Appl Biochem Biotechnol (2011) 163:338–345
DOI 10.1007/s12010-010-9042-9

Development of DNA-Designed Avian IgY Antibodies for Quantitative Determination of Bovine Interferon-Gamma

Gholamreza Nikbakht Brujeni · Sayed Amir Hossein Jalali · Mohammad Kazem Koohi

Received: 5 April 2010 / Accepted: 12 July 2010 /
Published online: 22 July 2010

Abstract Interferon-gamma (IFN-γ), a cytokine produced by sensitized T lymphocytes, is one of the key elements in defining T helper 1 lymphocyte immune responses. Quantitative evaluation of IFN-γ expression could provide an important analytical tool for measurement of cell-mediated immunity and investigating immune responses to infectious diseases. Method of DNA-designed avian IgY antibodies was used for production of monospecific polyclonal antibodies that allows quantification of the recombinant bovine IFN-γ protein. IFN-γ cDNA was subcloned and expressed in mammalian expression plasmid (pcDNA3.1 (+)) under the control of the human cytomegalovirus promoter. Chickens were immunized by plasmid DNA, and eggyolk antibodies extracted from eggs were collected after immunization. IgY-specific antibodies were evaluated by an antigen capture enzyme-linked immunosorbent assay (ELISA) using recombinant IFN-γ. Based on the results, developed bovine IFN-γ capture ELISA could detect up to 1 ng/ml of IFN-γ by 64-fold diluted IgY. Monospecific anti-bovine IFN-γ antibodies generated in chickens are useful for quantifying different concentrations of recombinant bovine IFN-γ, which is expressed in cell culture.

Keywords Bovine interferon gamma · Capture enzyme-linked immunosorbent assay (ELISA) · DNA-designed IgY

G. Nikbakht Brujeni (✉)
Department of Microbiology and Immunology, Faculty of Veterinary Medicine,
University of Tehran, Tehran, Iran
e-mail: nikbakht@ut.ac.ir

S. A. H. Jalali
Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
e-mail: smjalali@ut.ac.ir

M. K. Koohi
Department of Toxicology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
e-mail: mkkoohi@ut.ac.ir

Introduction

Interferon-gamma (IFN-γ), the only member of the type II class of interferons, is a 19.4-kDa glycoprotein mainly secreted by sensitized T lymphocytes. It is known to have a key role in the immune response to many intracellular bacteria via its effect on macrophage activation and induction of class I and class II MHC. In vitro production of bovine IFN-γ by antigen stimulation of sensitized T lymphocytes have been considered as a sensitive and specific indicator of *Mycobacterium spp*. exposure in cattle [1, 2]. Quantification of IFN-γ in body fluids and tissues also can be a useful tool for assessment of inflammation or disease progression [1, 3–5].

Capture enzyme-linked immunosorbent assay (ELISA) is a sensitive assay for measurement of picogram to microgram quantities of substances, such as hormones, cell signaling chemicals, infectious disease antigens, and cytokines. Monoclonal antibodies are usually used for this method to increase its sensitivity and specificity [6]. However, preparation of monoclonal antibodies for capture ELISA is expensive, laborious, and requires great skill [7]. In order to find a feasible and straightforward preparation, this study was designed to produce anti-bovine IFN-γ antibodies in lying hens. Production of antibodies according to DNA-designed avian IgY antibodies technology was followed by genetic immunization of chickens with a plasmid encoding a given antigen. Specific and biologically active IgY antibodies are transmitted vertically from their serum into the egg and accumulate in the egg yolk [8]. This approach allows direct generation of antibodies from plasmid DNA and avoids the costly and tedious preparation of purified antigens required for conventional antibody productions [9].

The major low-molecular-weight serum immunoglobulin in chicken is IgY, which can be obtained easily from the egg yolk. As compared to mammalian IgG, chicken IgY has a slightly larger molecular mass (approximately 167 kDa) immunoglobulin repertoires [10]. Chicken egg yolk immunoglobulins (IgY) from immunized hens have considerable advantages for the production of polyclonal antibodies. It reacts with more epitopes on a mammalian antigen because of their evolutionary differences, and it is feasible to use for capturing or detecting such antigens. IgY also has the advantage in that, it avoids the interference in immunological assays caused by the complement system, rheumatoid factors, anti-mouse IgG antibodies, or human and bacterial Fc receptors [11]. These differences in molecular interaction bring advantages to the application of IgY antibodies; they have been used successfully in a variety of methods in different areas of research, diagnostics, medical application, and biotechnology [12, 13].

In this study, we took advantage of "DNA-designed" egg yolk antibody for the production of polyclonal, monospecific antibodies against the recombinant bovine IFN-γ (rBoIFN-γ), and then combined this antibody to develop a sensitive and specific capture ELISA for the quantification of rBoIFN-γ secretion in cell culture.

Materials and Methods

Cloning, Sequencing, and Expression of BoIFN-γ Gene

Total cellular RNA was isolated from the spleen tissue using RNX^{plus} reagent (Cinnagen Company, Tehran, Iran) following the manufacturer's procedure. First-strand cDNA synthesis was performed using a RevertAid™ M-MuLV Reverse Transcriptase Kit (Fermentas, GmBH, Germany). BoIFN-γ cDNA production was conducted using a BoIFN-γ-specific primer, 5′-GTGATATCCTGAAGCGCCA-3′ (start from 1,097 bp), complementary to the 3′ untranslated region (UTR) of cattle IFN-γ mRNA (Accession no: NM174086). BoIFN-γ cDNA

amplification was performed in two steps (nested polymerase chain reaction (PCR)), using the same 3′ primer with addition of a specific PCR primer corresponding to the 5′ UTR of the BoIFN-γ sequence, 5′-CATAACACAGGAGCTACCG-3′ (start from 40 bp). In the first step, PCR was carried out for 30 cycles: one cycle of 94 °C for 3 min, 30 cycles of 94 °C for 60 s, 56 °C for 60 s, and 72 °C for 2 min, with a terminal step of 10 min at 72 °C using Bio Rad thermal cycler (Bio Rad, Mexico). BoIFN-γ cDNA open reading frame (ORF) was synthesized with 5 μl of the first-step amplification product after a second round of PCR, utilizing a specific forward primer flanked on its 5′ side by a HindIII restriction site and Kozak site, 5′-GAAGCTTAC C**ATG**AAATATACAAGCTATTTCTTAGCTTTACTGCTCTTGTGGG-3′, and a specific reverse primer flanked on its 5′ side by a XbaI restriction site, 5′-GGTCTAGA**TTA**CGTT GATGCTCTCCGGCCTCG-3′, following steps: one cycle of 94 °C for 3 min, 30 cycles of 94 °C for 60 s, 57 °C for 45 s, and 72 °C for 2 min, with a terminal step of 10 min at 72 °C in thermal cycler device (Bio Rad, Mexico). In this step, fragment of 516 bp was amplified.

To facilitate sequencing, PCR products were initially cloned into pDrive cloning vector using the Qiagen PCR cloning kit (Qiagen, Milden, Germany), and recombinant plasmids were transformed into *Escherichia coli* DH5α. Three positive clones were sequenced by Macrogen Inc. (Seoul, South Korea) in an ABI 3730 XL automatic DNA sequencer to confirm sequence identity of the cloned cDNA encoding the BoIFN-γ gene. The BoIFN-γ cDNA sequence in this step included 432 bp corresponding to mature protein and 69 bp signal peptide.

BoIFN-γ cDNA ORF portions of the recombinant pDrive plasmid was directly subcloned into the 5′ HindIII and 3′ XbaI restriction enzyme sites of the pcDNA3.1(+) expression cloning vector (Invitrogen, Carlsbad, CA , USA). Recombinant pcDNA3.1 plasmid (pcDNA3.1-IFN-γ) was isolated from transformed *E. coli* DH5α and purified using the plasmid DNA purification kit (Qiagen, Milden, Germany). Plasmid pcDNA3.1-IFN-γ was sequenced bidirectionaly to verify correct insertion and sequence fidelity.

Vector pcDNA3.1-IFN-γ was used to transfect COS-7 cells. Diethylaminoethyl (DEAE)-dextran method was applied for COS-7 cell transfection, and culture supernatant was collected after 24 h growth [14]. Collection of supernatant was followed every 48 h for 2 weeks.

ELISA for rBoIFN-γ Expression Assay

Sandwich ELISA for the detection of rBoIFN-γ expression in culture supernatant was performed using a bovine IFN-gamma ELISA kit according to the manufacturer's instruction (MABTECH, Hamburg, Germany). The standard curve allowed calculation of rBoIFN-γ concentration in supernatant using the relationship $f(y)=0.0003\ f(x)+0.0612$ (R2=0.9908), where $f(y)$ is the titer response as A450, and $f(x)$ is the rBoIFN-γ concentration.

Sequence Analysis

BoIFN-γ cDNA and protein sequences were analyzed by BLAST through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov; Altschul et al. 1997). The sequence alignments were made using Clustal software, and also, bovine IFN-γ protein prediction was done using the BioEdit version 7.0.5.3 software package (http://jwbrown.mbio.ncsu.edu/Bioedit/bioedit.html).

Production of Polyclonal Antibodies Against rBoIFN-γ

White leghorn chickens at 24 weeks of age were obtained from the local breeding unit, the laboratory animal resources of Amin Abad (Tehran, Iran). DNA immunization was carried out

by the modified method described by Christine Rollier [15]. Chickens received 300 μg of pcDNA3.1-IFN-γ (dissolved in saline buffer) injected intramuscularly in three sites (anterior quadriceps of both legs and breast), and booster doses were given 4 and 6 weeks later at the same sites. Eggs were collected 7 days after the third immunization to extract IgY antibodies. Isolation of IgY was carried out according to the method previously described by Deog Yong Lee [13].

Titration of Anti-rBoIFN-γ Antibodies

Optimization of the antibody titer was conducted using a checker board titration of ELISA. In each microplate well, 100 μl of the diluted rBoIFN-γ, ranging from 1 pg ml^{-1} to 10,000 pg ml^{-1}, was coated by overnight incubation at 4 °C. After incubation, unbound antigens were removed, and the plate was washed three times using phosphate-buffered saline (PBS) and Tween-20, 0.5%. Then the wells were blocked by 100 μl of PBS containing Tween 20 (0.5%) and 5% BSA. After three additional washing steps, 100 μl of egg yolk antibodies (anti-BoIFN-γ IgY) was added to the wells using four serial fourfold dilutions, starting at 1:64 dilution of egg extracts and left for 30 min at 37 °C. The plate was then washed, and 100 μl horse radish peroxidase-labeled goat anti-chicken IgY (Sigma-Aldrich, MO, USA), pre-diluted to 1:500, was added to each well and left for 30 min at 37 °C. After washing steps, 100 μl/well 2,2′-azino-bis-3-ethylbenz-thiazoline-6-sulfonic acid (ABTS; KPL, USA) substrate was added, and the mixture was incubated in the dark place for 15 min at RT. The reaction was then stopped by the addition of 100-μl/well stopper solution (1% SDS). Absorbance at 405 nm was measured using a microplate reader (Stat Fax 2000, Awareness Technology, Inc., USA). Controls for this assay comprised sera from immunized chickens as positive control and from non-immunized chickens as negative control.

Antigen Capture ELISA

To develop an antigen capture ELISA for quantification of BoIFN-γ, a polystyrene plate was coated by egg yolk extract that was 1:64 diluted in coating buffer. The plate was blocked and washed as described above. Standard rBoIFN-γ (MABTECH, Hamburg, Germany), ranging from 1 pg to 10,000 pg diluted in PBS was added to plate and incubated for 2 hr at 37 °C. After washing steps, 100 μl of monoclonal antibody PAN-biotin (MABTECH, Hamburg, Germany) at 0.1 μg ml^{-1} in PBS-BSA 1% was added and incubated for 1 h. After additional washing steps, 100 μl Streptavidin-HRP (MABTECH, Hamburg, Germany), diluted 1:1000 in PBS–Tween, was added to each well and incubated at 37 °C for 1 h. The reactions were developed, and OD values were measured as mentioned before. Detection limit of the ELISA was determined at the lowest concentration of rBoIFN-γ shown P/N≥2. Quantification of bovine IFN-γ in different culture supernatants were also carried out using both of the commercial ELISA kit (MABTECH, Hamburg, Germany) and the above mentioned procedure.

Results and Discussion

This paper reports the ability of DNA-based immunization with plasmid expressing rBoIFN-γ protein for the production of anti-rBoIFN-γ antibodies in chickens. According to sequencing data analysis, the ORF of BoIFN-γ cDNA was 501 bp, and the nucleotide sequence of BoIFN-γ cDNA encodes a 166 aa protein that has one nucleotide substitution. Substitution was observed in nt A_{432} to G_{432}, but aa sequence has 100 homology with BoIFN-γ aa sequence from GB

database. Corresponding nucleotides and predicted aa bovine IFN-γ sequences with accession number FJ263670 are illustrated in Fig. 1. The predicted aa sequence of rBoIFN-γ was 100% homologous to bovine IFN-γ, 95% homologous to caprine and ovine IFN-γ, 86% homologous to camel, and 83% to lama IFN-γ. Homology to non-ruminant species was considerably less, with 77% homology to equine, 75% homology to feline and canine IFN-γ, 61% homology to human IFN-γ, 44% homology to mice IFN-γ, and 35% homology to chicken IFN-γ. Recombinant IFN-γ of all four ruminant species contained 166 aa residues, and the greatest aa homology occurs between these sequences. Its high sequence homology might be the best explanation for the preparation of proper anti-IFN-γ antibody that is able to react against IFN-γ from other ruminant species. It may also suggest further applications of rBoIFN-γ or directly pcDNA3.1-IFN-γ for cytokine therapy in different animals.

ELISA test showed that COS-7 cells successfully transfected with pcDNA3.1-IFN-γ containing cytomegalovirus (CMV) promoter by using DEAE transfection method and secreted rBoIFN-γ protein in supernatant with maximum level at 96 h (9,438 $pg.ml^{-1}$) and 144 h (9443 $pg.ml^{-1}$) after transfection, and secretion continued for 2 weeks (Fig. 2). Stoeckle et al. (1996) [16] have demonstrated that human IFN-γ can be secreted in vitro by transfected HeLa cells and fibroblasts using a replication defective adenovirus for >4 weeks. To express caprine IFN-γ protein in COS-7 cells, Beyer et al. (1998) [17] have used vectors containing CMV and caprine arthritis encephalitis virus long terminal repeat (CAEV LTR) promoters and employed Lipofectamine transfection method. In their experiment, protein concentration in supernatant had been calculated as 2,100 pg ml^{-1} for CMV and 400 pg ml^{-1} for CAEV LTR promoters . Our results have revealed that rBoIFN-γ could be secreted in a higher concentration (9,438–9,443 $pg.ml^{-1}$). It is possibly related to the higher number of IFN-γ transcripts, due to intracellular amplification of the plasmid containing SV40 origin and greater intrinsic promotional activity of the CMV promoter.

A variety of gene transfer and expression systems are available, but there has been intense research into the development of efficient vector systems and transfection methods. These methods basically are different in their use of chemical, viral, bacterial, and synthetic

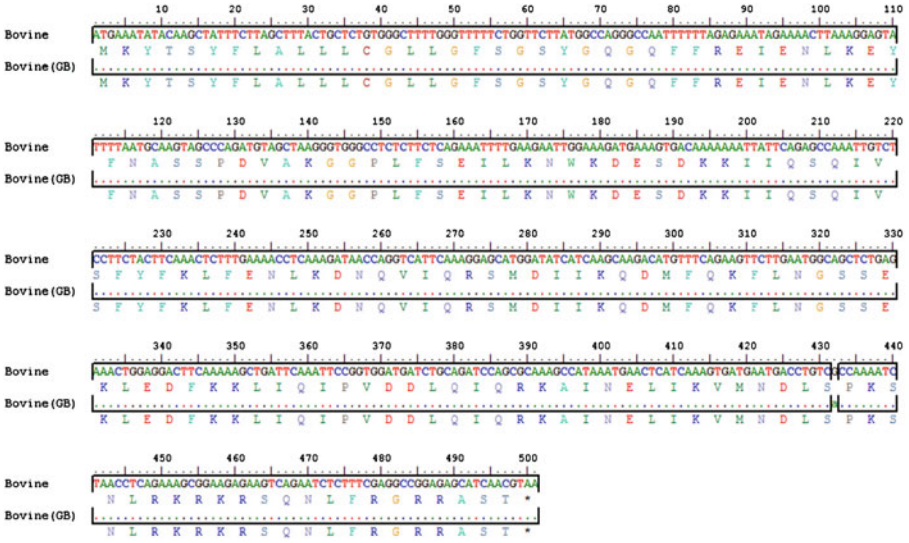

Fig. 1 Nucleotide and predicted aa sequences of rBoIFN-γ and alignment with bovine data base sequence

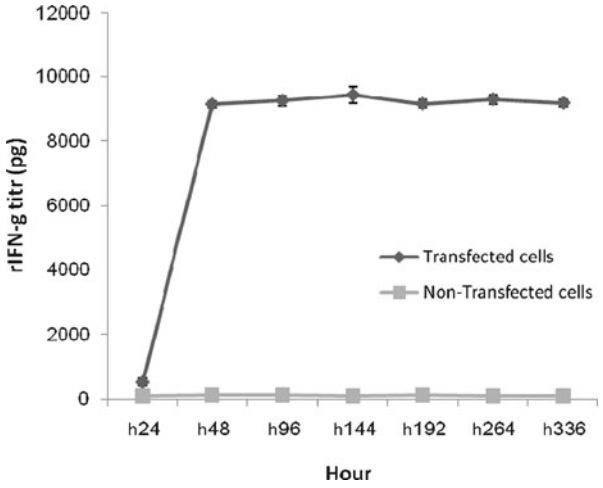

Fig. 2 Protein expression assay, ELISA test has shown for rBoIFN-γ expression in cell culture supernatant after 48 h of transfection for 336 h

delivery vectors. Although this research has mainly concerned expression of recombinant IFN-γ from bovine on mammalian cell lines that were transfected with synthetic delivery vectors by a chemical method, but selecting a system for production of recombinant proteins cannot always predict how well the protein will be expressed in the selected host. In general, DEAE-dextran DNA transfection is ideal for transfection and is suitable for overexpression of recombinant protein in transient transfections. The major advantages of the technique are its relative simplicity and speed, limited expense, and remarkable transfection efficiency. Since IFN-γ has been shown to possess numerous immunomodulatory capabilities and antiviral activity, the development of the efficient production system described in this report is an important breakthrough to establish IFN-γ reagent for bovine diseases. At present, recombinant DNA techniques have made it possible to produce IFN-γ on a large scale, which will help make it economically feasible to bring IFN-γ into general use for control and treatment of many infectious diseases in animals.

The result of in vivo expression of rBoIFN-γ protein showed that this protein was expressed successfully and induced a good immune response in chickens. Immunization of hens with pcDNA3.1-IFN-γ plasmids provides polyclonal antibody due to strong immunogenicity and sensitive epitope recognition.

It has been illustrated that normally, 100~200 mg/ml of IgY per yolk could be recovered in egg [13, 18]. Therefore, rBoIFN-γ-specific IgY production might be estimated at 2 to 20 mg per yolk, because the constitution of specific IgY is between 2% to 10% [19, 20]. Indirect ELISA for titration of IgY antibody to rBoIFN-γ has shown that 64-fold diluted and 1:256 diluted IgY responded 1 to 1,000 pg ml^{-1} of rBoIFN-γ. Based on the results, optimal IgY concentration was about 64 diluted (Fig. 3). However, there was a difference of tendency in IgY titer for using coated protein in a microtiter plate.

The DNA-designed IgY against rBoIFN-γ has many advantages compared with protein immunization, including no more need for purified protein for immunization, quality of antibodies generated, large yield, and easy scalability of IgY production, noninvasive production of antibodies, the elimination of adjuvant such as Freund's, and advantages of IgY as immunodiagnostic reagent [21]. However, protein continues to be expressed by DNA plasmid for a longer term stimulation of antibody production [22]. DNA-designed

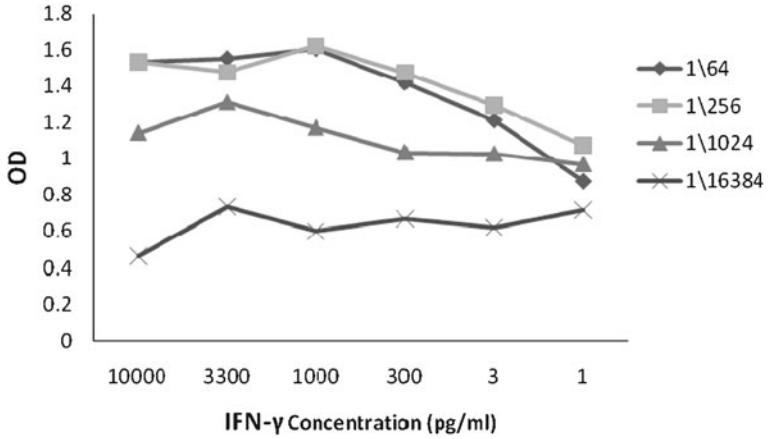

Fig. 3 Antibody titer of chicken IgY against rBoIFN-γ. Concentration of rBoIFN-γ protein ranged from 1 to 10,000 pg ml^{-1} and IgY was four times serial-diluted. IgY was up to 1:64 dilutions for reaction with rBoIFN-γ protein

IgY also has particular value as immunodiagnostic tools for immunofluorescence, immunohistochemistry, ELISA, western blotting, and immunoelectrophoresis [23].

Nonetheless, DNA-designed IgY was used instead of a monoclonal antibody for the capturing antibody, the sensitivity could still reach the nanogram level. However, in comparing with the chicken immunized by white rhinoceros IFN-γ protein, genetic immunization lead to enhance the sensitivity of IgY response (from micro-gram to nano-gram) [24], but the change in sensitivity of IgY was not observed with immunization by recombinant porcine interleukin-6 protein in chicken [13]. On the other hand, the antibody response elicited by plasmid immunization seems to be affected by multiple factors [25] and needs further studies on protein characteristics that can improve immunogenicity of recombinant proteins.

Capture ELISA conditions were optimized with anti-BoIFN-γ IgY antibodies on the basis of the titration. In the optimized condition, the optimal antigen capture ELISA could

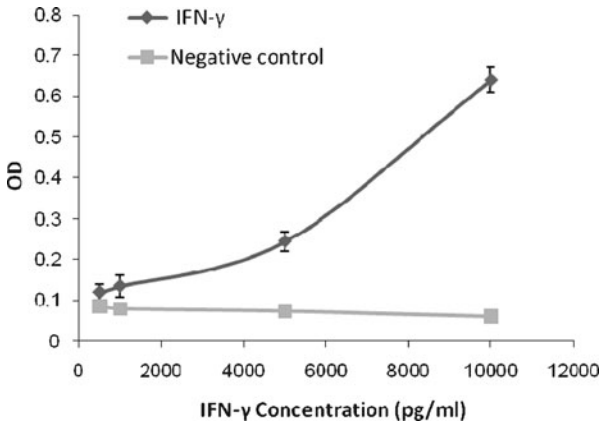

Fig. 4 Antigen capture ELISA for detection of rBoIFN-γ. Concentration of rBoIFN-γ ranged from 500 to 10,000 pg ml^{-1} and was normalized by PBS as negative control. Developed capture ELISA has detected about 1 ng ml^{-1} of rBoIFN-γ concentration in supernatant of cell culture

reliably detect concentrations of rBoIFN-γ, ranging from 500 to 10,000 pg ml^{-1} (Fig. 4). Comparison between commercial ELISA kit (MABTECH, Hamburg, Germany) and our in-house procedure demonstrated that with no significant differences, IgY antibodies produced against bovine IFN-γ could be used for quantification of bovine IFN-γ in culture supernatant. The production of IgY anti-bovine IFN-γ also can be used in different T cell assays like ELISPOT and ELISA, and it does not seem to have any limitation with assays of different body fluids.

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