1, varicella-zoster virus (VZV), pseudorabies virus (PrV), bovine herpesvirus 1 (BoHV-1) and equine herpesvirus 1 (EHV-1), are also dispensable in vitro, although they are involved in primary virus attachment to heparan sulfate or chondroitin sulfate moieties of proteoglycans on the host-cell surface (Cohen & Seidel, 1994; Mettenleiter et al., 1990; Okazaki et al., 1991; Osterrieder, 1999; Robbins et al., 1986; Spear et al., 1992), which facilitates subsequent binding to specific receptors (Spear et al., 2000). However, the presence of proteoglycans at the cell surface is not essential for infection with HSV-1 or PrV (Gruenheid et al., 1993; Karger et al., 1995). Besides their function during entry, gC proteins of PrV and EHV-1 have also been shown to be involved in viral egress (Osterrieder, 1999; Schreurs et al., 1988). Furthermore, gC proteins of EHV-1, HSV-1, PrV and VZV have been identified as virulence factors (Mettenleiter et al., 1988; Moffat et al., 1998; Osterrieder, 1999), and an immune-evasion function mediated by gC binding to complement component C3b has been described for several alphaherpesviruses (Friedman, 2003; Friedman et al., 1984; Huemer et al., 1993). The chicken pathogen Marek’s disease virus (MDV) also expresses a gC homologue, which was shown to be dispensable or even detrimental for replication in cell culture (Tischer et al., 2005). Although the tight cell association of MDV in vitro hampers investigation of entry or egress functions of gC, recent studies indicated that gC, together with other virus proteins, is relevant for horizontal spread of MDV in vivo (Jarosinski et al., 2007).

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**Fig. 1.** Construction of virus recombinants. (a) Map of the ILTV genome containing unique (U1, U2) and inverted repeat (IRs, TRs) sequences. KpnI restriction fragments and conserved glycoprotein genes are indicated. (b) Enlarged section of the gC gene (UL44) region, showing the positions of viral transcripts (dotted arrows) and ORFs (pointed rectangles). (c) In ILTV-ΔgCG, codons 1–367 of UL44 were replaced by an expression cassette encoding enhanced green fluorescent protein (EGFP) flanked by the human cytomegalovirus immediate-early promoter (P-HCMV) and the simian virus 40 polyadenylation signal (SV40-A⁺), which was subsequently deleted to obtain ILTV-ΔgG. Relevant restriction sites, as well as designations of transfer plasmids and of ILTV mutants (in italics), are indicated.
The ILTV gC gene encodes a 414 aa protein containing an N-terminal hydrophobic signal sequence, five potential N-glycosylation sites and a transmembrane domain close to the C terminus. ILTV gC is an abundant virion component, as identified by the use of monospecific antisera and monoclonal antibodies (mAbs) (Kingsley et al., 1994; Veits et al., 2003c). Interestingly, it lacks a positively charged region within its N-terminal part that is conserved in other alphaherpesvirus homologues and considered to be responsible for interaction with cell-surface proteoglycans (Cardin & Weintraub, 1989; Kingsley et al., 1994). Consistently, adsorption and penetration of ILTV in vitro were not affected significantly by heparinase treatment or pre-incubation with soluble heparin or chondroitin (Kingsley & Keeler, 1999).

To investigate the functions of ILTV gC in more detail, we deleted the gC gene of a virulent ILTV strain. Replication of the deletion mutant and a corresponding rescuant in cell culture, as well as virulence and immunogenicity in chickens, were compared with the properties of the parental wild-type virus.

RESULTS

Genome structure and protein expression of ILTV recombinants

For functional analyses of the gC gene, deletion mutant ILTV-ΔgC (Fig. 1c) and rescuant ILTV-gCR (Fig. 1b) were isolated. ILTV-ΔgC exhibits an almost-complete deletion of the gC ORF, UL44 (codons 1–367 of 415), whereas the adjacent ORFs UL43 and UL21 were not affected. Expression of gC was analysed by Western blot (Fig. 2a) and indirect immunofluorescence (IIF) (Fig. 2b) analyses. As expected, a gC-specific mAb (Veits et al., 2003c) detected its 60 kDa target protein in the cytoplasm of chicken embryo kidney (CEK) cells infected with wild-type ILTV-A489 or ILTV-gCR, but not in cells infected with ILTV-ΔgC (Fig. 2, upper panels). Reactions of a monospecific antiserum against pUL47 of ILTV (Helferich et al., 2007a) are shown as controls (Fig. 2, lower panels).

In vitro replication of gC-deleted ILTV

Isolation and propagation of gC-deleted ILTV in non-complementing chicken hepatoma (LMH) and CEK cells revealed that gC is not essential for productive in vitro replication. However, plaque diameters of ILTV-ΔgC on CEK cells were slightly, but statistically significantly (P ≤ 0.003), reduced to approximately 80% of the sizes of wild-type and rescued viruses (Fig. 3a). In contrast, growth kinetics of ILTV-ΔgC were similar to those of ILTV-A489 and ILTV-gCR (Fig. 3b), and final titres were not statistically significantly different (P ≥ 0.167). Separate kinetic analyses of intracellular and extracellular virus titres also did not reveal differences between ILTV-A489, ILTV-ΔgC and ILTV-gCR (results not shown). Thus, ILTV gC is obviously not relevant for virion maturation or virus release, but may be involved in cell-to-cell spread.

Functions of ILTV gC during virus entry

Whilst gC of many alphaherpesviruses is involved in primary attachment of virions to heparan or chondroitin sulfate moieties of proteoglycans at the cell surface (Mettenleiter et al., 1990; Okazaki et al., 1991; Osterrieder, 1999; Spear et al., 1992), different results have been obtained for ILTV (Kingsley et al., 1994; Kingsley & Keeler, 1999). We were now able to verify this by parallel investigations of gC-negative and gC-positive ILTV, as well as of corresponding PrV recombinants (Fig. 4a). Plating efficiency of gC-positive PrV on rabbit kidney (RK13) cells was reduced by approximately 100-fold after virus adsorption in the presence of high concentrations of soluble heparin or chondroitin sulfate, whereas these effects were much less pronounced for gC-deleted PrV (Fig. 4a). In contrast, heparin had no, and chondroitin sulfate only...
minor, effects on wild-type or gC-deleted ILTV on CEK cells (Fig. 4a). To test whether ILTV gC is at all involved in virus entry, the kinetics of virus adsorption to CEK cells at 4°C and of penetration at 37°C were investigated for ILTV-A489, ILTV-gC and ILTV-gCR. Whilst no differences were observed between adsorption kinetics of the three viruses (not shown), penetration of gC-deleted ILTV was delayed slightly compared with the wild-type and gC-rescued viruses (Fig. 4b). After 10–30 min at 37°C, penetration rates of ILTV-gC were statistically significantly lower than those of ILTV-A489 and ILTV-gCR (P<0.05).

**Virulence and protective efficacy of ILTV-ΔgC in chickens**

To investigate the suitability of gC-deleted ILTV for vaccine use, 10-week-old chickens were infected with 10^5 p.f.u. ILTV-A489, ILTV-ΔgC or ILTV-gCR per animal by intratracheal and ocular administration. Animals were observed daily for clinical symptoms, which were scored as described previously (Helferich et al., 2007b). A table containing the clinical scores of all animals is provided as Supplementary Table S1 (available in JGV Online). Two days after infection, the chickens infected with wild-type or revertant ILTV developed typical signs of ILT, such as gasping, coughing, expectoration of bloody mucus and
conjunctivitis. The symptoms were most severe between 4 and 5 days after infection (p.i.) (Fig. 5a) and total clinical scores of 1.91 or 1.98 were determined (Table 1). Approximately 50% of the animals of both groups died between 3 and 6 days p.i. Most of the chickens infected with ILTV-ΔgC also developed respiratory symptoms, but they were less severe and appeared 1–2 days later than in the other groups (Fig. 5a; Supplementary Table S1). A total clinical score of only 0.97 and a reduced mortality rate of 21% (Table 1) indicated moderate attenuation of ILTV-ΔgC. Due to the size of the animal groups, the differences between gC-deleted and wild-type or gC-rescued ILTV were not statistically significant with respect to total morbidity (P=0.241) or mortality (P≥0.118 in one-sided tests) rates. However, the severity of disease, as determined by maximum clinical scores of all animals, was statistically significantly lower in the group infected with ILTV-ΔgC than in chickens infected with ILTV-A489 (P=0.016) or ILTV-gCR (P=0.011), whereas no statistically significant differences were observed between the latter two groups (P=0.839). Comparison of mean clinical scores led to similar results.

Infectious ILTV could be reisolated from tracheal swabs of most animals of all groups taken on days 3–5 p.i. However, titres of ILTV-ΔgC were lower than those of ILTV-gCR and ILTV-A489 (Table 1). In animals necropsied on days 4 and 5 p.i. with ILTV-A489 or ILTV-gCR, severe haemorrhages were found in larynx and trachea, whereas animals infected with the deletion mutant ILTV-ΔgC exhibited only a moderate serous inflammation of these organs. Histopathology revealed complete necrosis and desquamation of the tracheal mucosa and a severe heterophilic/necrotic bronchopneumonia in animals infected with wild-type or gC-rescued ILTV (see Supplementary Fig. S1, available in JGV Online). In contrast, chickens infected with ILTV-ΔgC showed fibrinous exudates with a moderate amount of heterophils and erythrocytes in the lumen of the trachea, but only minor necrosis of tracheal epithelium. Viral proteins could be detected by immunohistochemistry in the tracheae of chickens infected with either virus, but only ILTV-gCR and ILTV-A489 were also found in the lungs of most infected animals (Supplementary Fig. S1).

After 4 weeks, all surviving chickens, as well as naive control animals, were challenged with 10⁷ p.f.u. virulent ILTV-A489 and observed for clinical signs (Fig. 5b). As expected, all non-immunized animals developed severe respiratory symptoms and conjunctivitis, leading to a mean clinical score of 1.71, and 27% of the controls died between 3 and 6 days after challenge (p.ch.) (Table 1). In contrast, all previously infected animals survived challenge infection (Table 1) and, in all immunized groups, the severity of clinical signs was statistically significantly lower than in non-immunized controls (P<0.002). However, several chickens still exhibited mild chronic respiratory distress after primary infection with ILTV-A489 or ILTV-gCR (Fig. 5b), resulting in mean clinical scores of 0.35 or 0.04, respectively (Table 1). In contrast, the animals previously immunized with ILTV-ΔgC had recovered completely before challenge, and neither clinical symptoms nor pathological alterations in necropsied chickens were observed after challenge infection (Fig. 5b; Table 1). Whilst all control animals shed considerable amounts of challenge virus, which could be reisolated from tracheal swabs taken on days 3, 4 and 5 p.ch., no infectious virus was detected in chickens previously immunized with ILTV-ΔgC, ILTV-gCR or ILTV-A489 (Table 1).

**Host immune response against gC-negative and gC-positive ILTV**

The ILTV-specific antibody response was analysed by IIF tests of chicken sera prepared before and 21 days p.i., as well as 17 days p.ch. As expected, the sera of naive animals did not react with ILTV-infected chicken cells, whereas the sera of all convalescent animals were positive after infection.
with either virus (Table 1). Further, all sera taken 21 days p.i. and 17 days p.ch. were shown to contain antibodies against the highly immunogenic ILTV glycoprotein gC by IIF tests on cells transfected with a corresponding expression plasmid. Sera of chickens infected with ILTV-gC or wild-type ILTV-A489 also reacted with cells transfected with a gC expression plasmid, whereas the sera of animals infected with ILTV-ArgC were negative. Interestingly, the sera of the animals immunized with ILTV-ArgC prepared 21 days p.i. and 17 days p.ch. exhibited unchanged reaction patterns, indicating that replication of the gC-positive challenge virus was not sufficient to induce detectable seroconversion (Table 1).

Blood samples of three chickens each infected with either ILTV-ArgC or wild-type ILTV-A489 were taken before and at 11 different times p.i. and p.ch., and lymphocyte populations were investigated by flow cytometry, which revealed several considerable differences (Fig. 6). Whilst the numbers of antigen-presenting cells, such as macrophages and monocytes, decreased after wild-type infection, increased blood concentrations of these cell types were observed during the first 7 days after infection with ILTV-ArgC (Fig. 6d). In contrast, the amounts of B cells (Fig. 6c), as well as of CD4- and CD8-positive T lymphocytes (Fig. 6a, b), were similar at early times after infection, but from day 21 p.i. increased to higher levels in chickens infected with ILTV-ArgC than in wild-type-infected animals. Consistently, challenge infection induced faster and more pronounced lymphocyte proliferation in chickens previously immunized with ILTV-ArgC (Fig. 6a–c). The antigen specificity of these lymphocytes remains to be investigated.

**DISCUSSION**

The present study describes the first ILTV recombinant lacking the conserved alphaherpesvirus glycoprotein gC. As in all other hitherto-investigated members of the *Alphaherpesvirinae* (Cohen & Seidel, 1994; Mettenleiter, 1986; Tischer et al., 1991; Osterrieder, 1999; Robbins et al., 1986; Tischer et al., 2005), gC proved to be non-essential for *in vitro* replication of ILTV. Although gC-deleted ILTV exhibited slightly delayed penetration kinetics and smaller plaques in cultured chicken cells, these defects did not lead to a statistically significant reduction of virus titres compared with wild-type or gC-rescued ILTV.

 Whilst the gC homologues of several alphaherpesviruses are involved in virus attachment by binding to heparan sulfate- or chondroitin sulfate-containing proteoglycans at the host-cell plasma membrane (Spear et al., 2000), ILTV gC obviously is not. This finding is in agreement with the absence of a positively charged region in the ectodomain of ILTV gC, which is conserved and required for heparin interaction in other gC homologues (Kingsley et al., 1994). Moreover, the present study revealed that infectivity of either gC-deleted nor wild-type ILTV is inhibited by soluble heparin or chondroitin sulfate, indicating that no other viral protein has adopted the binding function of gC. This confirmed an earlier publication that already postulated a heparin-independent entry pathway for ILTV.

**Table 1. Summary of animal experiments**

For morbidity, mean clinical scores (0, healthy; 1, slightly ill; 2, ill; 3, severely ill; 4, dead) of all animals of the group over 10 days after primary infection (p.i.) or after challenge (p.ch.) are shown in parentheses. ILTV shedding was determined by virus reisolation from tracheal swabs; mean virus titres of all animals of the group are given in parentheses. ILTV-specific antibodies were detected by IIF tests on ILTV-infected CEK cells; gC-specific antibodies were detected by IIF tests on LMH cells transfected with pRc-IgC; gJ-specific antibodies were detected by IIF tests on LMH cells transfected with pRc-IgJ. Note that different animal numbers in the same columns are due to death or exclusion of chickens from certain tests (see main text). NT, Not tested.

<table>
<thead>
<tr>
<th>Time (days p.i./ p.ch.)</th>
<th>ILTV-ArgC</th>
<th>ILTV-gC</th>
<th>ILTV-A489</th>
<th>Controls</th>
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<tbody>
<tr>
<td>Primary infection (10⁵ p.f.u per animal)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Mortality (%)</td>
<td>3–9</td>
<td>3/14 (21)</td>
<td>7/14 (50)</td>
<td>6/14 (43)</td>
</tr>
<tr>
<td>Morbidity (clinical score)</td>
<td>1–10</td>
<td>12/14 (0.97)</td>
<td>14/14 (1.98)</td>
<td>14/14 (1.91)</td>
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<tr>
<td>ILTV shedding (p.f.u. ml⁻¹)</td>
<td>3–5</td>
<td>13/14 (3.9 × 10⁵)</td>
<td>12/12 (1.4 × 10⁵)</td>
<td>13/13 (3.7 × 10⁵)</td>
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<tr>
<td>gl-Ab specific Abs</td>
<td>21</td>
<td>14/14</td>
<td>7/7</td>
<td>11/11</td>
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<tr>
<td>Challenge infection with ILTV-A489 (10⁵ p.f.u. per animal, 28 days p.i.)</td>
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<tr>
<td>Mortality (%)</td>
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<td>0/5</td>
<td>0/6</td>
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<tr>
<td>Morbidity (clinical score)</td>
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<td>4/6 (0.35)</td>
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<td>0/5</td>
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<td>0/12</td>
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<tr>
<td>gl-Ab specific Abs</td>
<td>17</td>
<td>12/12</td>
<td>5/5</td>
<td>9/9</td>
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(Kingsley & Keeler, 1999). Future studies will be required to determine whether ILTV uses alternative receptors for primary attachment or simply omits a corresponding step, which, at least for HSV-1 and PrV, seems to be dispensable for successful infection (Gruenheid et al., 1993; Karger et al., 1995).

However, the delay in penetration of gC-negative ILTV indicates an accessory function of this glycoprotein during virus entry. Delayed penetration independent of primary attachment to proteoglycans has also been described for gC-deletion mutants of PrV (Mettenleiter, 1989; Rue & Ryan, 2003). The penetration defect of gC-deleted ILTV might explain its reduced plaque sizes and, therefore, the present study provides no unequivocal evidence for functions of gC during virion maturation or egress, as have been shown for EHV-1 and PrV (Osterrieder, 1999; Schreurs et al., 1988).

Because of problems with the currently used conventionally attenuated ILT live-virus vaccines (Guy & Garcia, 2008), gC-deleted ILTV, like other genetically engineered ILT recombinants (reviewed by Fuchs et al., 2007), was tested for its suitability as a novel vaccine. After experimental intratracheal and ocular infection of chickens with gC-deleted ILTV, severity of clinical symptoms, mortality rate and quantity of virus shedding were reduced considerably compared with wild-type and rescued virus. However, residual virulence of ILTV-ΔgC was significant. Thus, the minor in vitro growth defects of gC-negative ILTV correlated with a moderate attenuation in vivo. Insufficient attenuation in animals has been also reported for gC single-deletion mutants of BHV-1 and PrV (Kaashoek et al., 1998; Mettenleiter et al., 1988).

Although gC has been identified as one of the major antibody-inducing proteins of ILTV (Veits et al., 2003c), immunogenicity was not reduced detectably in the absence of gC. Chickens immunized with ILTV-ΔgC were protected equally well against challenge with virulent ILTV as animals that had survived primary infection with wild-type or gC-rescued viruses, and the absence of challenge virus shedding might indicate induction of sterile immunity. These findings are in line with the hypothesis that cell-mediated immune responses against hitherto-unknown proteins are crucial for protection against ILTV infection (Fahey & York, 1990; Guy & Garcia, 2008).

Interestingly, quantitative analyses of blood lymphocyte populations of infected chickens revealed that, compared with wild-type virus, ILTV-ΔgC induces increased numbers of potential antigen-presenting cells during the acute phase of infection, and increased B- and T-cell numbers at later times. To some extent, this might be explained by the observation that the general condition of the animals is less affected by the mutant. However, a specific contribution of

Fig. 6. Lymphocyte numbers in chickens infected with ILTV-ΔgC (●) or ILTV-A489 (○). Blood samples were taken at the indicated times after infection. After antibody labelling, CD4-positive (a) and CD8-positive (b) T cells, B cells (c, MHC II⁺/Bu1A⁺) and antigen-presenting cells (d, MHC II⁺/Bu1A⁻) were identified by flow cytometry. The mean ± SD cell numbers (ml blood)⁻¹ of three animals each are shown. The time of challenge infection is indicated by a dotted vertical line.
gC deletion to the enhanced immune response is also conceivable. The gC homologues of several alphaherpesviruses have been shown to bind the central complement component C3/C3b and to inhibit complement activation (Friedman, 2003; Huemer et al., 1993). Activation of complement can lead to antibody-independent lysis or phagocytosis of virus particles (Welsh et al., 1975; Sölder et al., 1989). Furthermore, complement components opsonize the antigens and stimulate macrophages and other cells of the innate and adaptive immune system directly or indirectly (Frank & Fries, 1991). As the complement system plays an important role in defence of bacteria, its inhibition by herpesvirus gC should also facilitate secondary infections. This might explain the protracted respiratory symptoms observed in several animals infected with wild-type or gC-rescued, but not with gC-negative, ILTV. Although complement-binding activity of ILTV gC has not yet been demonstrated directly, the present results suggest that gC possesses an immune-evasion function and that its deletion might improve the efficacy of ILTV live vaccines.

On the other hand, the dispensability of antibodies against major envelope proteins of ILTV for protective immunity should enable their use for serological differentiation between field-virus-infected and vaccinated animals (DIVA; van Oirschot, 1999). In our trial, sera of all chickens immunized with wild-type or gC-rescued ILTV reacted specifically in IIF tests with gC-expressing cells, whereas sera of animals immunized with ILTV-AgC were negative. Corresponding results had been obtained with gJ-deleted or gJ-rescued ILTV and gJ-expressing cells (Fuchs et al., 2005). Unlike ILTV-AgC, the gJ-negative virus was almost avirulent in chickens, but also exhibited significant in vitro replication defects that might interfere with cost-efficient production of a putative vaccine. Furthermore, although ILTV-AgJ protected experimentally infected specific-pathogen-free (SPF) chickens against disease after challenge (Fuchs et al., 2005), the strong attenuation of the mutant might lead to reduced efficacy after mass application to conventional poultry. Most chickens immunized with ILTV-AgJ developed gJ-specific serum antibodies after wild-type challenge, which might indicate limited virus replication (Fuchs et al., 2005). In contrast, animals infected with ILTV-AgC remained negative for gC-specific antibodies, which confirmed efficient inhibition of challenge virus replication.

It remains to be tested whether reduced virus doses or other administration routes reduce the clinical symptoms caused by ILTV-AgC without affecting its protective efficacy. This has been shown for dUTPase-deleted ILTV, which appeared less pathogenic after ocular than after intratracheal immunization (Fuchs et al., 2000; Pavlova et al., 2009). Nevertheless, to obtain a safe vaccine based on ILTV-AgC, additional virulence-determining gene(s) must be deleted. Several ILTV proteins, such as dUTPase, thymidine kinase (Schnitzlein et al., 1995), secreted glycoprotein gG (Devlin et al., 2006), tegument protein pUL47 (Helfrich et al., 2007b) or iltvirus-specific pUL0 (Veits et al., 2003b), have been identified as virulence factors that can be deleted to obtain sufficiently attenuated viruses. Presumably, none of these deletions supports DIVA diagnostics but, combined with deletion of gC, might lead to marker vaccines permitting differentiation of successfully immunized from infected chickens. To facilitate detection of gC-specific serum antibodies, competitive diagnostic tests utilizing available gC-expression constructs and gC-specific mAbs (Veits et al., 2003c) could be established.

**METHODS**

**Viruses and cells.** ILTV recombinants were generated by co-transfection of LMH cells (Kawaguchi et al., 1987) with genomic DNA of virulent ILTV strain A489 (Fuchs & Mettenleiter, 1996) and transfer plasmids. Viruses were propagated further in CEK cells as described previously (Fuchs & Mettenleiter, 1996). The β-galactosidase-expressing gG- or gG/gC-negative PrV mutants PrV-1112 and PrV-8411 (Mettenleiter & Rauh, 1990; Karger et al., 1995) were propagated in the rabbit kidney cell line RK13. 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 p.i. and cells were overlaid with medium containing 6 g methylcellulose l−1.

**Plasmid construction.** The gC ORF, UL44, is localized within the 21 kbp KpnI fragment B of the ILTV genome (Fig. 1a), which had been cloned in pILT-K30 (Ziemann et al., 1998a). To obtain pILT-K30S, a 4928 bp KpnI–Sad fragment of the original insert was subcloned (Fig. 1b). Subsequently, a 1154 bp TsiI–EcoRV fragment containing gC codons 1–367 was deleted (pILT-K30STE; Fig. 1c) or replaced by a 1611 bp fragment of pBl-GFP (Fuchs & Mettenleiter, 1999), which contained an expression cassette for enhanced green fluorescent protein (EGFP; pLT-K30G; Fig. 1c). Non-compatible fragment ends were blunted by treatment with Klenow polymerase (New England Biolabs) prior to ligation.

**Generation of ILTV recombinants.** To facilitate generation of gC-deleted ILTV, LMH cells were first co-transfected (FuGene HD reagent; Roche) with genomic DNA of ILTV A489, pILT-K30SG (Fig. 1c) and transactivator plasmid pRc-UL48, which enhances the infectivity of virion DNA (Fuchs et al., 2000). The autofluorescent deletion mutant ILTV-AgCG was plaque-purified from virus progeny, and DNA of ILTV-AgCG was used for subsequent co-transfections with pRc-UL48 and pLT-K30STE (Fig. 1c) or pLT-K30S (Fig. 1b), respectively, to obtain the non-fluorescent deletion mutant ILTV-AgC and rescue mutant ILTV-gCR. All ILTV recombinants were characterized by Southern blot hybridization of viral DNA and by amplification and sequencing of the gC gene region (results not shown).

**In vitro characterization of ILTV mutants.** For Western blot analyses, CEK cells were infected with ILTV at an m.o.i. of 5, incubated for 24 h at 37 °C and processed as described previously (Pavlova et al., 2009). A mAb against ILTV gC (Veits et al., 2003c) and a rabbit antiserum against ILTV pUL47 (Helfrich et al., 2007a) were used at dilutions of 1:200 and 1:200000, respectively.

For plaque assays, confluent monolayers of CEK cells grown on six-well plates were infected with serial virus dilutions. After 2 h at room temperature, the inoculum was replaced by medium containing 6 g methylcellulose l−1 and incubation was continued at 37 °C for 48 h. The cells were fixed and IIF tests were performed (Pavlova et al., 2009) using ILTV gC- or gl-specific mAbs (Veits et al., 2003c) diluted...
For multi-step growth analyses, CEK cells were infected at an m.o.i. of 0.01 at 4°C and shifted to 37°C after 1 h. After 2 h at 37°C, non-penetrated virus was inactivated by treatment with citric acid (Mettenleiter, 1989). At different times after infection, the cells were harvested together with the supernatants and lysed by freeze–thawing. Progeny virus titres were determined by plaque assays and mean titres of three experiments per virus were calculated.

To assess the effect of soluble glycosaminoglycans on virus adsorption, CEK or RK13 cells were grown to monolayers, incubated for 1 h at 4°C with MEM containing 10% FCS and 50 μg heparin ml⁻¹ (H3393; Sigma), 500 μg chondroitin sulfate ml⁻¹ (C4384; Sigma) or nothing, and infected with serial dilutions of ILTV-A489, ILTV-AgC, PrV-1112 or PrV-8411 in the same medium. After 3 h at 4°C, the cells were washed twice with PBS, overlaid with medium containing methylcellulose and incubated for 48 h at 37°C. ILTV plaques were visualized by IIF tests with gJ-specific mAb, and detection of PrV plaques was facilitated by X-Gal staining (Sanes et al., 1986). For each virus, titres obtained in the absence and presence of soluble heparin or chondroitin sulfate were compared in three independent experiments, and mean percentages were calculated.

To determine penetration kinetics, CEK cells grown in six-well plates were infected with 200 p.f.u. ILTV per well, incubated for 1 h at 4°C and then shifted to 37°C. At different times, non-penetrated virus was inactivated (Mettenleiter, 1989) and plaque assays were performed as described above. Percentages of penetrated virus were calculated by comparison with titres obtained without acid treatment. The mean results of three experiments are shown.

Animal experiments. Three groups of 10-week-old SPF White Leghorn chickens (Lohmann Tierzucht), each group containing 18–21 chickens, were infected by ocular and intrastrachal administration of 10⁵ p.f.u. ILTV-AgC, ILTV-gCR or ILTV-A489. During the following 10 days, the chickens were observed daily for clinical symptoms and classified as healthy (0), slightly ill (1), ill (2), severely ill (3) or dead (4) as described previously (Helfrich et al., 2007b). Mean clinical scores of each group were calculated for each day and for the entire monitoring period. On days 3, 4 and 5 p.i., tracheal swabs were taken and analysed for infectious ILTV by plaque assays on CEK cells as described previously (Pavlова et al., 2009). On days 0 and 21 p.i., sera were collected from all animals and tested for ILTV-specific antibodies by IIF tests on CEK cells infected with ILTV-A489, and for gC- or gJ-specific antibodies by IIF tests on LMH cells that had been fixed 2 days after transfection (FuGene HD reagent; Roche) with expression plasmids pcDNA-IgC or pRc-IgJ (Veits et al., 2009). For each virus, sera were collected on days 3 and 4 p.i. and used for histopathological analyses.

After 4 weeks, all immunized chickens, as well as naive control animals, were challenged by ocular and intrastrachal administration of 10⁵ p.f.u. ILTV-A489 per animal. All chickens were again monitored for clinical symptoms for a period of 10 days and scored as described above. Tracheal swabs were taken on days 3, 4 and 5 p.c.h. and two animals per group were necropsied on day 4 p.c.h. On day 17 p.c.h., all surviving animals were euthanized and dissected, and sera were prepared for antibody detection.

For analysis of lymphocyte populations, whole-blood samples were taken on days 0, 3, 5, 7, 10, 13, 21 and 28 p.i., as well as on days 5, 7, 10 and 17 p.c.h. (33, 35, 38 and 45 days p.i.), from three chickens each primarily infected with ILTV-AgC or ILTV-A489. Because of the additional stress, these animals were not considered for clinical evaluation and quantification of virus shedding. Lymphocytes were purified by Ficoll-gradient centrifugation using LSMTP 1077 (PAA). Cells were stained with specific mAbs (AbD Serotec) and fluorescein isothiocyanate- or phycocerythin-labelled goat anti-mouse IgG (H + L) secondary antibodies (Dianova) in PBS containing 0.1% NaN₃ and 0.1% EDTA. The following mAbs were used: mouse anti-chicken CD4 (clone 2-35), mouse anti-chicken CD8 (clone 11-39), mouse anti-chicken major histocompatibility complex II (clone 21-1A6), mouse anti-chicken Bu1A (clone LL2) and mouse anti-chicken CD45 (clone UM16-6) for differentiation of lymphocytes from thrombocytes. Antigen-presenting cells, which are MHC II-positive and Bu1A-negative, and B cells expressing both markers were differentiated by double staining. Labelled cells were analysed quantitatively in a FACSCalibur flow cytometer using CELLquest software (BD Biosciences).

Statistical analyses. Mean values and SD of virus titres and plaque sizes were calculated, and mean values were compared by Student’s t-tests using Microsoft Excel. Total mortality and morbidity rates of animal groups infected with different ILTV mutants were compared by Fisher’s exact tests (Oyvind Langsrud; http://www.langsrud.com/fisher.htm). Maximum clinical scores of all individuals were compared between groups by using Wilcoxon rank-sum tests (R version 2.8.1.; R Foundation for Statistical Computing; http://www.R-project.org). All tests were performed in a two-sided manner unless otherwise noted. Differences were considered statistically significant if probabilities of the null hypothesis were < 0.05 (P < 0.05).

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