J. Vet. Sci. (2002), 3(3), 207-212

JOURNAL OF
Veterinary
Science

Expression of Recombinant Porcine Interleukin-2 and Application of Its Antibody to Immunoassays

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Received February. 8, 2002 / Accepted July 20, 2002

Abstract

Interleukin-2 plays an important role in T lymphocyte proliferation and immune response regulations. In this study, porcine IL-2 cDNA was cloned from peripheral blood mononuclear cells, and recombinant porcine IL-2 (rpIL-2) was expressed in *Escherichia coli*. The size of rpIL-2 without signal peptides was about 15 kDa when determined by SDS-PAGE and Western blotting analysis. Anti-rpIL-2 antibody was produced from mice immunized with the purified rpIL-2, and its specificity was examined by Western blotting and ELISA. In the Western blotting assay, anti-rpIL-2 and anti-recombinant human IL-2 (rhIL-2) antibodies specifically recognized rpIL-2 and rhIL-2, respectively. However, anti-rpIL-2 antibody did not recognize rhIL-2, and anti-rhIL-2 antibody also did not react with rpIL-2 in the same assay. In ELISA, anti-rpIL-2 antibody strongly interacted with both rpIL-2 and rhIL-2, and anti-rhIL-2 antibody also efficiently recognized both proteins. Taken together, the specificity of anti-rpIL-2 antibody for rpIL-2 was demonstrated by Western blotting and ELISA. It was also shown that ELISA is more efficient than Western blotting in determining the species cross-reactivity of anti-rpIL-2 antibody.

Key words : porcine IL-2, expression, antibody, specificity, species cross-reactivity

Introduction

Interleukin-2 (IL-2) was originally identified as a T cell growth factor in the supernatants of lectin-stimulated human and mouse T lymphocytes [11, 21, 26]. IL-2 plays a number of important roles in the regulation of immune responses, including the proliferation of activated T lymphocytes, the generation of cytotoxic T lymphocytes (CTL), increased natural killer (NK) cell activity, and the production of interferon and antibodies [8, 10, 14, 23, 28, 30]. The IL-2 of several species, such as human, pig, and bovine, induces the proliferation of their own T cells and also shows species cross-reactivity, by supporting the growth of other animals T cells [5, 6, 7].

IL-2 cDNAs from several species have been cloned, including human, mouse, pig, bovine, and ovine [2, 12, 13, 18, 29]. The molecular weight of their IL-2 is about 15-17 kDa [5, 23, 25]. The production of porcine IL-2 from phytohemagglutinin (PHA) and concanavalin A (Con A)-stimulated T lymphocytes has been observed, and its characteristics determined by biological and biochemical studies [9, 16]. There are relatively few reports on porcine IL-2 expression in the baculovirus or *E. coli* system [5, 15, 17]. Iwata *et al*. (2000) reported upon the production of IL-2 antiserum from mice using a glutathione S-transferase (GST)-IL-2 fusion protein as an antigen. In addition, no study to the best of our knowledge has shown the development of immunoassays for detecting porcine IL-2 in blood.

Swine viral diseases caused by pseudorabies virus and circovirus are characterized by inducing the suppression of host immune functions [3, 24]. In the case of pseudorabies virus infection, immune suppression is mediated by the inhibition of IL-2 production [3]. Since IL-2 is deeply involved in the regulation of cellular immune responses against viral infection, its modulation status may be correlated with some viral disease developments. Because of its efficiency and sensitivity, ELISA is broadly used to determine cytokine variations in animal blood. The main purpose of this study was to develop a porcine IL-2-specific ELISA system. In order to do this, we expressed rpIL-2 in the *E. coli* system, produced mono-specific polyclonal antibody from mice, and examined the specificity of the antibody by Western blot and ELISA. The ELISA system developed in this study may be further applied to study the modulation of IL-2 expression in virus-infected pigs.

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Materials and Methods

Cloning of porcine IL-2 cDNA

Porcine IL-2 gene was amplified by PCR using cDNA as a template and a pair of primers as described elsewhere [4]. Briefly, single-stranded cDNA was synthesized from 5μg of total RNA prepared from phytohemagglutinin (PHA)-stimulated porcine peripheral blood mononuclear cells (PBMC) using a Superscript Preamplification System for First Strand cDNA Synthesis Kit (Gibco BRL, Grand Island, NY). The open reading frame (ORF) region of IL-2 was then amplified for 30 cycles with a primer set #1 (Table 1) under the following PCR conditions; 94℃ for 30 sec, 53℃ for 30 sec, and 72℃ for 45 sec. The pure PCR product was cloned into TOPO TA cloning vector (Invitrogen, Carlsbad, CA) producing pTopoIL-2. The identity of the IL-2 gene was confirmed by DNA sequencing and restriction enzyme analysis.

Expression of recombinant porcine IL-2 protein

In order to subclone IL-2 cDNA into an expression vector, a new pair of primers, set #2 (Table 1), was synthesized. The forward and backward primers included the restriction enzyme sites of *Bam* HI and *Hind* III at the 5' end of the primers, respectively. IL-2 cDNA, without a signal sequence, was amplified for 30 cycles with primer set #2 and pTopoIL-2 as a template under the following PCR conditions; 94℃ for 30 sec, 5 5℃ for 30 sec, and 72℃ for 45 sec. The PCR product was cloned into TOPO TA cloning vector, and the insert DNA was digested with *Bam* HI and *Hind* III. Digested insert DNA was purified with a gel extraction kit (Qiagen, Hilden, Germany) and ligated into the *Bam* HI- and *Hind* III-digested pQE-30 expression vector (Qiagen) that contains a sequence encoding a methionine and 6xHistidine at the N-terminus. The ligation reaction product was transformed into M-15 competent cells (Qiagen), and a clone containing IL-2 cDNA was selected and named as pQEIL-2. When the bacterial culture reached an OD_{600} of 0.6, the expression of IL-2 was induced by the addition of IPTG into the bacterial culture to a final concentration of 1mM. After 4 h of culture in a shaking incubator at 185 rpm, the bacteria were harvested by centrifugation at 4,000 × g for 20 min, and the bacterial pellet was stored at -70℃ until required for analysis.

Purification of rpIL-2

The bacterial pellet was resuspended in 45 ml of lysis buffer (100 mM NaH_2PO_4, 10 mM Triscl, 8 M Urea, 0.5% Triton X-100, 10 mM Imidazloe, pH 8.0) and sonicated 10 times for 30 sec (Bandelin, Germany). The clear cell lysate was passed three times through a column packed with ProBond Resin (Invitrogen) that specifically bound 6xHis-tagged recombinant protein. The column was washed three times with 15 ml of wash buffer (100 mM NaH_2PO_4, 10 mM Tris.Cl, 8 M Urea, 20 mM Imidazloe, and pH 8.0). The rpIL-2 was eluted with 10 ml of elution buffers (100 mM NaH_2PO_4, 10 mM Tris.Cl, 8 M Urea, 50 mM Imidazloe) having three different pHs, i.e., pH 6.7, 6.3, and 5.9.

Production of mono-specific polyclonal antibody

Mono-specific polyclonal antibody against rpIL-2 prepared in *E. coli* was produced from mice. Four weeks old ICR mice (Dae Han Biolink, Korea) were intraperitoneally and subcutaneously immunized with 100 μg of rpIL-2 mixed with Freund's complete adjuvant (Gibco BRL). The second immunization was conducted by injecting the same amount of rpIL-2 mixed with Freund's incomplete adjuvant 2 weeks after the first immunization. The third immunization was completed in the same way as the second, 3 weeks after the first injection. Blood samples were collected from the mice 1 week after the final immunization. Sera were separated from the blood and stored at -20℃ until use.

SDS-PAGE and Western blot

Lysates of bacteria induced or not induced with IPTG, and the column-purified rpIL-2 samples were analyzed by SDS-PAGE using 15% polyacrylamide gel. After SDS-PAGE, the protein bands were visualized by Coomassie Brilliant Blue staining.

The rpIL-2 and rhIL-2 prepared in two 15% polyacrylamide gels were electrophoretically transferred onto two separate nitrocellulose membrane papers (Bio Rad, Hercules, CA) using a semidry transfer method. The nitrocellulose membrane papers were blocked overnight with 5% skim milk in TBS. After three washes with TTBS (0.05% Tween 20), one membrane was incubated with mouse anti-rpIL-2 antibody, and the other with rabbit anti-rhIL-2 antibody for 2 h at room temperature. The membranes were washed three times with TTBS, and incubated with the alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (H+L) or goat anti-rabbit IgG (H+L) (Bio-Rad) for 2 h at room temperature. After washing, the reacted bands were

Table 1. Primer sets used for the cloning and subcloning of porcine IL-2 cDNA.

Primer Set	Nucleotide sequence	Size of PCR product
Set#1		
Forward	5′ −ATGTATAAGATGCAGCTCTTG−3′	465bp
Backward	5′ −TCAAGTCAGTGTTGAGTAGATG−3′	
Set#2		
Forward	5′ −GGATCCGCACCTACTTCAAGCTC−3′	417bp
Backward	5′ −AAGCTTTCAAGTCAGTGTTGAGTAG−3′	

visualized by color development with AP color developer (Bio-Rad) as described by the manufacturer.

ELISA

The specificity of the polyclonal antibody produced from the rpIL-2-immunized mice for recombinant porcine and human IL-2 was examined by ELISA. One hundred $\mu\ell$ of rpIL-2 (10 μg/1 ml) and rhIL-2 (10 μg/1 ml) resuspended in the coating buffer (14.2 mM Na_2CO_3, 34.9 mM $NaHCO_3$, 3.1 mM NaN_3, pH 9.6) was added to a microplate for ELISA (Greiner, Austria), and incubated overnight at 4℃. The plate was washed three times with PBST (0.05% Tween 20), and blocked with PBST containing 1% bovine serum albumin (BSA) for 1 h at 37℃. As the first antibody, 100 $\mu\ell$ of 1:100-diluted mouse anti-rpIL-2 polyclonal antibody or 1:1,000-diluted rabbit anti-rhIL-2 (Endogen, Woburn, MA) was added to the plate, and incubated for 1 h at 37℃. After washing with PBST, 100 $\mu\ell$ of goat anti-mouse IgG (H+L)-HRP conjugate (Bio-Rad) or goat anti-rabbit IgG (H+L)-HRP conjugate (Bio-Rad) was added to the plate, and incubated for 1 h at 37℃. Color was developed by adding 100 $\mu\ell$ of ABTS substrate solution (Bio-Rad) to the plate. After 30 min of incubation at room temperature, the OD value was measured at 405 nm using an ELISA reader (Molecular Devices, Sunnyvale, CA).

Results

Cloning and expression of rpIL-2 in *E. coli*

The ORF of porcine IL-2 composed of 465 bp encoding 154 amino acids was cloned into TOPO TA cloning vector. Subsequent DNA sequencing demonstrated that the cloned insert DNA was 100% identical with the reference porcine IL-2 cDNA sequence (data not shown) [13]. IL-2 cDNA without signal sequences was cloned into TOPO TA vector (Fig. 1), and then subcloned into an expression vector pQE30. Expression of rpIL-2 was induced by the addition of IPTG to the bacterial culture (Fig. 2).

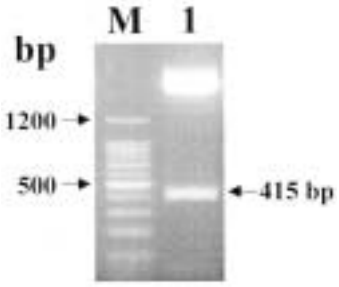

Fig. 1. Cloning of porcine IL-2 cDNA. Porcine IL-2 gene without a signal sequence was amplified by PCR with a forward primer containing a *Bam* HI site and a backward primer containing a *Hind* III site at their 5' ends. The PCR product was cloned into a TOPO TA cloning vector. Plasmid DNA having the correct insert was digested with *Bam* HI and *Hind* III, and analyzed in an 1.5% agarose gel.

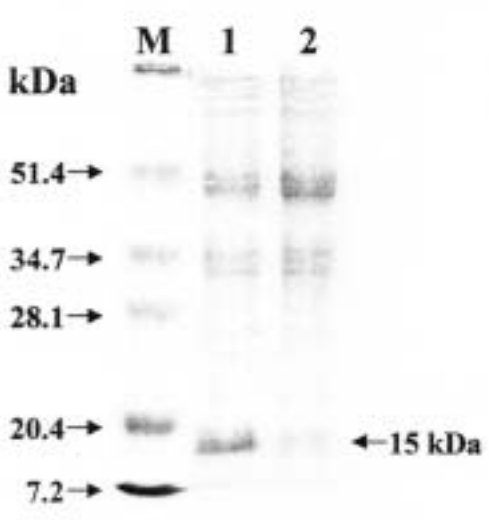

Fig. 2. Expression of recombinant porcine IL-2 protein in *E. coli*. Expression of rpIL-2 was induced by the addition of IPTG into bacterial culture, and analyzed in a 15% polyacrylamide gel. The expressed recombinant IL-2 was identified as a 15 kDa band in the gel. M: standard protein marker; Lane 1: induction with IPTG; Lane 2: no induction with IPTG.

Determination of protein purification conditions and the size of rpIL-2

The affinity column-purified rpIL-2 composed of 146 amino acids, including 134 amino acids of IL-2, and additional 6 amino acids and 6xHistidine-tags at the N-terminus of the vector, appeared as a single band of 15 kDa by SDS-PAGE analysis (Fig. 3). The fractions eluted by elution buffers of pH 6.7-5.9 showed rpIL-2 as a clear single band. However, elution buffers with pHs lower than pH 5.9 eluted contaminated proteins with the IL-2 protein under our experimental conditions (data not shown). These results indicate the importance of optimal pH in the elution step of recombinant protein. A comparison of the recombinant porcine and human IL-2 by SDS-PAGE demonstrated that two proteins were of almost the same size, 15 kDa (Fig. 4).

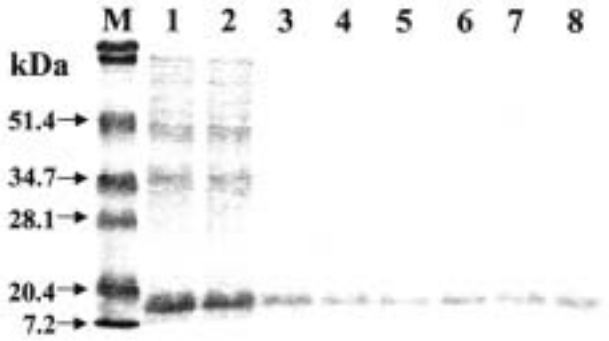

Fig. 3. Purification of recombinant porcine IL-2. Recombinant porcine IL-2 containing 6xHis at its N-terminus was purified using ProBond™ resin under the denaturing

conditions. M; standard protein marker; Lane 1: bacteria lysate; Lane 2: flow-through; Lane 3: first washing; Lane 4: second washing; Lane 5: third washing; Lane 6: first elution; Lane 7: second elution; Lane 8 third elution.

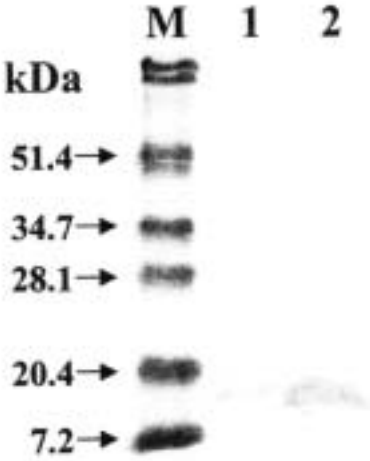

Fig. 4. Electrophoretic analysis of purified recombinant porcine IL-2 and recombinant human IL-2 in 15% SDS-polyacrylamide gel. M: standard protein marker; Lane 1: porcine IL-2; Lane 2: human IL-2.

Specificity and cross-reactivity of anti-rpIL-2 antibody

The mono-specific polyclonal antibody was produced from mice immunized with rpIL-2. To test the specificity of the antibody, Western blotting was conducted with porcine and human IL-2. Mouse anti-rpIL-2 antibody specifically and strongly interacted with rpIL-2 (Fig. 5A). However, it was too weak to identify a rhIL-2-specific band. On the other hand, the commercial rabbit anti-rhIL-2 readily recognized the rhIL-2 antigen (Fig. 5B), but did not clearly produce a rpIL-2-specific signal. These results indicate that the Western blotting assay does not efficiently demonstrate the species cross-reactivity of the antibodies for the recombinant IL-2 proteins. Therefore, ELISA was developed to demonstrate both specificity and cross-reactivity of the antibodies. Mouse anti-rpIL-2 antibody very strongly recognized both pig IL-2 and human IL-2 (Fig. 6). The OD values against rpIL-2 and rhIL-2 were 3.27±0.33 and 2.56±0.41, respectively. Rabbit anti-rhIL-2 antibody also specifically reacted with human and porcine IL-2 (Fig. 6). The corresponding OD values against rhIL-2 and rpIL-2 were 3.74±0.10 and 2.79±0.03, respectively. The ELISA results definitely reflected the specific interactions between the anti-rpIL-2 and anti-rhIL-2 antibodies and the porcine and human IL-2 proteins, and also showed species cross-reactivity.

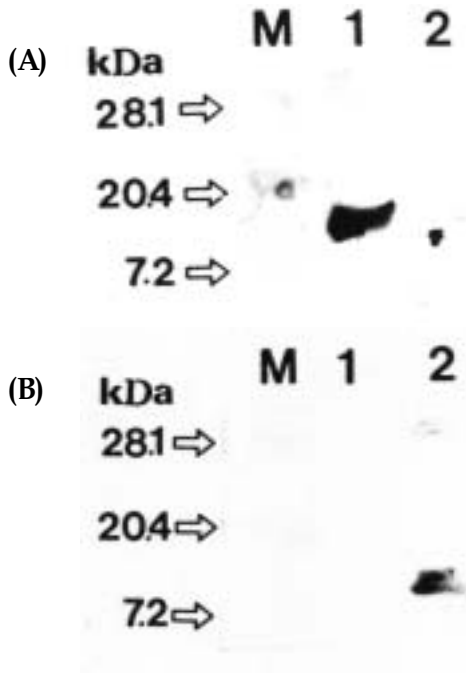

Fig. 5. Western blotting analysis of purified recombinant porcine IL-2 and recombinant human IL-2. Porcine and human IL-2 proteins were transferred onto two nitrocellulose membranes. One of them was incubated with mouse anti-rpIL-2 antibody (A), and the other with rabbit anti-rhIL-2 antibody (B). The membranes were incubated with goat anti-mouse IgG (H+L)-AP conjugate and goat anti-rabbit IgG (H+L)-AP conjugate, respectively. IL-2-specific bands were identified by the addition of AP color development buffer to the membranes. M: standard protein marker; Lane 1: porcine IL-2; Lane 2: human IL-2.

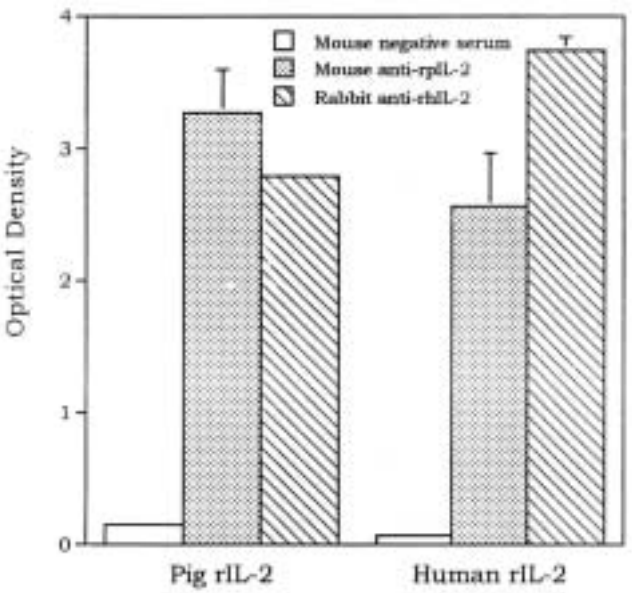

Fig. 6. Cross-reactivity of anti-rpIL-2 and anti-rhIL-2 antibodies with rpIL-2 and rhIL-2 in ELISA. Porcine and

human recombinant IL-2 was coated on the ELISA plate, and incubated with different antibodies, including mouse negative serum, mouse anti-rpIL-2 antibody, or rabbit anti-rhIL-2 antibody. Goat anti-mouse IgG (H+L)-HRP conjugate or goat anti-rabbit IgG (H+L)-HRP conjugate was used as secondary antibodies. After color development, by adding substrate solution, OD values were determined at 405 nm with an ELISA reader.

Discussion

IL-2 produced from activated T lymphocytes induces T cell proliferation, and regulates a broad range of cellular immune responses by interacting with its high affinity receptor [23, 28]. Some animal viruses, such as human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), and pseudorabies virus (PRV), induce a suppression of immune response by inhibiting IL-2 production during infections [3, 20, 27]. It may also be speculated that the porcine circovirus (PCV)-induced immunosuppression phenomenon is a consequence of the disturbance of cytokine-mediated immune responses [24]. As the first step in conducting a project designed to study the interaction between disease development and porcine cytokine modulation, we prepared recombinant porcine IL-2 and antisera against it to develop immunoassays, which would be applied to study the modulation of IL-2 expression in pathogen-infected pigs.

The size of rpIL-2 expressed in *E. coli* was about 15 kDa when determined by SDS-PAGE, as shown in other reports [5, 17]. The mono-specific polyclonal antibody produced from mice with rpIL-2 readily detected the recombinant protein of the same size in Western blotting assay, demonstrating the specificity of the antibody for the rpIL-2 antigen. However, the same antibody barely detected rhIL-2 in the same conditions of Western blotting assay. The commercial rabbit anti-rhIL-2 antibody also did not recognize rpIL-2 in Western blotting assay. The reason for the extremely poor recognition of human IL-2 by mouse anti-rpIL-2 antibody and porcine IL-2 by rabbit anti-rhIL-2 in Western blotting is attributable to the reactivity of the antibodies in the assay-specific conditions. On the other hand, both mouse anti-rpIL-2 and rabbit anti-rhIL-2 antibodies very efficiently recognized both rpIL-2 and rhIL-2 in ELISA demonstrating both the specificity and species cross-reactivity of the antibodies. However, mouse anit-rpIL-2 antibody always recognized rpIL-2 more strongly than rhIL-2 ($p<0.05$). Similarly, rabbit anti-rhIL-2 antibody produced higher OD values with rhIL-2 than that with rpIL-2 ($p<0.01$). The predicted amino acid sequences of human and porcine IL-2 cDNA exhibit 72% homology [13]. The OD values obtained by ELISA with two antibodies would reflect the difference of amino acid sequences of the two proteins.

Co-delivery of IL-2 with vaccines for pseudorabies virus (PRV), influenza virus, or bovine viral diarrhea virus (BVDV), induces enhanced and protective immune responses [1, 19, 22]. Therefore, it is highly promising that rpIL-2 prepared in this study, if it has biological or functional activity, may be used as an adjuvant in the development of efficient porcine viral vaccines. Based on the polyclonal antibody prepared in this study, we intend to produce monoclonal antibodies specific for several epitopes of rpIL-2. These reagents will be valuably used for evaluating the immunomodulatory effect of viral diseases and for performing more sophisticated immunological assays.

Acknowledgments

This study was supported by grant No. 0999-011-1-2 from the Agricultural Research and Promotion Center, and by grants from the Brain Korea 21 project, and from the Research Institute for Veterinary Science, Seoul National University, Korea.

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