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# Evaluation of immunological responses to a glycoprotein G deficient candidate vaccine strain of infectious laryngotracheitis virus

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ABSTRACT

Infectious laryngotracheitis virus (ILTV), an alphaherpesvirus, causes severe respiratory disease in poultry. Glycoprotein G (gG) is a virulence factor in ILTV. Recent studies have shown that gG-deficient ILTV is an effective attenuated vaccine however the function of ILTV gG is unknown. This study examined the function and *in vivo* relevance of ILTV gG. The results showed that ILTV gG binds to chemokines with high affinity and inhibits leukocyte chemotaxis. Specific-pathogen-free (SPF) chickens infected with gG-deficient virus had altered tracheal leukocyte populations and lower serum antibody levels compared with those infected with the parent virus. The findings suggest that the absence of chemokine-binding activity during infection with gG-deficient ILTV results in altered host immune responses.



## 1. Introduction

Infectious laryngotracheitis virus (ILTV) is an alphaherpesvirus that causes severe and economically significant respiratory disease in poultry worldwide [1,2]. The virus encodes glycoprotein G (gG) [3]. Glycoprotein G is conserved in most alphaherpesviruses and is a virulence factor in ILTV. Previous studies of a gG-deletion mutant of ILTV in specific-pathogen-free (SPF) chickens have shown that vaccination with gG-deficient ILTV prevents disease following subsequent challenge with virulent virus [4,5]. There are a number of potential advantages of using gG-deficient ILTV as a live attenuated vaccine. These include an increased level of safety compared with conventionally attenuated vaccines, and the potential to serologically differentiate between infected and vaccinated birds based on the presence or absence of anti-gG antibodies [4,5].

The function of ILTV gG has not investigated previously. Recent studies of gG from a number of different mammalian alphaherpesviruses have shown that gG functions as a viral chemokine-binding protein (vCKBP) *in vitro* [6–8]. Viral CKBPs form part of a strategy for evading the host immune response and have been identified and studied in some other large DNA viruses, especially poxviruses [9,10]. Glycoprotein G is the only vCKBP to be identified so far in the alphaherpesvirus subfamily [6]. Broad-spectrum chemokine-binding has been demonstrated by gG from a number of mammalian herpesviruses including equine herpesviruses 1 and 3 (EHV-1 and EHV-3), bovine herpesvirus 1 and 5 (BHV-1 and BHV-5) and feline herpesvirus 1 [7,8,11]. However, in some alphaherpesviruses, including herpes simplex viruses 1 and 2 and equine herpesvirus 4, chemokine-binding by gG could not be demonstrated [7]. There is no information regarding the presence of vCKBPs in non-mammalian herpesviruses. The functional significance of the capacity of gG binding to chemokines has not been extensively studied. To date, functional inhibition of leukocyte chemotaxis has only been demonstrated for EHV-1, BHV-1 and BHV-5 gG [7,12]. *In vivo* studies of gG as a vCKBP using gG-deficient viruses have been restricted to investigations of EHV-1 in mouse models of respiratory infection. The effects of EHV-1 gG on viral replication, viral virulence and inflammatory cell responses have varied greatly depending on strain of mouse and dose of virus used [12,13]. Currently there is no information regarding the role of gG as vCKBP in its natural host.

Although no previous studies have evaluated the role of ILTV gG as a vCKBP, deletion of gG from the ILTV genome has been shown to result in significant attenuation during infection of the natural host, and to induce protection against disease following challenge with virulent virus. An increased local inflammatory cell response was seen in the trachea of birds infected with the gG-deficient ILTV mutant, but no effect on viral replication was observed. Reinsertion of gG into the ILTV genome was shown to restore the virulent phenotype [10]. A separate study has also demonstrated that chickens inoculated with gG-deficient ILTV develop a significantly lower concentration of anti-ILTV antibody in serum compared to birds

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**Table 1**
Primers used in this study.

| Primer | Sequence (5′-3′)[a] |
|---|---|
| INSgGfwd | TGAAGAACTAGTATGAGCGGCTTCAGTAACAT |
| INSgGrev | TGGGCGCTGCAGCTAGTGATGGTGATGGTGATG CTGCTGGAGCGTAGAGGG |
| gGfs | CGTCGGGATACCTGATTTCG |
| gGrs | GCTGGTAGGCGTAGATGCCG |
| exgG2f | TTAGAATTCCCGGTACTGGACGG |
| exgG2r | CGCGCGGCCGCTCCCATAGGTAAAG |

[a] Underlined nucleotides indicate restriction endonuclease cleavage sites used for cloning, double underlined nucleotides indicate the sequence encoding a polyhistidine tag in primer INSgGrev.

inoculated with a vaccine strain of ILTV that encodes gG [5,14]. These findings strongly suggest that ILTV gG has an immunomodulatory role *in vivo*.

To examine this hypothesis and better understand the immune response to vaccination with gG-deficient ILTV, this study investigated the vCKBP activity of ILTV gG *in vitro* and during infection of the natural host. The interactions between recombinant baculovirus-expressed ILTV gG and recombinant chemokines were investigated *in vitro* by surface plasmon resonance (SPR) technology. The functional significance of ILTV gG as a vCKBP was investigated by assessing the ability of ILTV gG (derived from the supernatants of ILTV-infected cells) to inhibit avian heterophil chemotaxis. Immune responses to infection with gG-deficient, wildtype, or gG-rescuant ILTV were assessed by analysing leukocyte populations in the tracheal mucosa of inoculated chickens. In addition, concentrations of anti-ILTV antibodies in serum were assessed in birds inoculated with the different viruses.

## 2. Materials and methods

### 2.1. Virus strains and propagation

The construction of a gG-deletion mutant of ILTV (ΔgG ILTV) and a gG-rescuant virus (ΔgGR ILTV) from the virulent CSW-1 strain (wt ILTV) has been described previously [14]. All virus strains were propagated in the chicken hepatoma cell line LMH [15] as previously described [16]. Previous studies have shown that there is no difference in the growth kinetics of these ILTV strains propagated under these conditions [14].

Virus supernatants for Western blotting were obtained from LMH cells infected at a multiplicity of infection of approximately 0.2 plaque forming units per cell. The supernatant was harvested when the cells showed complete cytopathic effect. When supernatants from infected cells were used in chemotaxis assays, detached cells were removed by centrifugation and the supernatant was concentrated 4-fold using Ultra-4 Centrifugal Filter Devices (Amicon) with a nominal 10,000 kDa molecular weight limit.

### 2.2. Western blotting

Polyclonal anti-ILTV gG antibodies for use in Western blotting were generated by the immunisation of rats with recombinant ILTV gG tagged with glutathione-S transferase (gG-GST) produced in *Escherichia coli*. A region of the gG gene, approximately 700 bp in length, was amplified from wt ILTV DNA by PCR using the primers exgG2f and exgG2r. These primers included sites for digestion with *Eco*RI and *Not*I, respectively (Table 1). After restriction endonuclease digestion of the vector and PCR product, the PCR product was ligated into pGEX-4T-1 (GE Healthcare Life Sciences). The sequence was verified using Big Dye Terminator (BDT) Version 3.1 chemistry (ABI PRISM) and the sequencing primers gGfs and gGrs (Table 1). Inclusion bodies containing gG-GST were extracted using Bacterial Protein Extraction Reagent (Thermo Scientific) from *E. coli* cultures (JM109 strain, Promega) transformed with this plasmid. Inclusion body proteins were separated by sodium dodecyl sulfate 5.0–12.5% polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The region of the gel containing the recombinant protein was excised and homogenized. Four rats were each immunised with 200 μl of homogenized gel slice in Freund's complete adjuvant. Serum was collected after twice boosting with 200 μl of the same homogenized gel slice in Freund's incomplete adjuvant. A pool of sera from these rats was diluted 1:1000 in phosphate buffered saline (PBS) containing 2.5% skim milk powder and 0.05% Tween 20 before use in Western blots.

To demonstrate the presence or absence of ILTV gG in cell culture supernatants, supernatants from mock-infected LMH cells, and LMH cells infected with wt ILTV, ΔgG ILTV or ΔgGR ILTV were separated by SDS-PAGE under reducing conditions and the proteins transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon, Millipore). After blocking membranes overnight at 4 °C in 5% skim milk powder in PBS, blots were probed with rat antiserum to ILTV gG for 1 h at room temperature. After three washes in PBS containing 0.05% Tween 20, bound antibody was detected with a 1:1000 dilution of horseradish peroxidase-conjugated (HRP-conjugated) rabbit anti-rat immunoglobulin G (Dako) and visualized using enhanced chemiluminescent (ECL) substrate (Amersham).

### 2.3. Expression and purification of recombinant ILTV gG in a baculovirus system

Recombinant histidine-tagged ILTV gG (gG-His) was generated using the BAC-TO-BAC baculovirus expression system (Life Technologies). The coding region of ILTV gG was amplified from wt ILTV DNA by PCR using the primers INSgGfwd and INSgGrev. These primers included sites for digestion with *Spe*I and *Pst*I, respectively (Table 1). Primer INSgGrev also encoded a polyhistidine tag. After restriction endonuclease digestion of the vector and PCR product, the PCR product was ligated into pFastBac1 (Life Technologies). The sequence was verified using BDT 3.1 chemistry and the sequencing primers gGfs and gGrs (Table 1). The resultant plasmid was used to transform DH10Bac cells (Life Technologies) to generate recombinant bacmid DNA encoding gG-His. Recombinant bacmid DNA was extracted and used to transfect Sf9 insect cells using Cellfectin (Invitrogen) to generate recombinant baculovirus particles.

Proteins in supernatants from Sf9 insect cells infected with recombinant baculovirus were separated by SDS-PAGE, transferred to PVDF membranes and the presence of gG-His was demonstrated by probing the membrane with rat antiserum to ILTV gG as described above. Large-scale cultures (400 ml) of infected Sf9 cells were prepared. Recombinant gG-His was purified from the supernatant of Sf9 cells 72 h after infection using Ni-NTA Agarose (Qiagen) after first dialyzing the supernatant against lysis buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 10 mM imidazole, pH 8.0). The purified protein was subjected to SDS-PAGE and stained with Coomassie blue to determine purity. Western blot was used to confirm the identity of the purified protein. The concentration of purified protein was assessed using the Bradford assay (BioRad).

### 2.4. SPR analysis of protein–protein interactions

Interactions between recombinant ILTV gG-His and recombinant chemokines were investigated *in vitro* using SPR technology. The following recombinant purified human (h) and mouse (m) chemokines were purchased from Peprotech: hCCL1, hCCL2, hCCL3, hCCL4, hCCL5, hCCL7, hCCL8, hCCL11, hCCL14, hCCL15, hCCL18, hCCL19, hCCL20, hCCL21, hCCL22, hCCL23, hCCL24, hCCL26, hCCL27, hCCL28, hCXCL1, hCXCL2, hCXCL3, hCXCL4,