Article

Preparation of ChIL-2 and IBDV VP2 Fusion Protein by Baculovirus Expression System

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This study aims to produce an effective subunit vaccine against infectious bursal disease virus (IBDV). The genes of chicken interleukin-2 (ChIL-2) and IBDV viral protein 2 (VP2) were amplified and fused by splice overlap extension-polymerase chain reaction (SOE-PCR). The fusion gene was digested by EcoR I/Kpn I and inserted into pBacPAK8 vector, resulting in recombinant transfer plasmid pBacPakVP2-IL2. The recombinant plasmid was transfected into Sf-9 cells accompanied with hybrid nuclear polyhedrosis virus (HyNPV) genome DNA and lipofectin. Plaque-purification indicated that we had got the recombinant Hy-VP2-IL2. Fusion protein VP2-IL2 was expressed effectively both in insect cells and *bombyx mori*. The expression of fusion protein was confirmed by ELISA, SDS-PAGE and Western blotting assay, respectively. This efficient system allows us to meet the need for inexpensive vaccines required by the poultry industry. *Cellular & Molecular Immunology*. 2005;2(3):231-235.

Key Words: IBDV, ChIL-2, fusion gene, fusion protein

Introduction

Infectious bursal disease virus (IBDV) causes a severe disease of young chickens, which targets the bursa of Fabricius (1). IBDV destroys immature B cells in the bursa, and results in severe immunosuppression of the humoral immune system (2). Chickens around world are constantly exposed to IBDV, which leads to billions dollars losses in the poultry industry. The genome of IBDV consists of two segments of dsRNA. Segment B encodes viral protein 1 (VP1, 90 kD), the viral polymerase, segment A encodes a polyprotein that is cleaved into viral proteins VP2 (47 kD), VP4 (28 kD) and VP3 (32 kD). Segment A also encodes putative VP5, a 17 kD protein present in IBDV-infected cells (3). VP2 is the major host protective antigen and elicits neutralizing antibodies and protection (4, 5), thus VP2 was chosen to be vaccine candidate against IBDV infection. The VP2 gene has been expressed by *Escherichia coli*, *Saccharomyces cerevisiae*, fowl poxvirus, herpesvirus and baculovirus. The vaccinations with these expression products have exhibited variable levels of protection (6, 7).

However, traditional inactivated or attenuated vaccines could not provide enough protection against these antigenic variants and were very virulent (vv) IBDV (8). Recent studies focused on the importance of T cells in IBDV pathogenesis have shown that cell-mediated immunity may be more important in IBDV infections than previously thought (9).

Interleukin-2 (IL-2), initially known as T-cell growth factor, is a powerful immuno-regulatory lymphokine which produced by lectin- or antigen-activated T cells (10). It is secreted by mature T lymphocytes upon stimulation and certain T-cell lymphoma cell lines constitutively. When eukaryotic expression plasmid (pCI-IL-2) or recombinant chicken IL-2 (rChIL-2) expressed in *E. coli* was co-administrated with the H_5 subtype inactivated avian influenza virus (AIV) vaccines, both can enhance the efficiency of H_5 vaccine (11). ChIL-2 could enhance protective immunity against avian pathogens, which would introduce a new weapon into the control of infectious diseases of poultry.

In this study, we constructed fusion transfer vector containing the IBDV VP2 and ChIL-2 genes in tandem with six histamines. The purpose of the present study was to express the fusion protein in *bombyx mori*. After purification and identification, the newly developed fusion protein may offer an available vaccine candidate which is efficient, convenience and cheap for poultry industry.

Materials and Methods

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Insect cells and silkworm
Sf-9 insect cells were cultured in TC-100 medium (Invitrogen, USA) supplemented with 10% (v/v) FCS and 0.5% streptomycin and 0.5% gentamicin at 27°C in a humidified incubator. A hybrid strain of silkworm (commercial name, 54A) was used in this experiment. The larvae were reared with mulberry leaves at 23-25°C.

Construction of recombinant transfer vectors
The VP2 gene was amplified by PCR from the pcDNA3VP2 using the P1 and P2 primers while ChIL-2 gene was obtained from pET-IL-2 using P3 and P4 primers. The P1 primer (5'-GCG GTA CCA TGA CAA ACC TGC AAG AT-3') and P4 primer (5' GCG AAT TCT TAA TGA TGA TGA TGA TGA TGT TTT TGC AGA TAT CTC ACA AA-3') appended Kpn I and EcoR I sites (underlines) onto the 5' and 3' ends of the PCR product, respectively. The P2 (5'-AGA GCC GCC ACC GCC GCT GCC ACC GCC ACC CTT ATG GCC GGA TTA T-3') and P3 (5'-GGC GGT GGC GGC TCT GGC GGT GGT GGT TCT ATG TGC AAA GTA CTG ATC TTT-3') primers included nucleotides encoding linkers (underlines) or their complementary sequences. VP2 and IL-2 fragments were obtained after 30 cycles of amplification and they were purified from a 0.7% agarose gel using a gel extraction kit (Shanghai Sangon, China). A "fusion assembly" PCR (10 cycles of 94°C, 30 s; 50°C, 40 s; 72°C, 1 min) was then carried out in a standard 50 μl reaction containing 50 ng of the ChIL-2 and VP2 PCR products. Subsequently, 2 μl fusion assembly PCR production was added to another 50 μl reaction by the addition of BSA and $MgCl_2$. The "fusion amplification" PCR was then carried out for 25 cycles (94°C, 30 s; 55°C, 40 s; 72°C, 2 min). The fusion PCR product was digested with Kpn I and EcoR I, cloned into suitably digested baculovirus transfer vector pBacPak8. To confirm the nucleotide sequences of the cloned inserts, the positive clone was confirmed by DNA sequence analysis.

Viral infection
The recombinant baculovirus Hy-VP2-IL2 was obtained by co-transfecting pBacPakVP2-IL2 with wild type HyNPV DNA (12). Recombinant transfer plasmid pBakPakVP2-IL2 (1 μg) was mixed with 15 μg of the HyNPV DNA according to the method of Summers and Smith (13) in a polystyrene conical tube. Fourteen microliters of lipofectin (GIBCO, BRL) was added and the final volume was adjusted to 40 μl with sterile distilled water. The mixture was incubated at room temperature for 15 min and used to co-transfect log phase Sf-9 cells. After 24 h of incubation in FCS-free TC-100 medium, the culture medium was replaced by fresh medium containing 10% FCS. The cells were allowed to grow at 27°C for 5 days and the medium was collected as the primary viral stock for the screening of recombinant viruses. Recombinant virus carrying the fusion gene was plaque-purified as described by Gruenwald (14).

Production of fusion protein in silkworm larvae
Silkworm larvae in early fifth-instar were used for infection by recombinant viruses. Before inoculation, the larvae were placed on ice for 5-10 min. Twenty microliters of the viral solution (2×10^6 pfu/ml) was injected subcutaneously into the body of the larvae. Half an hour after the injection, the larvae were fed with mulberry leaves and reared at 23-25°C.

Immunofluorescence microscopy analysis
The method for direct observation through negative staining was performed as previously described (15). Infection cells were washed twice with phosphate buffered saline (PBS), fixed with 3.7% paraformaldehyde for 10 min at room temperature and washed three times with PBS. Cells were permeabilised with 0.1% Triton in PBS for 5 min at room temperature, washed with PBS and blocked with 1% BSA in PBS for 30 min at room temperature. Slides were incubated with IBDV polyclonal antibody and chicken interleukin-2 polyclonal antibody respectively for 1 h in a moist chamber at room temperature. Anti-rabbit FITC-conjugated antibody (Promega, USA) was diluted 1:100. Images were captured with the reflected Light Fluorescence Microscope (Olympus, Japan).

SDS-PAGE and Western blotting analysis
The sample was mixed with loading buffer, then boiled for 5 min, centrifuged for 1 min and 20 μl of clear supernatant was loaded on to a 12.5% slab gel. Following electrophoresis, the gel was either stained by Coomassie brilliant blue, or used for Western blotting analysis by transferring onto PVDF membrane (Amersham, USA). The PVDF membrane was then soaked in blocking solution containing 5% skim milk and 0.05% Tween-20 in PBS overnight at 4°C. After washing with PBS containing 0.05% Tween-20, the membrane was incubated with IBDV polyclonal serum as primary antibodies. Following washing, the membrane was incubated with an HRP-conjugated goat anti-rabbit IgG and colour-developed in DAB substrate. After washing, PVDF membrane was examined with IL-2 polyclonal serum, the same secondary antibody and substrate.

Results

Plasmid construction
IBDV VP2 and ChIL-2 fusion gene was acquired with SOE-PCR (Figure 1A), which was appropriately 1,900 bp. The fusion gene products were cloned into the vector pBacPak8 resulting pBacPakVP2-IL2, which was verified by restriction endonuclease digestion (Figure 1B) and DNA sequencing (data not shown).

Production the recombinant baculovirusl
The pBacPak8 vector is a baculovirus transfer vector designed for protein production in insect cells or larvae. The expression vector recombinant with wild type viral DNA yields recombinants, which are polyhedrin negative, produces foreign gene products. After three rounds of plaque-purification, the recombinant baculovirus Hy-VP2-IL2 was successfully constructed.

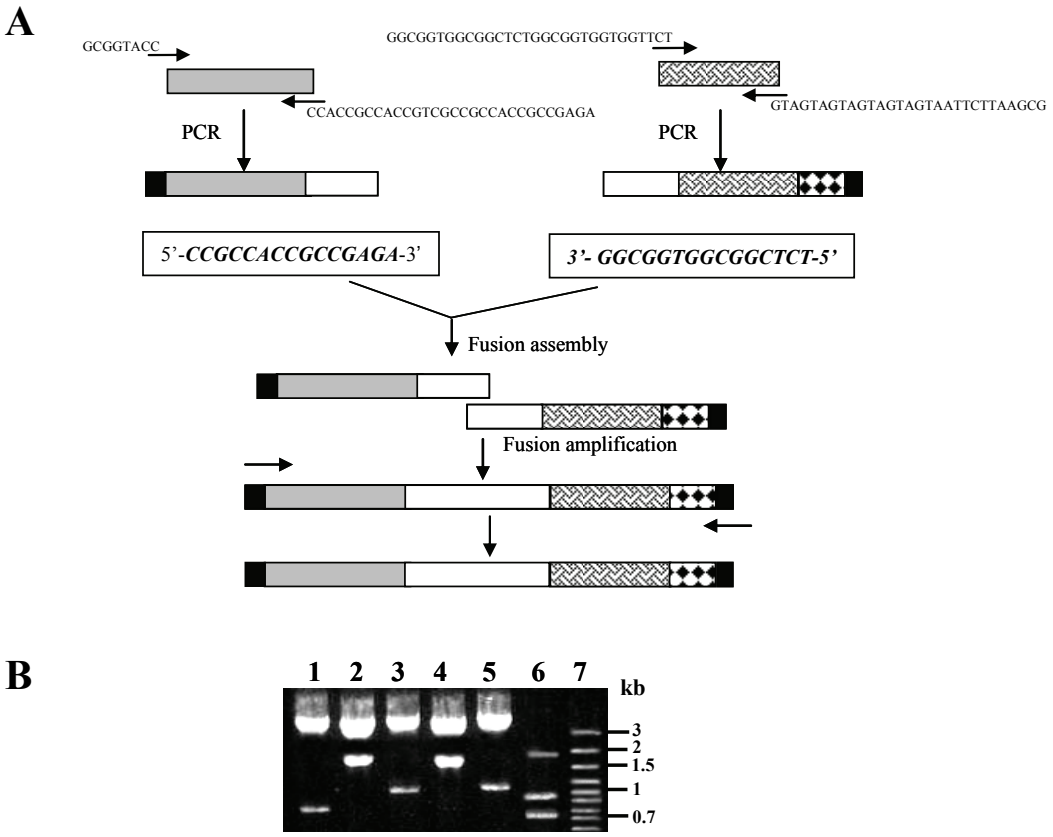

Figure 1. Recombinant plasmid constructed by SOE-PCR and digested by restriction endonuclease. (A) Construction of VP2-IL2 fusion gene. The PCR primers used to amplify the VP2 and IL-2 genes were shown with the sequences of their 5' tails. During "fusion assembly" PCR, the 15-bp boxed linker sequences anneal; fusion amplification was followed to produce the fusion gene product. (B) Verification of pBakPakVP2-IL2 by Sac I, Kpn I/EcoR I, Xba I/BamH I, Bgl II and BamH I, which were shown in Lanes 1-5. Lanes 6 and 7 showed the DL2000 and 100 bp DNA marker.

Sf-9 cells infected by wild type baculovirus had no fluorescence when they reacted with IBDV polyclonal serum (Figure 2A) or primary antibody or ChIL-2 polyclonal serum (Figure 2C). Sf-9 cells were infected by recombinant baculovirus shown brilliant fluroscence in reacting with IBDV polyclonal (Figure 2B) serum and ChIL-2 polyclonal serum (Figure 2D). Fusion protein expressed in the Sf-9 cells had the dual immunity to either IBDV antibody or ChIL-2 antibody.

SDS-PAGE and Western blotting

Harvesting the superstratum of Sf-9 cells which were infected by recombinant baculovirus Hy-VP2-IL2. The newly moulted fifth-instar silkworm larvae were used for infection and expression. Haemolymph were collected to measure the fusion protein expression peak by ELISA everyday post-infection. The results shown that peak arrived on the 4th day post-infection (date not shown). The silkworm larvae haemolymph was collected on the same day. The haemolymph was identified by SDS-PAGE and stained by Coomassie Brilliant Blue. Western blotting showed that VP2-IL2 fusion protein had expressed successfully in insect larvae haemolymph. As shown in Figure 3, a dominant band of approximately 60 kD, which is corresponds to the molecular weight of fusion protein, was observed in the Hy-VP2-IL2 infected samples.

Discussion

When infectious bursal disease appeared in chickens in 1962, the disease ignited as "Gumboro disease" after the geographic location of the first recorded outbreaks. From then on IBDV is a constant threat in the poultry industry worldwide (16). In order to argument the protection against IBDV infection, many methods were tried. DNA vaccines were prepared using immune stimulating complexes (ISCOM) as adjuvant, which could improve the antibody response of DNA vaccine (17). Traditional vaccines of IBDV have focused on stimulating the B cell reaction. However, T cell mediated immunoreactions play more important roles than ever thought (9). Plasmid DNA expressing VP2 of IBDV together with ChIL-2 induced better protection than vaccination with VP2 plasmid alone (18). In this paper, we designed the

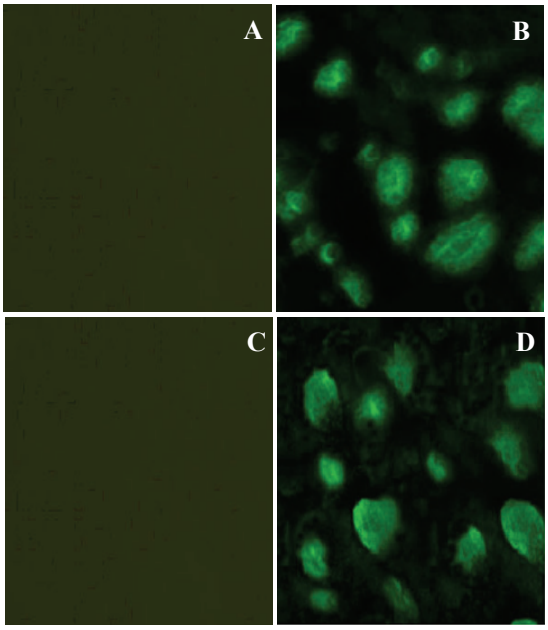

Figure 2. Analyzing the immunofluorescence of Sf-9 cells infected by wild type or recombinant baculovirus. A and B showed the fluorescence image where IBDV polyclonal antiserum acts as primary antibody. C and D figured out fluorescence image where ChIL-2 polyclonal antiserum acted as primary antibody. Sf-9 cells were infected by HyNPV in A and C while Hy-VP2-IL2 in B and D.

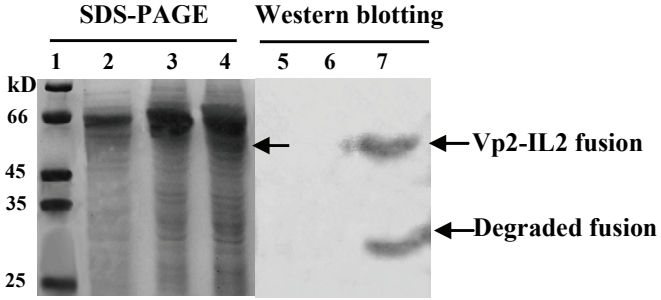

Figure 3. Identification of VP2-IL2 fusion gene expressed in larvae of silkworm infected with recombinant HyNPV by SDS-PAGE and Western Blotting. Lane 1, protein marker l; Lane 2, haemolymph of healthy larvaes; Lane 3, haemolymph of larvaes infected with HyNPV; Lane 4, haemolymph of larvaes infected with Hy-VP2-IL2; Lane 5, haemolymph of healthy larvaes; Lane 6, haemolymph of larvaes infected with HyNPV; Lane 7, haemolymph of larvae infected with Hy-VP2-IL2.

VP2-IL2 fusion protein linked by $(Gly_4Ser)_4$ and chose the HyNPV-silkworm system to express them. The data presented above showed that high levels of expression of fusion protein in a functionally active state could be achieved in silkworm larvae.

The main reason that we chose VP2 and ChIL-2 genes fused and expressed in baculovirus system were below: (1) IL-2 is a lymphokine that is responsible for the proliferation and differentiation of native T cells as well as for the activation of cytolytic T cells and natural killer cells; (2) the short half-life of IL-2 in circulation is expected to be prolonged by fusion techniques; (3) fusion protein can make up the limitation of traditional IBDV vaccines in T-cell mediated immunoreactions. Expression of the fusion protein can ensure that both the antigen and the vaccine are delivered to the same antigen presenting cell (APC) so allowing the direct activation of the APC; (4) the silkworm has become an ideal multi-cellular eukaryotic model system for basic research. At the same time we have chosen the HyNPV instead of traditional BmNPV or AcNPV, because HyNPV owns both characters of BmNPV and AcNPV. That is to say, recombinant baculovirus Hy-VP2-IL2 can grow in Sf-9 cells (or Sf21 cells) and BmN cells, even the silkworm larvae. This should be the new convenience method when using the baculovirus-insect cells or larvae system.

Cytokines are natural modulators of the immune system and offer the potential chance of further improving the protective immune response of conventional vaccines against avian pathogens of economic importance to the poultry industry. But many questions still remained. For example, how much partical protection against IBDV can be achieved by using the fusion protein as vaccination? How long is the half-life of the fusion protein in prolonged circulation? How to balance the dosage of fusion protein between high immunity efficiency and low clinical side-effects?

In conclusion, we succeeded in preparing recombinant VP2-IL2 fusion protein from haemolymph of silkworm *B. mori* larvae infected with Hy-VP2-IL2. The method for producing the fusion protein VP2-IL2 will be suitable for large-scale preparation of vaccination antigen against IBDV and may be applicable to produce other recombinant protein. The use of the fusion protein generated in larvae is safe, effective and inexpensive. These findings may offer a potential vaccine candidate for controlling infectious bursal disease in poultry.

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