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Expressions of Bovine IFN-γ and Foot-and-Mouth Disease VP1 antigen in *P. pastoris* and their effects on mouse immune response to FMD antigens

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Abstract

As a highly contagious disease in cloven-hoofed animals, foot-and-mouth disease virus (FMDV) may cause a considerable social-economic loss in those countries affected. IFN-γ has a wide range of antiviral and immune modulating functions. Thus, to study the immune enhancing effects of recombinant Bovine IFN-γ (rBoIFN-γ) on a recombinant FMDV vaccine, BoIFN-γ, FMDV VP1 and BoIFN-γ/VP fusion genes were cloned, expressed, co-expressed in pichia pastoris (*P. pastoris*) respectively, and subsequent immune effects have been evaluated in this study. The results showed that the genes encoding for BoIFN-γ, VP1 and BoIFN-γ/VP1 are successfully expressed in *P. pastoris* and their products are directly secreted into the cultural supernatant at a high level of 1.0 g/L analyzed by thin-layer scanning. In addition, rVP1 alone could induce both humoral and marginal cell-mediated immune responses in mice, while the group with co-inoculations of rBoIFN-γ could markedly enhance both humoral and cell-mediated immune responses; even more dramatic immune responses were observed with the group inoculated with the fusion product, rBoIFN-γ/VP1. The fusion product could be further investigated for its utility of FMDV vaccine development.

Keywords: BoIFN-γ; FMDV; Immune adjuvant; *P. pastoris*

1. Introduction

Foot-and-mouth disease virus (FMDV) is a member of the family *Picornaviridae*, genus *Aphthovirus* that causes a highly contagious disease in cloven-hoofed animals and may exert destructive social-economic impacts on the countries affected [1]. Up to date, the vaccination with inactivated FMDV is a major means to prevent and control this disease in most developing countries. However, the inactivation processes are technically challenging and can sometimes lead to incomplete inactivation, which is considered as a risk to massively employ the conventional vaccines which only stimulates limited immune responses [2]. Though newly developed genetic recombinant vaccines have become alternatives characterized by the advantages of being safer and more economic, their general disadvantage is the failure to induce more effective and complete immune responses in inoculated animals [3]. Therefore, it is necessary to develop various immune adjuvants to enhance immune effects of vaccines. Despite of several commercialized adjuvants available such as the complete Freund's adjuvant (CFA), aluminum salts, QS21, ISCOM, etc., but their

Abbreviations: FMDV, foot and mouth disease virus; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RT-PCR, reverse transcriptase PCR; PBMC, peripheral blood mononuclear cell; LB, Luria Bertani; BSA, bovine serum albumin; KDa, kilo-dalton; OD, optical density; MW, molecular weight; HPRT, hypoxanthine phosphoribosyl transferase; MTT, 3-(4,5-dimethylthiazol-2-yi)-2,5-diphenyltetrazolium; CFA, complete Freund's adjuvant; DTH, delayed-type hypersensitivity; LPA, lymphocyte proliferation assays

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immune effects and toxicities are still in questions [4]. Recently, the concept of adjuvant has been expanded to include the employment of recombinant cytokines in combination with newly developed vaccines, this group of adjuvants is considered as genetic or molecular adjuvants [5–7].

It is observed that FMDV-carrier animals often have significant levels of neutralizing antibodies, but no immunopathological change is found in possibly infected tissues [8], which suggests that cell-mediated immunity may be involved in the clearance of persistent FMDV, and thus, it has been hypothesized that the initiation of FMDV persistence is correlated with the amount of the interferons produced in cells [9]. The ability of FMDV to form cytopathic effect (CPE) in the cell culture is apparently correlated with the suppression of the alpha/beta interferon [10]. These evidences indicate the inhibitory effect induced by IFNs.

To investigate the immune modulation effects of the recombinant BoIFN-γ on FMDV vaccination, the coding regions for BoIFN-γ gene and FMDV capsid protein VP1 or fused product from both genes were expressed in yeast expression system, *Pichia pastoris*, respectively, and subsequently, immune responses of each regimen have been used to evaluate the ability to induce immune responses in BALB/C mice.

2. Materials and methods

2.1. Reagents and antigens

RNA isolation and reverse transcription reagent Kits were purchased from Promega (Madison, Wisc., USA), *ExTaq* DNA polymerase and all restriction enzymes were purchased from TaKaRa (Dalian, China), *P. pastoris* expression vector, pPICZαA, was from Invitrogen (Carlsbad, CA, USA), horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, MTT and TMB were from Sigma (St. Louis, USA). Eight-week-old female BALB/C mice were purchased from the Institute of Genetics of Chinese Academy of Sciences.

FMDV O-serotype inactivated vaccine in oil emulsion was acquired from Zhongmu Ltd. (Beijing, China), and the 146S antigen component was obtained from the purified as described previously and stored at 4 °C [4,11]. The concentration of the 146S antigen was determined by the Bradford protocol as described previously [4].

2.2. Cloning of BoIFN-γ and FMDV VP1 genes

After isolation of peripheral blood mononuclear cells (PBMC) from Holstein cow and stimulated with Con A (10 μg/ml) for 2 h in vitro, total RNA was extracted and reverse transcribed into cDNA by using RNA isolation kit and reverse transcription reagent kit (Promega Inc.) according to the manufacturer's instructions. The BoIFN-γ gene was amplified from the cDNA using an upstream primer (5′-GATGGTACCTGCCACCTGCCTCACACCC-3′, containing K*pn* I site underlined) and a downstream primer (5′-CGATCTAGAGTCCTTTCTCCTGAAACTCTC-3′, containing X*ba* I site underlined), an *ExTaq* DNA polymerase, 4 × dNTP and suitable buffer in a programmed thermal cycler with 32 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min.

For the co-expression of FMDV VP1 and BoIFN-γ in *P. pastoris*, the VP1 fragment was amplified from the plasmid pMD18-VP1 (gifted from Jin Huali, China Agricultural Univesity) using an upstream primer (5′-GCAGAATTCACCACCTCTGCGGGTGAGTCT-3′ with an E*coR* I site underlined) and a downstream primer (5′-GACGGTACC*GGTGGCGGTGGCGGT*CAGAAGCTGTTTTGCGGGT-3′ with a K*pn* I site underlined) and five glycine residues as a linker in italic under the conditions of a six initial cycle of denaturation at 94 °C for 1 min, annealing at 51 °C for 1 min and extension at 72 °C for 1 min; then annealing temperature was elevated to 55 °C to continue 28 cycles, and a final extention at 72 °C for 10 min.

The PCR products of BoIFN-γ and VP1 were both purified by low melting-point agarose gel and digested with K*pn* I and X*ba* I, or E*coR* I and K*pn* I, respectively. The expression vector pPICZαA (Invitrogen) was also digested with K*pn* I and X*ba* I, or E*coR* I and K*pn* I, or E*coR* I and X*ba* I, respectively [12]. All the digested fragments were ligated by T_4 DNA ligase to yield three constructs, designated as pPICZα/BoIFN-γ, pPICZα/VP1 and pPICZα/BoIFN-γ/VP1, respectively. Constructs were transformed into *E. coli* JM109 in a low salt LB plate with 25 μl/ml of zeocin selection, followed by the identification procedures using both restriction enzyme digestions and a PCR. The further confirmation is performed by sequencing with the pPICZαA sequencing primers.

2.3. Expressions of BoIFN-γ, VP1 and BoIFN-γ/VP1 in P. pastoris GS115

Five to ten micrograms of each plasmid was linearized by S*ac*I digestion, and then transformed into 80 μl of competent GS115 cells by electroporation using GenePulser (Bio-Rad, CA, USA) under the conditions of 1500 V, 25 μF, 200 Ω and 4.96 ms. The recombinant transformants were identified according to the manufacturer's protocol (Invitrogen).

Each of confirmed colonies was inoculated into 25 ml of cultural media, BMGY (pH 5.6) [13], and shaken vigorously (≥300 rpm) at 28–30 °C until the OD_{600} value reached 2–6. The cultures were centrifuged at room temperature and the collected pellets were suspended in 100–200 ml of induction culture medium, BMMY (pH 5.6) [13], till the OD_{600} value reached 1.0. The expression was induced initially at 28–30 °C with ≥300 rpm shaking by addition of methanol every 24 h to achieve a final concentration of 1.0%. One milliliter of the culture was sampled at various time points during 1–5 days to determine the optimal conditions as described previously [13,14].