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Expression of bioactive recombinant bovine interferon-γ using baculovirus

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SUMMARY

The precise role of bovine interferon-γ (BoIFN-γ) in disease and therapy is still poorly defined. Clearly it is involved in defence against parasites, bacteria, viruses and possibly tumor cells. This paper reports the expression of BoIFN-γ in a baculovirus system to generate a fully functional recombinant protein. Bovine interferon-γ cDNA was cloned from mitogen stimulated peripheral blood mononuclear cell (PBMC) RNA utilizing the reverse transcription-polymerase chain reaction (RT-PCR). The cDNA open reading frame (ORF) encoding for a putative 166 amino acid protein (22KDa) was cloned and expressed into baculovirus transfer vector pBlueBac 4.5/V5 His. This vector was co-transfected with *Autografa californica* multiple nuclear polyhedrosis virus (AcMNPV) DNA into *Spodoptera frugiperda* cells (Sf9) and the recombinant virus, named AcBoIFN-γ, was then recovered. Recombinant BoIFN-γ (rBoIFN-γ His) was accumulated in the serum-free medium of AcBoIFN-γ-infected cells. The nickel affinity spin column purified rBoIFN-γ His was shown to be a glycosylated 20-22 KDa protein as confirmed by SDS-PAGE glycan determination and showed antiviral activity *in vitro* against the bovine viral diarrhoea-mucosal disease virus (BVD/MD). The production of this bioactive rBoIFN-γ His will allow us to explore this cytokine as a potential vaccine adjuvant or therapeutic agent for bovine diseases.

KEY WORDS: baculovirus/bovine IFN/expression/insect cells.

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INTRODUCTION

Interferons are a group of immunoregulatory proteins belonging to the cytokines family. They are capable of regulating a wide spectrum of biological functions, including immune and inflammatory responses, tissue repair and antiviral properties. In particular, interferon-γ (IFN-γ) has been reported to induce antiviral activities against many viruses in vitro (Sen and Ransohoff, 1993) and is used for medical treatments against persistent virus infections in humans and experimentally in animals (Fleischman *et al.*, 1980; Gribaudo *et al.*, 1987) IFN-γ is produced by activated T lymphocytes and natural killer (NK) cells in response to antigenic or mitogenic stimulus. It is a powerful activator of mononuclear phagocytes, increasing their ability to destroy intracellular microorganisms and tumor cells. It induces many types of cells to express class II MHC molecules and can also increase the expression of class I, and facilitates differentiation of both B and T lymphocytes. IFN-γ is a powerful activator of NK cells

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and also activates neutrophils and vascular endothelial cells (Billiau, 1996). The gene that encodes IFN-γ in cattle is found on chromosome 5 (Chaudhary *et al.*, 1993; Walrand *et al.*, 1989; Weissmann and Weber, 1986).
Recently, recombinant DNA techniques have made it possible to produce cytokines on a large scale, which will make it economically feasible to bring them into general use for the control of many infectious animal diseases (Walrand *et al.*, 1989; Digby and Lowenthal, 1995; Tizard, 1995; Murakami *et al.*, 2001; Di Napoli *et al.*, 2004; Ruttanapumma *et al.*, 2005). These reagents are valuable tools for dissecting the immune response in order to understand how cytokines control the responses leading to protective effector mechanisms. Moreover, the availability of recombinant cytokines can be useful in the diagnosis of infectious diseases that present a cell-mediated immunity reactivity.
In this paper, we describe the cloning, expression and purification of bovine interferon-γ (BoIFN-γ) as a fully functional recombinant protein using the baculovirus gene expression system. The biological activity of the rBoIFN-γ produced was evaluated and its possible practical use as a therapeutic molecule in some infections of importance in the field of veterinary sciences is also discussed as adjuvant in the formulations of new innovative vaccines or as base molecules for the development of kits for laboratory diagnosis.

MATERIALS AND METHODS

Construction of the bovine IFN-γ cDNA recombinant transfer vector

Bovine PBMC were obtained from a 2-year-old healthy cow and isolated by lysing buffer then stimulated with 5µg/ml concanavalin A (ConA) (Sigma-Aldrich, Italy). In brief, 1ml of EDTA-treated blood was added to 20 ml of Lysing buffer (17 mM TrisHCl, pH 7.2, 144 mM NH_4Cl) and incubated for 30 min at room temperature (RT). After centrifugation at 1600 x g for 10 min at RT, PBMC were washed three times in RPMI-1640 medium supplemented with 10% FCS, 1% L-glutamine, 200 U/ml penicillin, 200 U/ml streptomycin and counted. Viability was evaluated by trypan blue dye exclusion. Aliquots of $2x10^5$ freshly isolated viable PBMC in 200 µl of complete medium containing 5µg/ml Con A were plated in 96-well plates. After incubation for 72 hours at 37°C in a humidified 5% CO_2 incubator, the cells were harvested and mRNAs were extracted using Quick prep micro mRNA purification kit (Amersham Bioscience, UK). First strand cDNA synthesis and reverse transcription reaction were completed using a Ready to go RT-PCR beads two-step kit (Amersham Bioscience, UK). Oligonucleotide primers used for reverse transcriptase-polymerase chain reaction (RT-PCR) (forward: 5'-GCGGATCCCACCATGAAATATA-CAAGCTATTTCTTA-3' and reverse 5'-CCG-GAATTCGTTGATGCTCTCCGG-3') were designed from the sequence data by Cerretti *et al*. 1986, (Gene Bank: M29867) to include the entire coding region of the BoIFN-µ to construct cDNAs which included the open reading frame carrying BamHI restriction site at the 5' end and EcoRI at 3' end. RT-PCR was performed using Ready to go RT-PCR beads two-step (Amersham Bioscience, UK). In brief, first-strand cDNAs were generated by using Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase with specific antisense primers and then double strand cDNAs were amplified by polymerase chain reaction (PCR) with sense primers. Each of the 35 cycles of PCR involved 1 min of denaturation at 95°C, 1 min of annealing at 55°C, and 2 min of extension at 72°C. The PCR products were ligated into baculovirus transfer vector pBlueBac4.5/V5 His (Invitrogen, UK) to construct the parent plasmid pBBIFN-µ. Recombinant plasmids were isolated from transformed *E.coli* DH5α (Stratagene, UK) following selection for ampicillin resistance. Ligation of IFN-γ cDNA into ten clones was confirmed with restriction enzyme digest and detection of the approximately 518-bp product in ethidium bromide-agarose gels. The confirmed clones were sequenced by the dideoxy method with a Dye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems, Italy).

Generation of the recombinant baculovirus

Log phase Sf9 cells ($2.5x10^6$ cells/ml) were seeded in a 60 mm plate with serum free Sf-900 II medium (Invitrogen, UK) and then co-transfected using a mixture containing 4µg of the plasmid pBBIFN-γ, 0.5 Ìg of linearized Bac-N-Blue DNA (Invitrogen, UK), 20 µl of Cellfectin reagent

(Invitrogen, UK), in 1 ml of serum-free Sf-900 II medium. The cells were then incubated at RT for 4 h. At the end of the incubation the cells were allowed to grow for 5 days at 28°C before the medium was harvested. Recombinant viruses exhibiting the ,-galactosidase-positive phenotype were selected and isolated by plaque assay with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Roche Diagnostics, Italy) and amplified three rounds before being used for expression. Single blue plaques were selected as recombinant virus (AcBoIFN-γ) and analyzed by PCR to establish the presence of IFN-γ insert and to confirm the isolation of a pure recombinant plaque. The recombinant plaque was used to generate a large scale, high-titer viral stock in Sf9 cell cultures.

Expression of recombinant bovine IFN-γ (rBoIFN-γ His)

For production of recombinant protein, Sf9 cell cultures were carried out in tissue culture plates and in spinner culture flasks. For monolayer culture in the culture plate, the Sf9 cells were seeded at $3x10^6$ cells/60 mm plate and recombinant virus with a titre of $2.8x10^7$ plaque-forming units/ml was used to infect the Sf9 cells at a virus-to-cell ratio of 5 m.o.i. The cells were then allowed to grow in serum-free medium (Tsujimoto *et al*., 1983; Doverskog *et al*., 2000) in the presence of proteases inhibitor (10μg/ml of PMSF, 0.5 μg/ml of Leupeptin, 0.5 μg/ml of Aprotinin and 1μg/ml of Pepstatin A) at 28°C for 5 days before the medium was harvested for rBoIFN-γ His purification. The protocol for expression in spinner flask culture was identical except that cells were seeded at $2.5x10^6$ cells/ml and were under constant stirring at 60 rpm. To evaluate the production of rBoIFN-γ His in the cultured infected Sf9 cells, aliquots of each cell culture were harvested at different times post-infection (p.i.) and analyzed by an indirect immunofluorescence assay (IFA). In brief, $2x10^5$ cells were applied to a slide, air dried and fixed in 1% fresh paraformaldehyde in PBS for 15 min at RT. The slides were then incubated for 1 hour at RT with anti-V5 monoclonal antibody (Invitrogen, UK) at a dilution of 1/200 in PBS containing 1% BSA, and after three washes in PBS, incubated for 30 min at RT with FITC-conjugated goat anti-mouse IgG (Dako, Denmark) at a dilution of 1/40 in PBS. After another three washes in PBS, the slides were mounted in glycerol/PBS (1:1 vol/vol) and examined under an UV-light microscope.

Purification and analysis of the rBoIFN-γ His

AcBoIFN-γ-infected Sf9 cell culture medium was collected from day 1 through day 6 p.i., clarified by centrifugation, and concentrated by Microcon concentrators (Millipore, Italy); the medium was then loaded on a nickel chelated column (Talon, Clontech Laboratories, CA) according to the manufacturer's instructions to purify the expressed protein rBoIFN-γ His under native conditions. The affinity purified protein was gel filtered with a PD-10 column (Amersham Pharmacia Biotech, Italy) in PBS to remove the imidazole which was used in the elution of the protein from the Talon column. The purity of rBoIFN-γ His was then evaluated by 15% SDS-PAGE and Comassie brilliant blue staining and their density was measured by Versadoc densitometer (Bio-Rad Laboratories, Italy).
The proteins on the gel were then blotted onto nitrocellulose membrane (Hybond-C, Amersham Bioscience, UK) for Western blot analysis. The membrane was blocked for 2 h at RT with blocking reagent (Roche Diagnostics, Italy) and was further incubated for 1 h with anti-V5 monoclonal antibodies (Invitrogen, UK) diluted 1:5,000 in blocking buffer followed by incubation for 1 h with peroxidase-conjugated anti-mouse IgG antibodies (Dako, Glostrup, Denmark) diluted 1:1.000 in blocking buffer. The membrane was then treated with 4-chloro-1-naphthol (Bio-Rad Laboratories, Italy). To detect the glycosylation of the expressed proteins, a Dig Glycan Detection kit (Roche Diagnostics, Italy) was used according to the manufacturer's instructions. In brief, the purified rBoIFN-γ sample was applied to SDS-PAGE and blotted onto nitrocellulose membrane. Then the glycosylated polypeptides were detected with the kit.
The concentration of purified rBoIFN-γ His was determined by a quantitative ELISA assay (LiStarFISH-Milan, Italy).

RESULTS

To clone a cDNA of a BoIFN-γ gene, PMBCs were isolated by Lysing buffer from peripheral blood of healthy cattle. This simple and time-efficient

technique required a total time of less than 1hour and the bovine PMBCs isolated by this method were highly purified (>97%) as determined by Wright staining and with a viability >90% after up to 18 hours in culture, as determined by trypan blue exclusion.
RT-PCR amplification of BoIFN-γ mRNA with two primers located at 94-591 nt. yielded a product about 518 bps, which was the expected size when examined on a 2% agarose gel. This product was cloned into the BamHI and EcoRI sites to place the gene under the control of the polyhedrin promoter of the pBlue Bac 4.5/V5 His transfer vector. After sequence confirmation by the dideoxy terminator method, the recombinant baculovirus was obtained by transfecting the Sf9 cells with the linear viral DNA (BacNBlue-Invitrogen, UK) and the transfer vector that contains the BoIFN-γ gene (Fig. 1a).
Single blue plaque was isolated (Fig. 1b) and amplified three rounds before the titre of the recombinant virus was determined by plaque assay. For expression in spinner flasks, a higher viral titre ($2x10^8$ PFU/ml) was attained by fourth-round amplification.
The expression conditions were optimized in 60 mm tissue culture plates and plaque-forming unit to cell ratios (MOI) of 1, 5, 10 and 20 were used in expressions that were allowed to proceed for 6 days. As control of the rBoIFN-γ expression in each batch of transfected cells, aliquots of Sf9 culture were analyzed by IFA at 12, 24, 48, 72, 96, 120 h post-infection (Fig. 2).
The optimal expression yield in the culture medium was observed at the MOI of 5. Recombinant BoIFN-γ expression also increased with the incubation time, but the best expression levels, which averaged 150-200 AU/ml, were observed at day 4 of incubation.

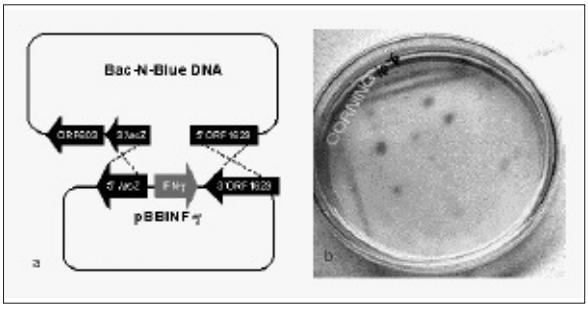

FIGURE 1 - *The generation of recombinant baculovirus expressing bovine interferon-gamma. a) Recombination between pBBIFN-γ DNA and Bac-N-Blue DNA. b) Recombinant blue plaques in Sf9 cells co-transfected with pBBIFN-γ DNA and Bac-N-Blue DNA.*

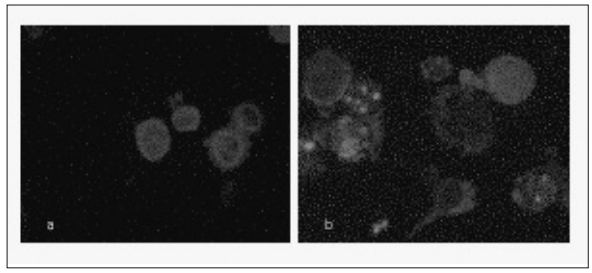

FIGURE 2 - *Indirect immunofluorescence analysis of Sf9 cells infected with AcBoIFN-γ. a) Sf9 control cells; b) rBoIFN-γ His expression in Sf9 infected cells (48 h p.i.).*

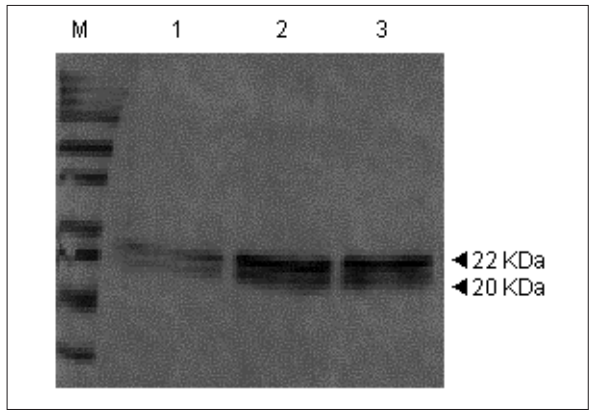

FIGURE 3 - *Western blot analysis of purified rBoINF-γ His: lane 1, BoINF-γ 48h p.i. with AcBoINF-γ; lane 2, BoINF-γ 72h p.i. with AcBoINF-γ; lane 3, BoINF-γ 96h p.i. with AcBoINF-γ; M, molecular weight markers.*

rBoIFN-γ was loaded onto the Talon metal affinity spin column and the bound protein was eluted with 150 mM Imidazole in 50 mM Na_3PO_4, 500 mM NaCl, 10% Glycerol, 0.1% Nonidet-P40. Following concentration with a Microcon concentrator the recombinant protein was analyzed by Western blot analysis and, as shown in Fig. 3, there were two significant bands at 20 and 22 KDa position, which were the expected size for the glycosylated forms of BoIFN-γ.
To analyse the antiviral activity of rBoIFN-γ an in vitro cytopathic effect reduction assay was used (Sentsui *et al*. 2001; Wu *et al*., 2002). In brief, 50 µl of a serially 2-fold diluted sample were added to each well of 96-well flat-bottom microplates in triplicate per dilution $2x10^6$ bovine turbinate (BT) cells/well in EMEM supplemented with 3%

FCS, 1% L-glutamine, 200 U/ml penicillin, 200U/ml streptomycin (Flow Laboratories, Irvine,UK) were seeded on the previous day, and further incubated at 37°C for 24 h in a humidified 5% CO_2 incubator. After washing twice with PBS, each well was challenged with 100 µl of EMEM containing 10^7 PFU of bovine viral diarrhoea-mucosal disease virus (BVD/MD) at a final multiplicity of infection (MOI) of 0.1 and incubated for 1h with gentle rocking at 37°C, then unattached virus was then removed and each well was overlayed with 100 µl 0.5% methyl cellulose in medium. After an additional 18 to 24 h of incubation, methyl cellulose was removed and wells were stained for 3 min with 0.5% crystal violet in 70% methanol. The stain was removed by immersing the plates in water several times and plaques were then counted and rBoIFN-γ activity of individual samples was calculated (Cerretti *et al*., 1986).
In vitro growth of BVD/MD was suppressed by more than 0.45 AU/ml of rBoIFN-γHis in BT cells (Flow Laboratories, Irvine, UK).

DISCUSSION

The high levels of expression of foreign genes reported for baculovirus systems (King and Possee, 1992; O'Reilly *et al*., 1992), the fact that foreign genes undergo posttranslational modification, and the ability to scale up protein production encouraged the use of this system for expression of recombinant bovine IFN-γ. The plasmid pBlue Bac 4.5/V5 His, used in our work, is a baculovirus transfer vector used for expression of recombinant proteins in lepidopteran insect cell lines. It utilizes the polyedrin promoter from *Autografa californica* multiple nuclear polyhedrosis virus (AcMNPV) for high-level expression of the gene of interest (Crawford and Miller, 1988). In addition, it contains the following features:

1) a C -terminal peptide encoding the V5 epitope and a 6xHis tag for detection and purification respectively;
2) the SV40 polyadenylation signal for increased transcription termination efficiency and mRNA stability;
3) the early-to-late promoter for expression of full-length β-galactosidase in recombinant virus;
4) the ampicillin gene and the pUC origin for selection, replication, and maintenance of the vector in *E. coli*;
5) this vector is derived from pJVETL-Z and contains the 5' polyedrin mRNA leader sequence of the baculovirus transfer vector pVL941. Sf9 cells infection with recombinant AcBoIFN-γ resulted in the expression of 20-22kDa proteins, as detected in the culture supernatants by Western blotting analysis and within the cells by IFA.

Glycosylation of recombinant IFN-γ indicated that the protein had undergone posttranslational processing and this is important in terms of extending the biological half-life of the protein. Thus, baculovirus expression could potentially yield a product with high specificity, a potentially greater in vivo half-life than yeast or *E.coli* derived proteins, and be easily scaled up to produce large amounts of protein for clinical use. Usually the expression level of a foreign gene does not vary from experiment to experiment, provided the infection conditions are optimal (cell viability of >98%, cells in exponential growth, use of high titre virus stocks to establish one step growth). Using a nickel chelated column, rBoIFN-γ His was purified with about 95% purity and the titre of purified rBoIFN-γ His was 150-200 AU/ml.
Although it is difficult to quantify the baculovirus derived interferon, other than in terms of arbitrary units, it can be concluded that recombinant IFN-γ can be stably expressed in the baculovirus system and that the protein product appears to be biologically active showing antiviral activity *in vitro* against the BVD/MD.
The ultimate aim of this work was to take the recombinant protein towards clinical application. We have thus developed an efficient expression system for glycosylated and biologically active recombinant bovine IFN-γ. This system has many advantages for the purification of rBoIFN-γ, and such purified material may be suitable for use in vitro for the production of polyclonal and monoclonal antibodies and for use in vivo to assess the potential of bovine recombinant interferon-gamma as a viral vaccine adjuvant in cattle.

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