Journal of Animal and Veterinary Advances 2 (12): 648-653, 2003

Expression and Functional Characterization of Recombinant Turkey IFN-γ

Chien C. Loa, Ming K. Hsieh, Ching C. Wu and Tsang L. Lin
Department of Veterinary Pathobiology, Purdue University, West Lafayette, Indiana 47907-2065, USA

Abstract: The turkey interferon gamma (IFN-γ) protein was expressed in a prokaryotic expression system as histidine-tagged fusion protein and functional activity of the recombinant protein was examined. The turkey IFN-γ gene was subcloned from a recombinant pCR3.1-IFNγ construct to the prokaryotic expression vector pQE30. The recombinant expression construct (pQE-IFNγ) was identified by polymerase chain reaction and sequencing analysis. Expression of histidine-tagged fusion protein with molecular mass of 23 - 24 kd was determined by Western blotting analysis with monoclonal antibodies specific to the histidine-tag or chicken IFN-γ. The protein recovery was 7 mg from 500 ml of bacterial culture. The purified recombinant protein was capable of activating macrophage and inducing production of nitric oxide. The functional activity of the recombinant protein could be neutralized by a monoclonal antibody specific to chicken IFN-γ. These results indicated that the expressed protein was a biologically functional turkey IFN-γ protein.

Key words: Bioassay, expression, macrophages, nitric oxide induction, neutralization, recombinant interferon-γ, Turkey

Introduction

Cytokines are proteins produced by cells of immune system in response to infections. Cytokines play pleiotropic roles in the regulation of immune responses including activation and differentiation of naïve immune cells to effector cells and subsequently enhance the immune functions and production of other cytokines. As natural modulators of immune responses, cytokines are potential candidates of replacements for in-feed antibiotics and chemicals as growth promotants. Although antibiotics and chemicals have been widely used as additives in the animal feed to control the early infections and improve the production, the residues of these additives in commercial meats cause serious concern of public health and may result in emerging resistant mutants of pathogenic microorganisms (Hilton *et al.*, 2002).

Among the cytokines, interferon-gamma (IFN-γ) had been proved as promising therapeutic agent and growth promotant. Administration of chicken IFN-γ alone enhanced body weight gain of broilers (Lowenthal *et al.*, 1997). The increase of weight gain could be 7.9 %. Treatment of chickens with recombinant chicken IFN-γ protein demonstrated protective effect against *Eimeria* infection and improvement of weight gain loss associated with this disease (Lowenthal *et al.*, 1997; Lillehoj and Choi, 1998) . In addition, IFN-γ could be potential vaccine adjuvant. Co-administration of antigen and chicken IFN-γ resulted in enhanced primary and secondary antibody responses that persisted at higher levels in comparison with chickens received antigen alone (Lowenthal *et al.*, 1998). Immunization of cat with antigen and recombinant cat IFN-γ protein significantly enhanced antibody titers to a level comparable with that evoked by commonly known saponin or aluminum adjuvants (Schijns *et al.*, 2002).

The use of IFN-γ as therapeutic agent in turkey is becoming more feasible with the recent cloning of turkey IFN-γ gene in different laboratories including ours (Lawson *et al.*, 2001 and Loa *et al.*, 2001). Although the cross-reactivity between turkey and chicken IFN-γ was demonstrated in the laboratory, the extents of this cross-reactivity in vivo are not clear. Comparatively, turkey type I IFN had lower level of cross-species activity than chicken type I IFN (Suresh *et al.*, 1995). Administration of turkey IFN-γ was reported to have acute immunostimulatory activity against organ invasion of *Salmonella enteritidis* in chicken (Farnell *et al.*, 2001). However, co-administration with turkey IFN-γ down-regulated the protective immune responses of turkeys to *Chlamydophila psittaci* stimulated by a DNA vaccine (Vanrompay *et al.*, 2001). Further investigation for the in vivo application of turkey IFN-γ in turkey or chicken is still required.

Production of large amount of protein is a critical first step for investigation and application of turkey IFN-γ as therapeutic agent, growth promoter, or vaccine adjuvant in vivo. In addition, sufficient quantity of protein is required for generation of monoclonal antibodies and development of enzyme-linked immunosorbent assay for detection of turkey IFN-γ, which is an important tool for evaluation of cell-mediated immunity. Although the application of turkey IFN-γ protein was demonstrated in a study with chicken (Farnell *et al.*, 2001), the expression and production of the protein has never been documented. In order to obtain a reliable source of biologically active protein and a large amount of protein, the objective of the present study was to subclone the turkey IFN-γ gene to a prokaryotic expression system, express, and characterize the recombinant IFN-γ protein.

Materials and Methods

Construction of IFN-γ Gene in the Expression Vector pQE30: The entire coding region of turkey IFN-γ was obtained from the recombinant pCR3.1-IFNγ construct (Loa *et al.*, 2001) by digestions with restriction enzymes Kpn I and Pst I. The digested product was separated from pCR3.1 vector by agarose gel electrophoresis and recovered from the gel by Zymoclean kit (Zymo Research, Orange, CA). The cleaned digested product was cloned to Kpn I and Pst I sites of the pQE30 expression vector (Qiagen, Chatsworth, CA) according to manufacturer's instruction. The pQE30 expression system allowed the expression of recombinant IFN-γ protein with a six histidine-tagged sequence on the N-terminal end. The construct was transformed to competent *Escherichia coli* strain M15 (Qiagen). Transformants were grown in LB medium containing 100 $\mu g\ ml^{-1}$ ampicillin and 25 $\mu g\ ml^{-1}$ kanamycin. Plasmids were purified by QIAquik mini-prep kit (Qiagen) and sequenced by Purdue Genomic Center (West Lafayette, IN) to confirm that the inserted IFN-γ gene was in frame with the vector. The correct construct was referred as pQE-IFNγ.

Expression of Recombinant IFN-γ Protein in *E. coli*: For expression of the recombinant protein, M15 bacteria transformed with pQE-IFNγ plasmid DNA were inoculated in a tube containing 25 ml of LB broth supplemented with 100 $\mu g\ ml^{-1}$ ampicillin, 25 $\mu g\ ml^{-1}$ kanamycin, and 1 % glucose and cultured overnight at 37°C in a shaking incubator (250 rpm). The 25 ml culture was transferred to a 1,000 ml flask containing 500 ml of LB broth supplemented with 100 $\mu g\ ml^{-1}$ ampicillin, 25 $\mu g\ ml^{-1}$ kanamycin and 1 % glucose. The flask was shaken at 37° C until the culture reached an OD 600 of 0.5. Protein expression was induced by addition of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). Before the addition of IPTG and at 1, 2, 3, or 4 hr after the addition of IPTG, 1 ml of the culture was collected and centrifuged. The bacteria pellet was resuspended in Laemmli sample buffer (Laemmli, 1970) and boiled for 5 min before analysis with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.

Extraction of Recombinant IFN-γ Protein from Bacteria Cell lysate: The bacteria were harvested by centrifugation at 10,000 x g for 10 min. The supernatant was discarded and the cell pellet was resuspended in Bugbuster reagent (Novagen, Madison, WI) with a volume of 1 ml for every g of pellet (wet weight). After complete resuspension of the pellet, a mixture of nuclease solution, Benzonase (Novagen), was added to remove the viscous nucleic acids at a volume of 1 μl for every 1 ml of Bugbuster reagent. The mixture was gently rotated at room temperature for 20 min. The lysate was then centrifuged at 16,000 x g for 20 min at 4°C. The supernatant and inclusion body pellet were analyzed by SDS-PAGE and Western blotting for the presence of recombinant IFN-γ protein.

Purification of Recombinant IFN-γ Protein by Chromatography with Nickel-NTA Column: The inclusion bodies containing the recombinant IFN-γ protein were resuspended in the same volume of Bugbuster reagent as that used to resuspend the bacterial pellet. Lysozyme was added to a final concentration of 200 $\mu g\ ml^{-1}$ and incubated at 25°C for 5 min. Addition of 5 volume of Bugbuster reagent at a dilution of 1:10 was followed by centrifugation at 10,000 xg for 20 min. The treated inclusion bodies were dissolved in Binding buffer containing 5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, 8 M urea, 14 mM 2-mercaptoethanol, and 0.3 % SDS at pH 7.9. The dissolved inclusion bodies were filtered through a 0.45 nm syringe filter (Millipore, Bedford, MA) and incubated with nickel chelating NTA slurry (10 mg protein ml^{-1} of gel) at 25°C for 1 hr. The mixture was loaded to a column and washed with 10 bed volumes of Washing buffer (20 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, and 8 M urea at pH 7.9). The recombinant IFN-γ protein was eluted from the column with Eluting buffer containing 1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, and 8 M urea at pH 7.9. Fractions eluted from the column were analyzed by SDS-PAGE on a 15 % polyacrylamide / bisacrylamide gel (Laemmli, 1970). Identity of the recombinant IFN-γ protein was confirmed by SDS-PAGE of fractions eluted from the column and Western blotting analysis of electrotransferred protein on nitrocellulose membrane (Millipore) with monoclonal antibodies (Mab) specific to histidine tag (Qiagen) or chicken IFN-γ (kindly provided by Dr. H. S. Lillehoj, USDA, Beltsville, MD).

SDS-polyacrylamide Gel electrophoresis and Western Immunoblotting: The samples were solubilized in sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 1 % SDS, 10 % glycerol, 0.001 % bromophenol blue, and 1 % 2-mercaptoethanol and boiled for 5 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out using the discontinuous buffer system (Laemmli, 1970). Polypeptide bands were revealed by staining the gel with coomassie brilliant blue G-250. For immunoblotting, polypeptides separated by SDS-PAGE were electrotransferred onto nitrocellulose membrane (Millipore) with transfer buffer containing 50 mM Tris, 384 mM glycine, and 20 % (v/v) methanol, pH 8.3. Electrotransfer was performed at 65 volts for 1 hr. The nitrocellulose membrane was incubated 1 hr in PBS buffer containing 0.05 % Tween 20 (PBS-T). After washing three times in PBS-T, membrane was incubated for 2 hr at room temperature with Mab specific to histidine tag (Qiagen) or chicken IFN-γ (Dr. H. S. Lillehoj) at 1:200 of dilution in PBS-T. Three times of washing were followed by addition of peroxidase-conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD). After

incubation of 2 hrs at room temperature, the membrane was washed three times and covered with the peroxidase substrate, 3,3'-diaminobenzidine (DAB). The blot was allowed to develop and the reaction was stopped by washing the membrane in distilled water.

Bioassay for Nitric Oxide-inducing Activity: Macrophage-activating activity of IFN-γ was measured as the ability to induce MQ-NCSU (Qureshi *et al.*, 1990) chicken macrophages to secrete nitric oxide as previously described (Loa *et al.*, 2001). Briefly, serial dilutions of recombinant IFN-γ samples were added at 100 μl per well to 96-well cell culture plates in triplicate. MQ-NCSU cells were added at a concentration of 1 x 10^5 cells / 100 μl per well in RPMI 1640 medium supplemented with 10 % fetal bovine serum. The cultures were incubated in a humidified CO_2 incubator at 41°C for 24 hrs. The contents of nitric oxide in the culture supernatants were analyzed by Greiss reagent (Promega, Madison, WI) according to the manufacturer's instruction. The titer of IFN-γ was determined as the dilution factor that conferred the production of nitric oxide equivalent to 6 μM.

Neutralization of Recombinant IFN-γ Protein: Recombinant Turkey IFN-γ protein was diluted to a concentration of 10 μg ml^{-1} and mixed with an equal volume of Mab specific to chicken IFN-γ (Dr. H. S. Lillehoj). The mixture was incubated at 37°C for 4 hr. The remaining IFN-γ activity was analyzed by the bioassay for nitric oxide-inducing activity.

Results

Construction and expression of IFN-γ gene in the expression vector pQE30: The entire open reading frame corresponding to turkey IFN-γ gene ligated to Kpn I and Pst I sites of plasmid pQE30 was confirmed by sequencing of both strands (Fig. 1). The reading frame of IFN-γ gene was in frame with the upstream six histidine-tagged sequence in the vector. Expression of the construct, pQE-IFNγ, in the host cell M15 was induced with IPTG. Time course studies of induction of the recombinant fusion protein by IPTG indicated that the expression of IFN-γ protein was initially observed at 2 hrs and increased from 2 to 4 hrs according to the analysis of Western blotting (Fig. 2B) with Mab specific to histidine tag, although the corresponding protein band was not detectable by staining of coomassie blue on SDS-PAGE (Fig. 2A). The induction with IPTG for 4 hr was selected in order to produce more IFN-γ protein.

Extraction and Purification of Recombinant IFN-γ Protein: Soluble and pellet (inclusion body) fractions obtained by centrifugation in the extraction were examined by SDS-PAGE and Western blotting analysis (Fig. 3, lanes 3 and 4). The results indicated that recombinant IFN-γ protein was not readily soluble in the buffer. Most of the protein was found in the inclusion

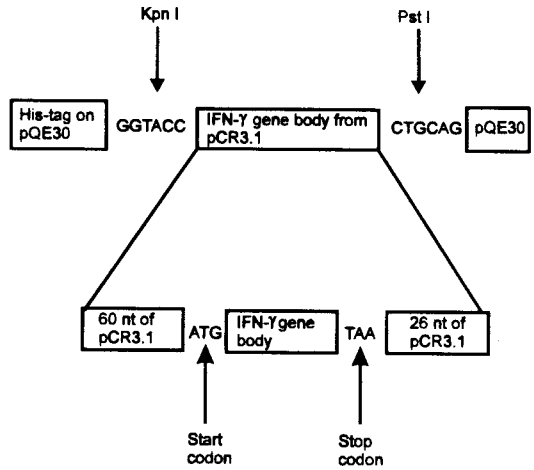


Fig. 1: Construction of pQE-IFNγ expression vector.

body. After treatment with lysozyme, the protein corresponding to the recombinant IFN-γ was further enriched according to SDS-PAGE and Western blotting analysis (Fig. 3, lane 5). The treated inclusion body was dissolved in the 8 M urea-containing buffer and purified by chromatography on a nickel-NTA column. The recombinant protein was eluted with 1 M imidazole-containing buffer. As shown in Fig. 3, SDS-PAGE analysis indicated the presence of two major protein bands with molecular mass about 23 and 24 kd in the purified recombinant protein (Fig. 3, lane 8). The molecular mass of 24 kd is similar to the expected histidine-tagged fusion IFN-γ protein. The protein band of 23 kd is probably result of degradation of the recombinant IFN-γ protein. There was another weak band with molecular mass about 44 kd. This weak band may represent the homodimeric form of the recombinant turkey IFN-γ protein as demonstrated for expression of recombinant chicken IFN-γ in the literatures (Farnell *et al.*, 2001 and Schijns *et al.*, 2002). These protein bands were recognized by Mab specific to histidine tag or chicken IFN-γ in the Western blotting analysis (Fig. 3B and 3C). Determination of protein recovery indicated that 7 mg of IFN-γ protein could be purified by chromatography on nickel-NTA column from 500 ml of bacterial culture.

Functional Characterization of Recombinant Turkey IFN-γ: The recombinant IFN-γ protein alone induced the secretion of nitric oxide from MQ-NCSU macrophage cells. The titer of each mg of the recombinant protein was 800 as determined by the nitric oxide-induction assay with serial dilutions of the purified IFN-γ (Fig. 4). The biological activity of recombinant turkey IFN-γ could be neutralized by a Mab against chicken IFN-γ (Fig. 5). The IFN-γ activity was markedly reduced from 0.217 to 0.071 with treatment of the antibody.

Discussion

The results of the present study demonstrated cloning

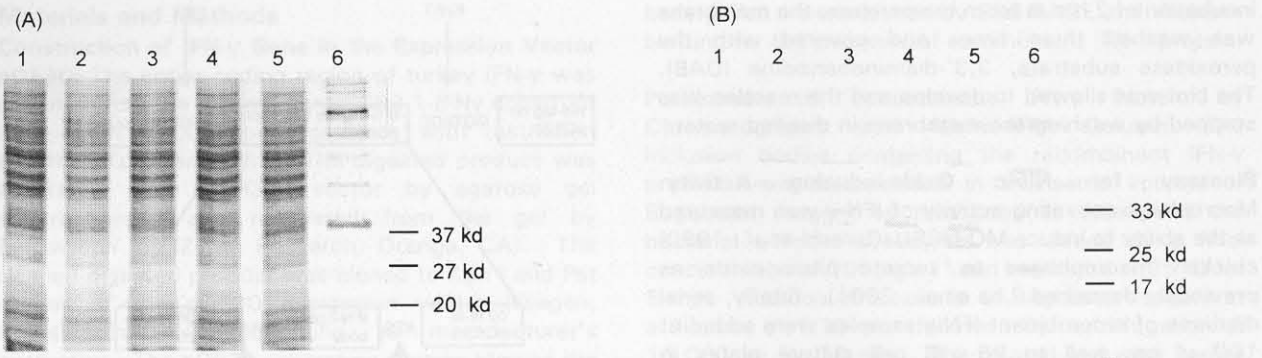
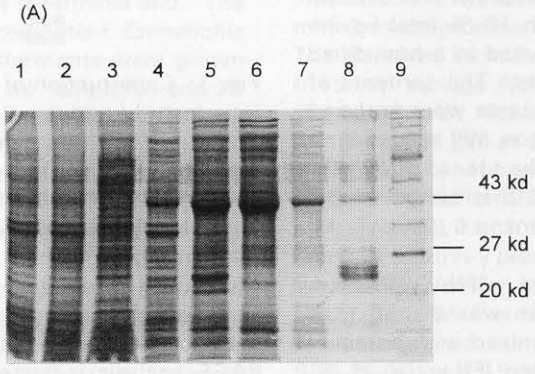
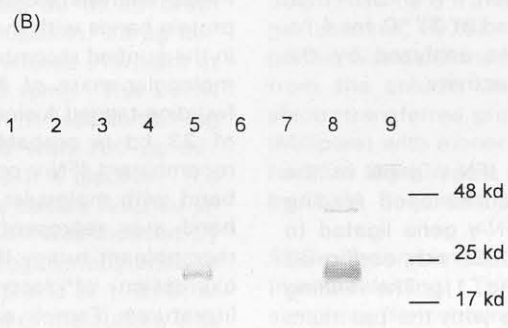


Fig.2: Induction of interferon gamma (IFN-γ) fusion protein expression by treatment of bacteria containing pQE-IFNγ with IPTG





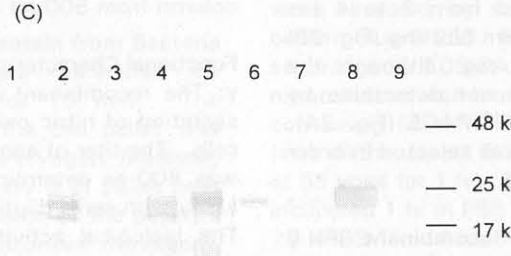


Fig.3: Purification of recombinant interferon gamma (IFN-γ) protein from M15 bacteria expressing pQE-IFNγ by chromatography on nickel-NTA column

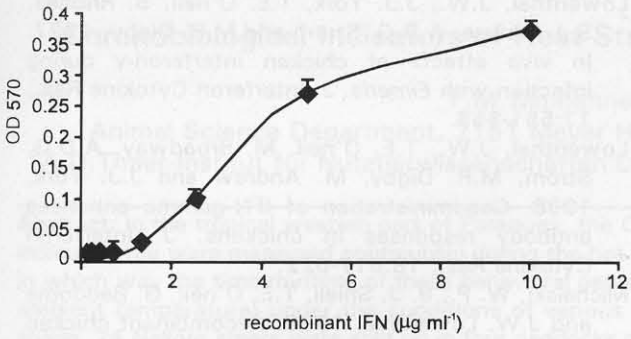


Fig. 4: Functional activity of recombinant turkey IFN-γ protein.

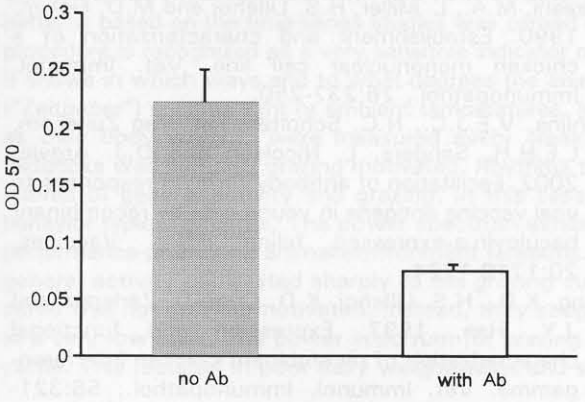


Fig. 5: Neutralization of recombinant turkey IFN-γ with monoclonal antibody specific to chicken IFN-γ.

and expression of a gene encoding turkey IFN-γ as a histidine-tagged fusion protein in *E. coli* as well as the purification by chromatography on nickel chelating agarose column. The recombinant IFN-γ protein was an efficient macrophage activating factor and induced production of nitric oxide. The functional activity of this recombinant protein was neutralized by a Mab specific to IFN-γ protein. These results indicated that the expressed protein was a functional turkey IFN-γ.

The size of turkey and chicken IFN-γ gene is the same (Loa *et al.*, 2001). It is speculated that the molecular mass of turkey and chicken IFN-γ protein should be similar based on the same size of genes. Variations in molecular weight of chicken IFN-γ have been reported in the literatures (Lawenthal *et al.*, 1997; Song *et al.*, 1997 and Lambrecht *et al.*, 1999) due to differences in glycosylation. The molecular mass of natural IFN-γ present in supernatants of chicken spleen cells stimulated with concanavalin A was 26 - 27 kd. Interferon gamma prepared from a hybridoma $CD4^+$ T cell cultures was found to be 22 - 23 kd (Song *et al.*, 1997). The molecular mass of recombinant chicken IFN-γ expressed by *E. coli* was about 17-18 kd (Lawenthal *et al.*, 1997; Song *et al.*, 1997 and Lambrecht *et al.*, 1999), which represented the monomeric form of non-glycosylated protein excluding the signal sequences.

According to these studies, the observed molecular mass at 23-24 kd of the expressed fusion IFN-γ protein in the present study is within the expected range. There are 38 additional amino acids for the histidine tag in the N-terminal of the expressed fusion IFN-γ protein plus 19 amino acids of the signal sequences. These extra amino acids increase the molecular mass of expressed target protein by approximately 6.3 kd. Degradation of a recombinant chicken IFN-γ protein expressed by pQE30 system was reported to be caused by cleavage of the 12 C-terminal amino acids (Lawenthal *et al.*, 1997 and Michalski *et al.*, 1999). The possible cleavage site is located in a highly conserved motif, Lys Arg Lys Arg, at 10 to 13 amino acids from the C-terminal end. Though the significance of this motif is not clear, it is conserved among IFN-γ proteins of all avian and mammalian species including turkey. It is possible that the same event of cleavage as reported for the recombinant chicken IFN-γ resulted in the observed degradation of recombinant turkey IFN-γ in the present study. It was demonstrated that the C-terminally truncated form of recombinant chicken IFN-γ had comparable biological activity with that of full-length form (Michalski *et al.*, 1999).

Detection of homodimeric form of chicken IFN-γ in the purified recombinant protein from *E. coli* was commonly demonstrated in the literatures (Lawenthal *et al.*, 1997 and Lambrecht *et al.*, 1999). Functional IFN-γ protein is a non-covalent homodimer (Ealick *et al.*, 1991). The feature characteristics of sensitivity to acid or heat of IFN-γ protein were probably because the disruption of homodimer formation.

Similar to the antiviral activity of cytokines IFN-α and IFN-β, IFN-γ has the capacity to inhibit viral replication. The type I IFNs (IFN-α and IFN-β) are secreted immediately after infection of somatic cells by virus. In contrast, production of IFN-γ awaits stimulation of $CD4^+$ (helper) T cells with MHC II-restricted presentation of antigens and is in a later release during viral infection. In addition, IFN-γ is a pleiotropic cytokine that plays key roles in modulating immune responses to infections while the type I IFNs do not participate in stimulation of immune responses. Interferon gamma is an important regulatory molecule in the type 1 T helper cell-driven cellular immune responses that are necessary for resistance to intracellular pathogens. The expression and purification procedures as described in the present study provide a simple and efficient method to obtain functional turkey IFN-γ in large quantity. The availability of recombinant protein facilitates the investigations of turkey IFN-γ as a potential candidate for immunotherapy and / or vaccine adjuvant in Turkey by modulating the immune responses. In addition, large amounts of turkey IFN-γ protein are necessary for production of Mab and the development of quantitative methods for monitoring this cytokine during the course of diseases in turkeys.

Acknowledgements

The authors thank Dr. H. S. Lillehoj for providing the monoclonal antibody to chicken IFN-γ. This work is supported by the United States Department of Agriculture.

References

Ealick, S. E., W. J. Cook, S. Vijay-Kumar, M. Carson, T. L. Nagabhushan, P. L. Trotta and C. E. Bugg, 1991. Three-dimensional structure of recombinant human interferon-gamma Sci., 252:698-702.

Farnell, M.B., M.E. Halawani, S. You, B.M. McElroy Hargis and D.J. Caldwell, 2001. In vivo biologic effects of recombinant-turkey interferon-gamma in neonatal leghorn chicks: protection against Salmonella enteritidis organ invasion. Avian Dis., 45:473-478.

Hilton, L.S., A.G.D. Bean and J.W. Lowemthal, 2002. The emerging role of avian cytokines as immunotherapeutics and vaccine adjuvants. Vet. Immunol. Immunopathol., 85:119-128.

Laemmli, U. K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227:680-685.

Lambrecht, B., M. Gonze, D. Morales, G. Meulemans and T. P. van der Berg, 1999. Comparison of biological activities of natural and recombinant chicken interferon-gamma. Vet. Immunol. Immunopathol., 70:257-267.

Lawson, S., L. Rothwell, B. Lambrecht, K. Howes, K. Venugopal and P. Kaiser, 2001. Turkey and chicken interferon-γ, which share high sequence identity, are biologically cross-reactive. Dev. Comp. Immunol., 25:69-82.

Lillehoj, H. S. and K. D. Choi, 1998. Recombinant chicken interferon-gamma-mediated inhibition of *Eimeria tenella* development in vitro and reduction of oocyst production and body weight loss following *Eimeria acervulina* challenge infection. Avian Dis., 42:307-314.

Loa, C.C., M.K. Hsieh, C.C. Wu and T. L. Lin, 2001. Molecular identification and characterization of turkey IFN-γ gene. Comp. Biochem. Physiol., 130B:579-584.

Lowenthal, J.W., J.J. York, T.E. O'neil, S. Rhodes, S.J. Prowse, A.D.G. Strom and M.R. Digby, 1997. In vivo effects of chicken interferon-γ during infection with *Eimeria*. J. Interferon Cytokine Res., 17:551-558.

Lowenthal, J.W., T.E. O'neil, M. Broadway, A.D.G. Strom, M.R. Digby, M. Andrew and J.J. York, 1998. Coadministration of IFN-gamma enhances antibody responses in chickens. J. Interferon Cytokine Res., 18:617-622.

Michalski, W. P., B. J. Shiell, T.E. O'neil, G. Beddome and J.W. Lowenthal, 1999. Recombinant chicken IFN-gamma expressed in *Escherichia coli*: analysis of C-terminal truncation and effect on biological activity. J. Interferon Cytokine Res., 19:383-392.

Qureshi, M.A., L. Miller, H.S. Lillehoj and M.D. Ficken, 1990. Establishment and characterization of a chicken mononuclear cell line. Vet. Immunol. Immunopathol., 26:237-250.

Schijns, V.E.J.C., N.C. Scholtes, H.I. Van Zuilekom, L.E.H.H. Sanders, L. Nicolson and D.J. Argyle, 2002. Facilitation of antibody forming responses to viral vaccine antigens in young cats by recombinant baculovirus-expressed feline IFN-γ. Vaccines, 20:1718-1724.

Song, K.D., H.S. Lillehoj, K.D. Choi, D. Zarlenga and J.Y. Han, 1997. Expression and functional characterization of recombinant chicken interferon-gamma. Vet. Immunol. Immunopathol., 58:321-333.

Suresh, M., K. Karaca, D. Foster and J.M. Sharma, 1995. Molecular and functional characterization of turkey interferon. J. Virol., 69:8159-8163.

Vanrompay, D., E. Cox, P. Kaiser, S. Lawson, M.V. Loock, G. Volckaert and B. Goddeeris, 2001. Protection of turkeys against Chlamydophila psittaci challenge by parenteral and mucosal inoculations and the effect of turkey interferon-γon genetic immunization. Immunology, 103:106-112.