Interleukin-18-mediated enhancement of the protective effect of an infectious laryngotracheitis virus glycoprotein B plasmid DNA vaccine in chickens

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The immunogenicity of an infectious laryngotracheitis virus (ILTV) glycoprotein B (gB) plasmid DNA vaccine and the immunoregulatory activity of chicken interleukin-18 (IL-18) were investigated in a challenge model. Two recombinant plasmids, pcDNA3.1/gB (pgB) and pcDNA3.1/IL-18 (pIL-18), containing gB and IL-18 were constructed. Chickens were intramuscularly administered two immunizations 2 weeks apart, and challenged with the virulent CG strain of ILTV 2 weeks later. All animals vaccinated with pgB alone or with a combination of pgB plus pIL-18 developed a specific anti-ILTV ELISA antibody and splenocyte proliferation response. The ratios of CD4⁺ to CD8⁺ T lymphocytes in chickens immunized with pgB plus pIL-18 were significantly higher than in those immunized with pgB alone. Co-injection of pIL-18 significantly increased the production of gamma interferon and IL-2, indicating that IL-18 enhances the T helper 1-dominant immune response. Challenge experiments showed that the morbidity rate in the pgB group (25 %) was significantly higher than that in the pgB plus pIL-18 group (10 %). The mortality rates in the pgB and pgB plus pIL-18 groups were 10 and 0 %, respectively, and the corresponding protection rates were 60 and 80 %. These results indicate that IL-18 may be an effective adjuvant for an ILTV vaccine.

INTRODUCTION

Infectious laryngotracheitis virus (ILTV) is a herpesvirus that causes an economically important chicken disease. After acute infection of the upper respiratory tract, the virus can establish latency in the central nervous system, and its subsequent reactivation can lead to infection of naive chickens (Fuchs et al., 2007). At present, infectious laryngotracheitis (ILT) is controlled using attenuated live viruses derived either by sequential passage in cell cultures or from embryonated chicken eggs. Current attenuated ILTV vaccines are not satisfactory because they do not provide protection against latent infection and recurrence or even spread of the disease (Dufour-Zavala, 2008). Therefore, there is a need for vaccines with greater efficacy and fewer side effects.

Various strategies have been adopted to develop genetically engineered ILTV vaccines. Okamura et al. (1994) successfully obtained a stable recombinant ILTV expressing LacZ. Schnitzlein et al. (1995) reported the successful construction of a thymidine kinase-negative ILTV mutant. A disadvantage of using recombinant ILTV as a vaccine is that complete prevention of latency may not be achieved. To overcome this problem, subunit preparations containing affinity-purified glycoproteins have been tested as alternatives and found to be successful (York & Fahey, 1991). However, because of the high costs of production and delivery, this vaccine is not suitable for immunization of large chicken flocks. Therefore, recombinant fowlpox virus vaccines against ILT appear more promising and have been shown to protect experimentally immunized chickens against subsequent challenge infections with a virulent ILTV (Sun et al., 2008). However, unlike conventional live ILTV vaccines, these virus recombinants require individual administration and are not suitable for mass vaccination.

DNA-based vaccination offers an attractive alternative; however, it has been recognized that DNA vaccines alone often generate only weak immune responses. Many studies have shown that the immunogenicity of an antigen can be enhanced by cytokines, including interleukin-2 (IL-2), gamma interferon (IFN-γ), IL-6 and IL-18 (Yoon et al., 2006; Zhang et al., 2007; Tang et al., 2008; Yin et al., 2009).
In this study, we evaluated the adjuvant effects of chicken IL-18 in a DNA immunization strategy using ILTV glycoprotein B (gB) as the vaccine antigen. By co-administration of gB with chicken IL-18, we showed that chicken IL-18 enhances the protective effect of gB inducing a Th1 (Th1)-type immune response as well as protective immunity.

**METHODS**

**Virus and experimental animals.** Specific-pathogen-free (SPF) White Leghorn chicken embryos were purchased from Shandong Institute of Poultry Science (Shandong, PR China). Chickens were hatched and housed in an SPF environment at the Laboratory Animal and Resources Facility, Henan Agricultural University. The virulent CG isolate of ILTV was propagated in the choriomeningitis membranes of 10-day-old SPF embryonated chicken eggs, and the membranes were harvested at 168 h post-inoculation. The 50% egg infectious dose (EID$_{50}$) was determined by inoculating serial tenfold dilutions of virus into 10-day-old SPF embryonated chicken eggs.

**DNA vaccine constructs.** A DNA fragment encoding the full-length gB gene was amplified from the DNA of the ILTV CG strain (GenBank accession no. DQ812546) as a template using sense (5′-GAGGAATATCTGGCATACGTTG-3′) and antisense (5′-GGGTTCCTCGAGTTATTCGTCTTC-3′) primers containing EcoRI and XhoI restriction sites (underlined), respectively. The PCR product was cloned into EcoRI/XhoI-cleaved mammalian expression vector pcDNA3.1 (+) (Invitrogen) to construct the recombinant plasmid pcDNA3.1/gB (pgB). The chicken IL-18 gene obtained by RT-PCR from chicken spleenocytes (GenBank accession no. AY775780) was cloned into pcDNA3.1 (+) between the EcoRI and XhoI restriction sites to produce the recombinant plasmid pcDNA3.1/IL-18 (pIL-18). Plasmids pgB and pIL-18 were sequenced to ensure correct insertions. The pgB and pIL-18 constructs were mixing the chosen plasmids prior to injection. Chickens in group 3, 4, 5 and 6 weeks after initial immunization. Sera were stored at –20°C until analysis. Total serum IgG specific for ILTV was assayed in 24-well, flat-bottomed plates. PBMCs isolated as described above were resuspended at 5 × 10$^6$ ml$^{-1}$ and 500 μl PBMCs was added to each well. The test wells were prepared by adding 500 μl ILTV purified by sucrose density gradient centrifugation containing 10$^{10}$ EID$_{50}$. Control wells were prepared by adding 500 μl complete RPMI alone (negative control). After 24 h incubation, culture supernatants were harvested to test for secretion of chicken IL-2, IL-4 and IFN-γ. Commercially available chicken IL-2, IL-4 and IFN-γ sandwich ELISA kits (Biosource) were used according to the manufacturer’s instructions. The concentrations of chicken IL-2, IL-4 and IFN-γ in the samples were determined from the standard curves.

**Cell population analysis.** PBMCs isolated as described above were adjusted to a concentration of 1 × 10$^6$ ml$^{-1}$ with PBS, and 100 μl of the resuspended samples (1 × 10$^6$ cells) was incubated for 1 h at room temperature with the following antibodies (double labelling): mouse anti-chicken CD3–Spectral Red and mouse anti-chicken CD4–PE or mouse anti-chicken CD8a–PE (BD Biosciences Pharmingen). The samples were analysed on a fluorescence-activated cell sorter.

**Virus challenge experiment.** At 15 days after the booster immunization, all of the chickens were challenged intratracheally with 150 EID$_{50}$ virulent ILTV CG strain. For further detection of virus, tracheal swabs were collected every day after challenge and placed in 0.6 ml PBS. The challenged chickens were examined daily for clinical signs, such as coughing, sneezing, ataxia, dyspnoea and death, for 2 weeks. Dead chickens were necropsied to confirm that they had died of ILTV infection. The challenged chickens generally began to show clinical signs from 2–10 days after challenge. Chickens in each group were euthanized at 14 days post-infection. Necropsies were performed immediately post-mortem, and tracheal swabs were collected for further detection of virus.

**Detection of virus in the trachea by PCR.** Virus in the tracheal swabs of the challenged chickens was detected by PCR. Viral DNA extracted using a commercial test kit (QiAmp DNA Mini kit; Qiagen) was subjected to PCR using primers specific for the thymidine kinase gene (forward primer: 5′-AGAACGATCTCCCG- ACGTT-3′; reverse primer: 5′-GGATGGGTGCTTACTCAC-3′). Total protection was defined as negative when virus was detected in the trachea.

**Peripheral blood lymphocyte proliferation assay.** An antigen-specific lymphocyte proliferation assay was performed as described previously (Froebel et al., 1999), with a slight modification. At 2 weeks after the final immunization, blood samples (2.5 ml per chicken) were collected via wing vein puncture in 2.5 ml syringes pre-loaded with 0.2 ml sodium heparin.

**ELISA.** Blood samples were collected by wing vein puncture at 0, 1, 2, 3, 4, 5 and 6 weeks after initial immunization. Sera were stored at –20°C until analysis. Total serum IgG specific for ILTV was measured by indirect ELISA according to a previously described method (Sun et al., 2008).

**Statistical analysis.** All data are presented as means ± SEM. The intergroup differences in humoral and cellular immune responses between groups were assessed using single-factor analysis of variance. The least significant difference test was used for between-group comparisons. P values of <0.05 were considered statistically significant.
RESULTS

Antibody responses to ILTV in chickens

After expression of the DNA constructs had been analysed in vitro (data not shown), chickens were inoculated intramuscularly twice in each quadriceps muscle separately with recombinant DNA plasmids.

The sera collected weekly from each group were assayed and the results are shown in Fig. 1. No specific antibody response to ILTV was induced by immunization of animals in these groups with pcDNA3.1(+) or PBS. The plasmid pgB with or without pIL-18 induced detectable antibodies to ILTV antigen in chickens 1 week after the initial injection. Compared with the chickens in the pcDNA3.1(+) and PBS group, the chickens in the pgB with or without pIL-18 groups showed significantly increased levels of anti-ILTV antibodies with subsequent vaccination (*P<0.05). However, the level of anti-ILTV antibodies in the animals vaccinated with pgB plus pIL-18 was higher than that in the animals vaccinated with pgB alone from week 2 after the first inoculation, although the difference was not significant (P>0.05).

gB-specific T-cell proliferation

To determine whether the T-cell proliferation response to the DNA vaccine encoding the gB gene might be boosted by the chicken IL-18-expressing vector, we examined PBMCs from vaccinated chickens for antigen-specific T-cell proliferation. As shown in Fig. 2, an enhanced T-cell proliferative response to gB was clearly observed in the groups immunized with pgB alone or with pgB plus pIL-18 when stimulated with purified ILTV gB protein, whereas the chickens vaccinated with pcDNA3.1(+) or PBS did not respond to the gB protein (*P<0.05). The level of the T-cell proliferative response in the group immunized with pgB plus pIL-18 was significantly higher than that in the group immunized with pgB alone (P<0.05). The concanavalin A positive-control sample showed a stimulation index of 4–5. These results indicated that higher levels of antigen-specific T-cell proliferative responses could be elicited by immunization with pgB plus pIL-18 than by immunization with pgB alone.

Levels of Th1 and Th2 cytokines

We investigated the effect of pgB with and without co-injection of chicken IL-18 on changes in Th1 and Th2 phenotypes. As shown in Fig. 3, the increase in the production of IFN-γ and IL-2 (Th1-type) after co-injection of pIL-18 was significantly higher than that after injection of pgB alone (P<0.05). In contrast, the IL-4 (Th2-type) level was decreased, but there was no significant difference (P>0.05). This profile of cytokine secretion suggested that chicken IL-18 enhances the induction of immune responses by promoting a Th1-dominant response.

Cell population analysis

Because activated CD4+ and CD8+ T lymphocytes are among the most crucial components of antiviral effectors, we assessed the cell subsets containing CD4+ and CD8+ T lymphocytes in the PBMCs of the vaccinated chickens (Table 1). Flow cytometric analysis of unstimulated cells was used to standardize the background responses, and there was little variation in the non-immunized chickens. The ratios of CD4+ to CD8+ T lymphocytes in the DNA vaccine-vaccinated groups were significantly higher (P<0.01) than in the groups vaccinated with pcDNA3.1(+) or PBS from
chickens was 70 and 75%, respectively, 14 days after challenge. None of the chickens immunized with pgB plus pIL-18 DNA vaccine died after challenge, whereas two of the 20 chickens injected with the pgB DNA vaccine died from the disease. The mortality rates of the groups inoculated with pgB and with pgB plus pIL-18 were significantly different (P<0.05).

To evaluate the level of protective response after challenge, tracheal swab samples were analysed by PCR. The results indicated that 20 and 40% of the birds vaccinated with the pgB plus pIL-18 and pgB plasmids, respectively, were positive for the presence of virus in the trachea. All chickens immunized with control vector pcDNA3.1(+) or PBS tested positive for virus in the PCR test. As shown in Table 3, the PCR results for all birds with clinical signs were positive, and the PCR results for some birds with no clinical signs were also positive.

A bird that did not show any clinical signs and had a negative result for PCR was defined as being protected. The protection rate in the group that was vaccinated with pgB alone (60%) was higher than that of the group administered with the empty vector or PBS (0%). The group vaccinated with the pgB plus pIL-18 DNA vaccine (80%) had the highest protection rate among all the vaccinated groups. Therefore, the protection rates of the pgB plus pIL-18 and pgB plasmids were significantly different (P<0.05), suggesting that plasmid pgB plus pIL-18 offered enhanced resistance against a virulent ILTV challenge.

DISCUSSION

gB is the most highly conserved herpesvirus structural glycoprotein and elicits neutralizing antibodies and cell-mediated immune responses to confer protective immunity against ILTV. Therefore, this protein is considered an important target when designing strategies for controlling ILT. Tong et al. (2001) reported that a recombinant fowlpox virus expressing the gB protein induced an antibody response in vaccinated chickens. Sun et al. (2008) also found that their constructed recombinant fowlpox virus co-expressing the fusion and haemagglutinin–neuraminidase genes of Newcastle disease virus and the gB gene of ILTV protected 100% of the chickens from death and 70% of the chickens from respiratory signs against ILTV challenge. A subunit vaccine made of a

**Table 1. Ratios of CD4+ to CD8+ T lymphocytes**

Data are expressed as means ± SEM. Data within a column with different letters are significantly different (P<0.05).

<table>
<thead>
<tr>
<th>Group</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
<th>Week 5</th>
<th>Week 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>pgB</td>
<td>1.50±0.09</td>
<td>1.88±0.29a</td>
<td>1.85±0.36ab</td>
<td>1.97±0.37ab</td>
<td>1.99±0.30ab</td>
<td>1.95±0.30ab</td>
</tr>
<tr>
<td>pgB+pIL-18</td>
<td>1.47±0.35</td>
<td>1.94±0.31a</td>
<td>1.97±0.22a</td>
<td>2.16±0.18a</td>
<td>2.07±0.05a</td>
<td>2.08±0.05a</td>
</tr>
<tr>
<td>pcDNA3.1</td>
<td>1.48±0.50</td>
<td>1.39±0.09c</td>
<td>1.49±0.04c</td>
<td>1.46±0.25c</td>
<td>1.44±0.10c</td>
<td>1.40±0.10c</td>
</tr>
<tr>
<td>PBS</td>
<td>1.43±0.43</td>
<td>1.38±0.22c</td>
<td>1.35±0.20c</td>
<td>1.41±0.24c</td>
<td>1.36±0.49c</td>
<td>1.36±0.49c</td>
</tr>
</tbody>
</table>

Fig. 3. Levels of cytokine production from PBMCs after gB stimulation in vitro. The test was repeated five times and values are expressed as means ± SEM. Statistically significant differences (P<0.05) are indicated by * (compared with negative control or blank control) or ** (compared with pgB alone).
205 kDa complex containing gB, which was purified from detergent extracts of virus-infected cells by lectin affinity chromatography, protected 100% of chickens against clinical disease and also against virus replication (York & Fahey, 1991). These studies prove that gB is a major protective immunogen of ILTV and is therefore a prime candidate for constructing recombinant vaccines.

DNA vaccinations have been shown to be a promising approach to protect animals and humans against pathogens because of their low cost of production, thermal stability and ability to induce a wide range of cellular and humoral immune responses (Tacket et al., 1999; Gurunathan et al., 2000). However, DNA vaccines induce animals to produce low antibody levels, which partially protect animals from challenge with a lethal dose of pathogen. Cytokines play important roles in the immune and inflammatory responses as indicators and regulators of the immune network (Cohen et al., 1998; Tovey & Lallemand, 2010). Many studies have shown that the immunogenicity of an antigen may be enhanced by recombinant cytokines (Min et al., 2001; Lin et al., 2005). However, the short half-life of recombinant cytokines and the side effects due to repetitive administration are still problematic (Barouch et al., 2004). Previous reports have shown that direct injection of cytokine genes into the

Table 2. Protection rates of different groups challenged by the virulent CG strain of ILTV

<table>
<thead>
<tr>
<th>Group</th>
<th>No. with clinical signs</th>
<th>Morbidity (%)</th>
<th>No. affected†</th>
<th>Mortality (%)‡</th>
<th>Protection rate (%)§</th>
</tr>
</thead>
<tbody>
<tr>
<td>pgB</td>
<td>5/20</td>
<td>25</td>
<td>8/20</td>
<td>2/20 (10)</td>
<td>60</td>
</tr>
<tr>
<td>pgB + pIL-18</td>
<td>2/20†</td>
<td>10†</td>
<td>4/20†</td>
<td>0/20 (0)†</td>
<td>80†</td>
</tr>
<tr>
<td>pDNA3.1(+)</td>
<td>13/20</td>
<td>65</td>
<td>20/20</td>
<td>14/20 (70)</td>
<td>0</td>
</tr>
<tr>
<td>PBS</td>
<td>17/20</td>
<td>85</td>
<td>20/20</td>
<td>15/20 (75)</td>
<td>0</td>
</tr>
</tbody>
</table>

*Morbidity was recorded each day after challenge and is presented as the percentage of chickens with clinical signs in each group.
†The number of chickens affected was determined as the number of PCR-positive birds from the tested chicken tracheal swabs.
‡Detectable ILTV in the tracheal swab samples was also determined by PCR.
§A bird that did not show any clinical signs and had a negative result for PCR was defined as being protected. The protection rate percentage was determined as the number of unaffected chickens/total number of chickens.
||Statistically significant differences (P<0.05) compared with pgB alone.

Table 3. Clinical signs and PCR results of the different groups challenged with the virulent CG strain of ILTV

+ , Positive PCR result; C, bird with clinical signs.
muscles could modulate immune responses (Zhu et al., 2003; Marshall et al., 2006; Yoon et al., 2006; Tang et al., 2008; Yin et al., 2009). IL-18 is a potent IFN-γ-inducing factor and is expressed by a wide range of cells, including various types of immunocompetent cells and non-immune cells. Similar to IL-12, the dominant function of IL-18 is to facilitate Th1 immune responses (Dinarello, 1999). Here, we selected chicken IL-18 as an adjuvant to improve the immunogenicity of an ILTV DNA construct in an ILTV challenge model.

The activation and proliferation of lymphocytes play a critical role in both the humoral and cellular immune responses induced by vaccination. Therefore, we also evaluated whether vaccination with pgB in the presence or absence of a chicken IL-18-expressing plasmid could influence the antigen-specific T-cell proliferation response. Our results showed that the T cells of chickens immunized with pgB alone exhibited a proliferative response. However, the level of proliferative response of T cells in the chickens co-injected with the chicken IL-18-expressing plasmid and pgB was significantly higher than that in the chickens injected with the pgB vaccine alone (P<0.05). This suggested that pIL-18 was able to stimulate T-cell proliferation. Similar results were also reported by Zhu et al. (2003) and Yin et al. (2009), who showed that co-delivery of an IL-18-expressing plasmid and DNA vaccine could enhance the CD8+ CTL response and T-cell proliferative response induced by the DNA vaccine. These data clearly showed that chicken IL-18 is a strong adjuvant that enhances vaccine potency.

In this study, protection against ILTV tracheal challenge was significantly increased by co-injection of pgB and pIL-18 compared with injection of pgB alone. Co-injection of pgB plus pIL-18 caused a significant decrease in the mortality and morbidity rates and the incidence of PCR-positive results for the presence of ILTV in the trachea; furthermore, the protection rate was improved. This protective immunity might be attributed to enhanced cell-mediated immunity, which is interpreted as increased splenocyte proliferation, increased levels of cytokine (IL-2 and IFN-γ) production and increased CD4+ to CD8+ ratios, resulting from the co-injection of pgB with pIL-18. We observed that the co-injection of pgB with pIL-18 induced significant IFN-γ production from PBMCs in vitro; this suggests that IFN-γ might play a critical role in protective immunity. Many studies have shown that IFN-γ has an effect on herpes simplex virus type 1 infection (Cantin et al., 1995; Zhu et al., 2003). IFN-γ plays a direct role in host resistance or induces an antiviral state in lymphocytes and macrophages (Landolfo et al., 1995). It has been shown that cell-mediated immunity to ILTV is also induced and is antigen-specific. It has also been confirmed that cell-mediated immunity to ILTV is believed to be a protective mechanism in ILTV infection.

The Th1-type immune response and IFN-γ are known to be significantly involved in the protective response against ILTV infection. In this study, the profile of cytokine secretion and T-lymphocyte proliferation responses suggested that IL-18 enhances the induction of immune responses by promoting a Th1-dominant response. These findings are consistent with the results of other studies, which used IL-18 plasmids as adjuvants in DNA vaccines and in which IL-18 enhanced the development of Th1-driven antigen-specific T-helper and cytolytic immune responses (Salagianni et al., 2007; Yin et al., 2009). Moreover, the IL-18 expression plasmid has been shown to have a positive effect on the magnitude and breadth of the immune response after successive vaccination, particularly with respect to the generation of significant numbers of antigen-specific CD4+ and CD8+ T cells (Marshall et al., 2006). Therefore, IL-18 appears to be a broadly effective Th1 adjuvant that might be useful in the development of ILTV vaccines. The present study demonstrated that the protection rate was significantly increased by co-injection of pgB and pIL-18 compared with injection of pgB alone when the animals were subjected to a challenge.

The route of DNA inoculation affects the ability to raise protective immunity. Compared with intravenous (wing vein), intramuscular, intratracheal (DNA drops administered to the trachea), subcutaneous (nape), intrabursal (injections just above the chicken’s vent) and intraorbital (DNA drops administered to the eye) routes, oral inoculation is an easy, convenient and economical way to deliver DNA vaccines for chickens. As the acid pH and harsh enzymic environment in the stomach and gastrointestinal tract can damage DNA molecules, oral vaccination has a relatively poor efficacy. DNA vaccines are often administered via intramuscular injection and are not suitable for mass vaccination for chickens. Therefore, application of DNA vaccines as aerosols should be studied in future.

In conclusion, these findings suggest that the co-injection of chicken IL-18 with the DNA product enhanced the immune response and protection. This study also demonstrated that the chicken IL-18 gene has great potential as a molecular adjuvant and is a promising candidate for the prevention of infectious diseases.

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