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High-Efficient Expression of Porcine IL-2 with Recombinant Baculovirus Infected Silkworm, *Bombyx mori*

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Abstract Biologically active porcine Interleukin-2(poIL-2) was produced from *in vitro* and *in vivo* baculovirus expression systems, namely the *Autographa californica* nuclear polyhedrosis virus (AcNPV)-cell culture system and the Hybrid nuclear polyhedrosis virus (HyNPV) - silkworm larva system. The concentration of the recombinant poIL-2(rpoIL-2) in the larvae hemolymph was 1 to 3 mg/mL, which was about 7 to 20 times those of the cell culture systems The level of this expression efficiency is equal to that with transgenic livestock, secretion products in milk.

Keywords: porcine IL-2, gene expression, baculovirus, silkworm, cytokine, mass-production

INTRODUCTION

Cytokines, which naturally occur in minute amounts, have great therapeutic value for diseases in that they activate or control the immune systems. In the field of agriculture, cytokine dosing was attempted to control diseases caused by an immature immune system in newborn livestock and stress-induced immune suppression. Large quantities of cytokine products must be prepared to conduct successful animal experiments. Because IL-2 is known to be involved in the induction of T cell and NK cell activation and proliferation, B cell proliferation and enhancement of immunoglobulin production, and activation of monocyte and macrophage [1], we investigated porcine IL-2 in order to develop a new therapeutic agent and vaccine adjuvant

Advances in biotechnology have made it possible to express foreign genes in several kinds of cell cultures. Foreign gene expression systems using *E. coli* are frequently applied to obtain recombinant proteins on a large scale. In addition, the baculovirus expression systems based on recombinant nuclear polyhedrosis viruses have also been widely used for efficient expression of foreign genes [2]. Among the nuclear polyhedorsis viruses, *Autographa californica* nuclear polyhedrosis virus (AcNPV) expression vector, originally developed by Smith *et al.* [3], was often employed for laboratory use Maeda et al. developed a different vector from a nuclear polyhedrosis virus (BmNPV) infecting a silkworm, *Bombyx mori*, and succeeded in a large scale expression of human α-interferon [4] These vectors have different characteristics, and they were not compared properly. Because baculoviruses have narrow host ranges, AcNPV cannot infect the host of BmNPV and vice versa. Recently, Mori *et al.* developed a hybrid baculovirus (HyNPVcp+) from AcNPV and BmNPV and a hybrid baculovirus lacking the cysteine proteinase gene (HyNPVcp-) [5,6]. To establish a system for large-scale porcine IL-2 expression in silkworm, we applied this hybrid virus to compare the AcNPV cell culture system and BmNPV silkworm system and to examine the effect of virus inoculation conditions on expression efficiency.

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MATERIALS AND METHODS

Construction of Porcine IL-2 Recombinant Baculoviruses

The full length poIL-2 cDNA was obtained by RT-PCR The primers were designed according to the poIL-2 cDNA sequence [7] with the addition of *Bam*HI restriction sites. The RNA was extracted from Concanavalin A activated peripheral blood lymphocytes. The RT-PCR products were cloned in pBluescript II. The inserted poIL-2 cDNA was confirmed by sequencing and excised by *Bam*HI digestion. The fragment is then re-cloned into the *Bam*HI site of the transfer vector pAcYM1 [8]. Resulting recombinant transfer vector was named pAcPIL2.

Nuclear polyhedrosis viruses, AcLacZ, HyNPVcp+ and HyNPVcp-, were used to establish poIL-2 recombinant isolates. HyNPVcp+ and HyNPVcp- were generous gifts from Dr. H. Mori, Kyoto Institute of Technology. A poIL-2 recombinant AcNPV was obtained by the method previously described [9] Briefly, the pAcPIL2 and DNA of AcLacZ [10], the LacZ gene recombinant AcNPV, linearized by Eco81I were co-transfected with Lipofectin (Gibco BRL, MD, U.S.A) to the *Spodoptera*

frugiperda cell line SF21AE. Plaques produced by the progeny viruses from the co-transfection were screened and recombinants exhibiting β-galactosidase-negative phenotype were selected. Recombinant virus was isolated by plaque purification three times. The resulted recombinant virus was named AcPIL2. Recombinant HyNPVcp+ and HyNPVcp- were prepared by the same method as described above, except for the use of the circular form of HyNPVcp+ and HyNPVcp- DNA and selection of the polyhedron negative phenotype. The resulted recombinant viruses were named HyPIL2cp+ and HyPIL2cp-

Recombinant poIL-2 Production

In order to produce rpoIL-2 with *Tricoplusia ni* derived cell line BTI TN 5B1-4 (TN5) [11], cells were infected with AcPIL2, HyPIL2cp+ or HyPIL2cp- at m.o.i. of 1.0 and cultured with serum free medium EX-CELL 401 (JRH Biosciences, KS, USA) at 28°C. To produce rpoIL-2 with *Bombyx mori* derived cell line, BmN [12] the cells were infected with HyPIL2cp+ or HyPIL2cp- at m o i of 1.0 and cultured with serum free medium Sf-900II SFM (Gibco BRL, MD, USA) at 28°C. In order to produce poIL-2 in the silkworm, the fifth instar larvae were injected by various amounts of HyPIL2cp- and fed at 25°C. The virus infected larvae hemolymph was recovered by cutting the abdominal legs in 0.1 M phosphate buffer pH 6.8, supplemented with 0.1% N-phenylthiourea. The culture fluid and the hemolymph were centrifuged at low speed, and the supernatants were stored at –80°C for further analysis

Biophysical and Biological Analyses of the rpoIL-2

The procedure for tricine-SDS-PAGE is described elsewhere [13] The protein bands were visualized with Coomassi brilliant blue (CBB) staining and their density were measured by the PDIS densitometer (Molecular Dynamics, CA, USA). The quantity of the products were calculated by comparing of the density to that of the protein molecular weight standards (Pharmacia LKB Biotechnology, Uppsala, Sweden). Tunicamycin treatment and N-terminal amino acid sequencing were performed as described previously [9].

Biological activity of the rpoIL-2 was determined with IL-2 dependent mouse T cell line CTLL-2 by MTT assay [14]. Diluted culture fluids or hemolymph was added to the RPMI medium supplemented with 10% FBS. CTLL-2 cells were incubated with this medium for 24 h. The cellular viability was expressed as $O.D._{570}$ of Formazan, produced due to the reduction of MTT (3-(4,3-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide).

RESULTS AND DISCUSSION

Recombinant poIL-2 Accumulation in AcPIL2- and HyPIL2- Infected Culture Fluids

We expressed rpoIL-2 with the most popular AcNPV baculovirus system. In the AcNPV recombinant virus

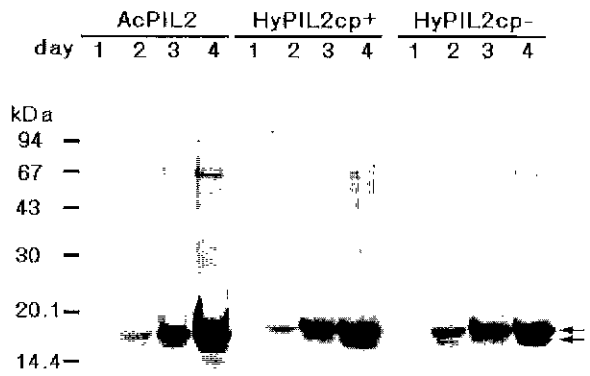

Fig. 1. Comparison of rpoIL-2 expression with AcPIL2, HyPIL2cp+ and HyPIL2cp- infected TN5 cells. Culture fluids were centrifuged and 20 μL each of the supernatants were resolved by tricine SDA-PAGE The gel was stained with CBB Arrows indicate rpoIL-2 migrated positions. Migration positions of molecular weight markers are indicated on left side of the panel.

(AcPIL2)-infected TN5 cell culture fluid, major 18.5 kDa protein and minor smaller protein were observed by CBB stained tricine SDS-PAGE (Fig. 1. AcPIL2). These proteins did not exist in the wild-type virus-infected TN5 culture fluid (date not shown). These products were accumulated from day 2 to day 4 post infection. The concentration of the major protein was about 150 μg/mL at day 4. The biological activity of the culture fluid was confirmed by the MTT assay with CTLL-2 cells More than 1×10^6 times diluted culture fluid was able to proliferate the CTLL-2 cells, but the wild-type virus-infected cell culture fluid could not (data not shown) Since the poIL-2 cDNA has secretion signal sequence, signal peptide cleavage in the baculovirus expression system was confirmed by the N-terminal amino acid sequence The sequence completely matched the native poIL-2 signal peptide cleavage site (Fig. 2). This data suggested that the rpoIL-2 had a mature form. Glycosylation of the rpoIL-2 was confirmed by tunicamycin treatment. The 18.5 kDa major product was not secreted and accumulated in the culture fluid (data not shown) because N-linked glycosylation was inhibited by tunicamycin These results indicated that the post-translational modification of the rpoIL-2 in the baculovirus system was similar to that in a mammalian expression system.

We compared the efficiencies of AcNPV-cell culture system and silkworm system using a porcine IL-2 gene recombinant HyNPV, HyPIL2cp+ and HyPIL2cp-. These viruses were inoculated to TN5 cells to compare the efficiency of AcPIL2. As shown in Fig. 1, rpoIL-2 accumulated in HyPIL2cp+ and HyPIL2cp- infected cell culture fluids in a similar manner as observed in AcPIL2-infected cell culture fluid. Hybrid viruses were therefore as efficient as AcNPV in rpoIL-2 producion. HyPIL2cp+ and HyPIL2cp- were then inoculated to BmN cells to examine whether the hybrid viruses work properly in a silkworm-derived system. As the SDS-PAGE data indicated, hybrid virus system also worked

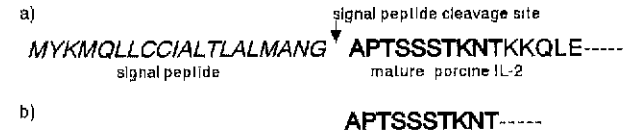

Fig. 2 N-terminal amino acid sequence of the rpoIL-2; (a) N-terminal amino acid sequence of poIL-2 precursor deduced from the cDNA sequence, (b) N-terminal amino acid sequence of rpoIL-2 Signal peptide sequence is indicated by italics. Signal peptide cleavage site is indicated with an arrow.

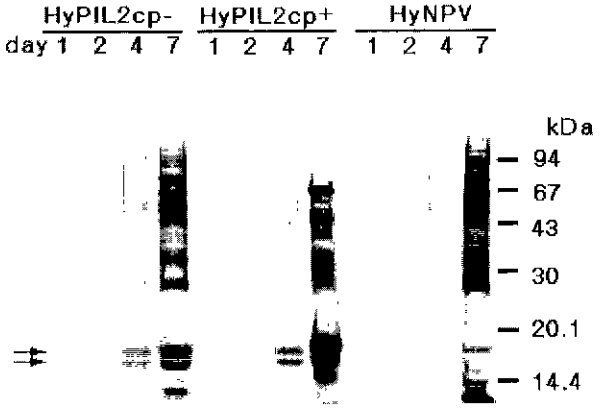

Fig. 3. rpoIL-2 expression by HyPIL2cp+ and HyPIL2cp- infected BmN cells. Culture fluids were centrifuged and 20 μL each of the supernatants were resolved by tricine SDS-PAGE. The gel was stained with CBB. Arrows indicate rpoIL-2 migrated positions

efficiently in the silkworm cells (Fig. 3). Recombinant poIL-2 accumulated to about 80 μg/mL in the culture fluid. Because rpoIL-2 in both HyPIL2cp+ and HyPIL2cp- infected cell culture fluids had the same molecular size, viral cysteine proteinase did not seem to digest the rpoIL-2. These results indicate that these hybrid virus worked efficiently in *Bombyx mori* system.

Recombinant poIL-2 Accumulation in HyPIL2cp- Infected Silkworm Larvae Hemolymph

Since HyPIL2cp+ and HyPIL2cp- showed no significant difference, we infected fifth instar silkworm larvae with HyPIL2cp-. Extra proteins with molecular weight similar to rpoIL-2 accumulated in the HyPIL2cp- infected cell culture fluid, were observed in the hemolymph of the larvae, by CBB stained tricine SDS-PAGE gel (Fig. 4). The biological activity of the HyPIL2cp- infected larvae hemolymph was confirmed by MTT assay with CTLL-2 cells. Hemolymph, diluted to more than $1X10^7$, proliferated CTLL-2 cells, but HyNPVcp- infected larvae hemolymph did not (Fig 5).

To determine the suitable condition for rpoIL-2 production, HyPIL2cp- of 5×10^5 to 5×10^2 plaque forming units (p.f.u.) was inoculated to the larvae of day 0, 2 and 4 of fifth instar. Hemolymph endogenous proteins increased significantly when the larvae were inoculated at day 4 (Fig. 4(b)). Recombinant poIL-2 accumulated in all HyPIL2cp- inoculated larvae group hemolymph

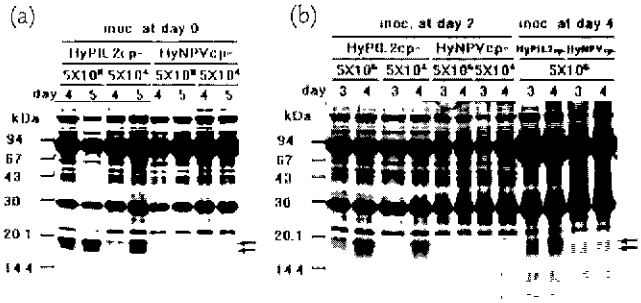

Fig. 4. SDS-PAGE of HyPIL2cp- infected silkworm larvae hemolymph. Different titers of HyPIL2cp- and HyNPVcp- were inoculated to silkworm larvae at different time (indicated above the gel panels) with different doses of HyPIL2cp- Hemolymph of the larvae were collected at day 3, day 4 or day 5 post infection. These samples were diluted 10 times and 20 μL of them were resolved by tricine SDS-PAGE The gel was stained with CBB; (a) Larvae hemolymph samples, HyPIL2cp- or HyNPV inoculated at day 0 of fifth instar, (b) Larvae hemolymph samples, HyPIL2cp- or HyNPV inoculated at day 2 or day 4 of fifth instar. Arrows indicate rpoIL-2 migrated positions

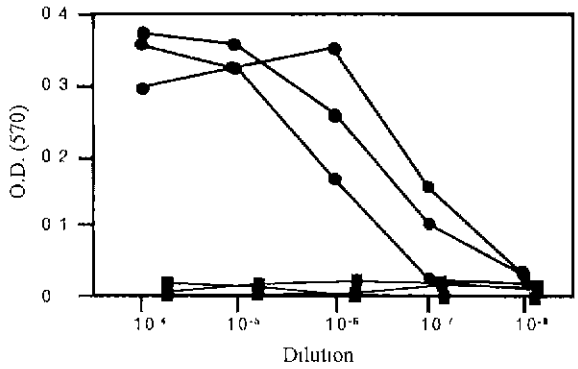

Fig. 5. Biological activities of rpoIL-2 accumulated in silkworm larvae hemolymph IL-2 dependent cell line, CTLL-2, was incubated with diluted hemolymph. Cell viability was measured by MTT assay (●) HyPIL2cp- infected larvae hemolymph, (■) HyNPV infected larvae hemolymph

samples. The concentration of the rpoIL-2 was about 1 to 3 mg/mL (Fig. 6(a), (b)), which is about 7 to 20 times higher than that of the AcPIL2 infected TN5 cell culture fluid. The larvae showing the highest concentration (ca. 3 mg/mL) were a group of 5×10^5 p.f.u. virus inoculated at day 0 of fifth instar (Fig. 6(a)). However, the recovered hemolymph volume from this group was low (Fig. 6(c)). Therefore, the total amount of the recovered rpoIL-2 from each larva was low in this condition (Fig. 6(e)). For the 5×10^2 p.f.u. virus inoculated day 0 larvae, results varied among individual larva and between the experiments (Fig. 6(a), (c), (e)). Though the number of experiments was limited, inoculate 5×10^4 or 5×10^5 p.f u. virus to day 2 of fifth instar larva was presumably efficient, thus providing more consistent results (Fig. 6(b), (d), (f)).

In this study, we have performed highly efficient production of porcine IL-2 with the silkworm, *Bombyx*

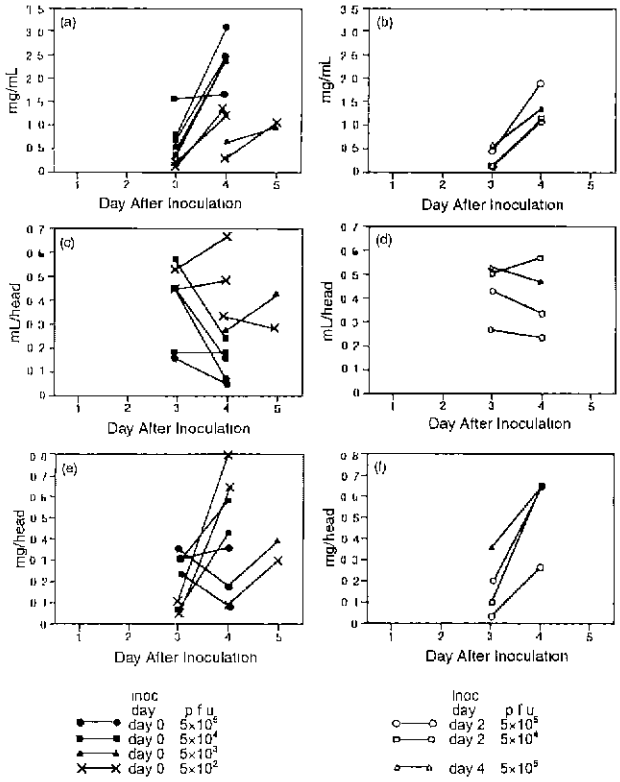

Fig. 6. Accumulation of rpoIL-2 in HyPIL2cp- infected larvae hemolymph and the hemolymph recovery Each silkworm larva groups were inoculated virus of 5×10^5 to 5×10^2 plaque forming units (p.f.u.) at day 0 to day 4 of fifth instar. Hemolymph samples were recovered on 3rd and 4th days or 4th and 5th days, post inoculation. Almost all of the larvae did not live beyond the period

mori The efficiency in this system was up to 20 times higher than that of the AcNPV expression system. Our findings demonstrate that the production efficiency is equivalent to the yield with transgenic livestock. Silkworm expression system is, in particular, highly suitable for the production of small proteins, such as cytokines, toxins and hormones, because hemolymph of silkworm seldom has endogenous proteins below 20 kDa. Furthermore, it is possible to confirm the quality of the product in short time, with low cost, in ordinal laboratory and facilities. These facts suggest that the silkworm expression system is highly suitable for mass-production of cytokines or rare proteins.

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