Cloning, Prokaryotic Expression, and Biological Analysis of Recombinant Chicken IFN-γ

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The full-length chicken interferon-gamma (CHIFN-γ) gene was amplified by reverse transcription-PCR using total RNA extracted from the spleen cells of white Leghorn chicken, a local Chinese breeding species. A truncated CHIFN-γ gene without the N-terminal signal peptide sequence was cloned into prokaryotic expression vector pET30a, resulting in a recombinant plasmid pET-30a-CHIFN-γ. After the recombinant plasmid was transformed into host cells BL21(DE3)pLysS, the expression of CHIFN-γ was induced by isopropyl β-D-thiogalactoside (IPTG). Rabbit antiserum was raised using the soluble CHIFN-γ as immunogen. Immunoreactivities of the CHIFN-γ and its antiserum were investigated using immunoblotting and ELISA. Moreover, the antiviral effect of the CHIFN-γ was analyzed. Our data indicate that the CHIFN-γ is biologically active.

Introduction

GENERALLY APPLICATION OF ANTIBIOTICS and vaccines is the major consideration for control and prevention of infectious diseases. However, the emergence of antibiotic-resistant pathogens, including bacteria as well as the low efficacy of some kinds of vaccines, prompts the necessity to search alternative or complimentary measures. Cytokines are natural mediators of host immune response. They can control the type and extent of a response after infection or vaccination. In other words, cytokines may be used as natural therapeutic agents.[1]

Interferon-gamma (IFN-γ) is a kind of cytokine and plays an important role against the replication of viruses, bacteria, and parasites.[2–6] In recent years, it has been used as an effective vaccine adjuvant in various animal models.[1,7–12] Chicken interferon-gamma (CHIFN-γ) was first cloned in 1995.[13] Partial biological properties of recombinant CHIFN-γ have been analyzed.[11,14] The predicted mature CHIFN-γ has 145 amino acids with a molecular mass of 16.9 kDa. Several reports have demonstrated the expression of CHIFN-γ in different expression systems such as *Escherichia coli* (*E. coli*), mammalian cells, and fowlpox virus.[2,11,13,14] The comprehensive structural characterization of recombinant CHIFN-γ was reported, and CHIFN-γ is now being considered as an excellent therapeutic candidate in poultry.[1,15]

In terms of cost and convenience, it is clear that the use of recombinant CHIFN-γ is favored over isolated native protein. In the current study, we cloned the gene encoding CHIFN-γ from white Leghorn chicken, a local breeding species. The expressed CHIFN-γ in *E. coli* was purified and used to prepare specific polyclonal antiserum. Both CHIFN-γ and its antibody were characterized by biological assays. The current study provides useful experimental material for further functional analysis of CHIFN-γ.

Materials and Methods

Cell culture

The African monkey kidney cells (Vero cells) were grown in Dulbecco's modified essential medium (DMEM) supplemented with 5% fetal bovine serum (FBS, ExCell Bio, Shanghai, China) at 37°C. Chicken spleen cells were prepared as described previously[15] and maintained in RPMI 1640 medium supplemented with 5% FBS at 40°C in a CO_2 incubator. Cells were cultured for different time points in the presence of various concentrations of Concanavalin A (Con A, Sigma, St. Louis, MO).

cDNA cloning of chicken interferon-gamma

Total RNA extraction from spleen cells of White Leghorns chicken was performed using TRIzol reagent (Invitrogen, Beijing, China), according to the manufacturer's instructions. Subsequently, reverse transcription (RT)-PCR was performed using a commercially available RT-PCR kit (TaKaRa, Dalian, Liaoning, China). For PCR, 100 ng of sense primer 5′-GGCCGAATTCATGACTTGCCAGACTTACA-3′ and antisense primer 5′-GGCCAAGCTTTTAGCAATTGTATCTC-3′ was used to amplify the CHIFN-γ. Underscored parts were *Eco*R I and *Hin*d III restriction enzyme sites, respectively. PCR profiles included 30 cycles of 95°C, 2 min; 50°C, 30 s; and 72°C,

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1 min. The PCR product was visualized in 1% agarose electrophoresis. The PCR product was subjected to DNA sequencing by automated sequence analysis (TaKaRa).

Expression and purification of CHIFN-γ

The PCR product was purified with a commercially available kit (Keygen Biotech, Nanjing, Jiangsu, China) and then used as a template to amplify a truncated gene encoding a signal peptide-deleted CHIFN-γ. The gene amplification using primer 5′-GGCCGAATTCCAACTTCAAGATGATA TAG-3′ and antisense primer 5′-GGCCAAGCTTTTAGCA ATTGTATCTC-3′ resulted in the deletion of the first 84 nucleotides in the N-terminal of the CHIFN-γ gene. Underscored parts in the primers were *Eco*R I and *Hin*d III restriction enzyme sites, respectively. The truncated gene was cloned into the multiple cloning sites *Eco*R I and *Hin*d III of prokaryotic expression vector pET-30a (Novagen, Madison, WI). The authenticity of insert was confirmed by automated sequence analysis (TaKaRa). The recombinant plasmid was then transformed into host cells *E. coli* BL21(DE3). Expression of the CHIFN-γ was induced using 1.0 mM isopropyl β-D-thiogalactoside (IPTG) at 37°C. The induced cells were pelleted at 12,000 rpm at 4°C for 2 min. The samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), after the pellets were treated with 2× SDS loading buffer. The soluble histidine (His)-tagged fusion CHIFN-γ was further purified using Ni-NTA affinity column (Qiagen, Hamburg, Germany), according to the manufacturer's instructions.

Western blot analysis

The unpurified and purified CHIFN-γ was subjected to SDS-PAGE, and then the proteins were transferred to a nitrocellulose membrane. The membrane was blocked with blocking buffer (5% non-fat dry milk and 0.05% Tween-20 in PBS) at room temperature for 1 h. The membrane was incubated with a monoclonal antibody against IFN-γ (1:25 diluted in PBS-0.05% Tween-20 [PBST]) at 37°C for 1 h, which was followed by incubation of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Boster, Wuhan, Hubei, China) diluted in PBST (1:1000) prior to diaminobenzidine enzyme-based color development in the dark.
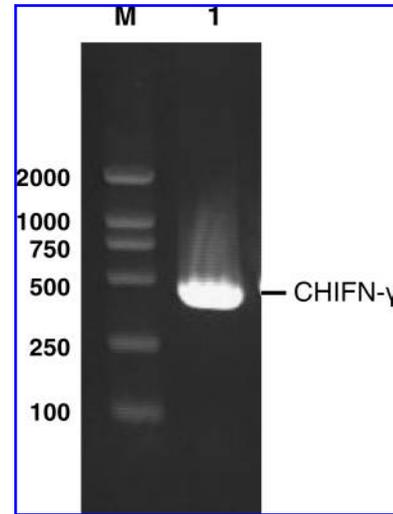
FIG. 1. Amplification of CHIFN-γ gene by RT-PCR. Total RNA extracted from Con A-stimulated chicken spleen cells was used as template for RT-PCR amplification of CHIFN-γ gene. M, DL2,000 DNA marker; lane 1, PCR product of CHIFN-γ amplification.

Preparation of specific polyclonal antiserum

A New Zealand rabbit was immunized with 1 mL purified CHIFN-γ (2 mg/mL) emulsified with an equal amount of Freund's complete adjuvant via subcutaneous injection. Fifteen days later, the rabbit was injected with the same antigen (0.5 mL) mixed with an equal volume of Freund's incomplete adjuvant three times at 10-days' intervals. Antiserum was isolated from neck artery blood of the immunized rabbit at day 42 post-inoculation.

Titration of the antibody using ELISA

The immunoreactivity between the CHIFN-γ and its polyclonal antiserum was analyzed using indirect ELISA, as previously described.[16] In brief, ELISA plates were coated with 100 μL CHIFN-γ (10 μg/mL) at 4°C overnight in carbonate-bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6).

FIG. 2. Sequences of CHIFN-γ. The nucleotide and amino acid sequences of the CHIFN-γ are provided.
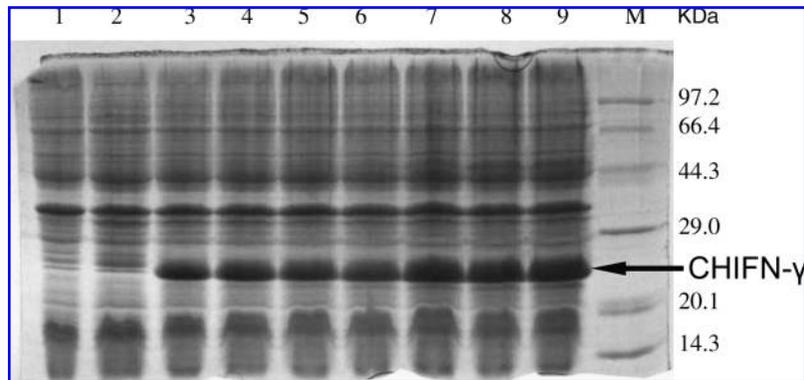
FIG. 3. SDS-PAGE analysis of bacterially expressed CHIFN-γ. The bacteria harboring the recombinant plasmid encoding the CHIFN-γ or empty vector were induced with 1 mM IPTG and the bacterial protein was analyzed by SDS-PAGE. Lane 1, empty vector control; lanes 2–9, expression of protein of interest at 0–7 h post-IPTG induction, respectively; M, protein molecular weight marker.

The wells were blocked with blocking buffer at 37°C for 2 h. After washing three times with PBST, the wells were incubated with the serially diluted polyclonal antiserum at 37°C for 1 h. After washing three times with PBST, the plates were incubated with HRP-conjugated goat anti-rabbit IgG (Boster, 1:5000 diluted in PBST) at 37°C for 1 h. O-phenylenediamine dihydrochloride (OPD) substrate (100 μL/well) was added and incubated for 15 min after washing with PBST. The optical density (OD) value was read at 490 nm using an ELISA reader, after stopping the reaction with 50 μL stop buffer (2 M H_2SO_4). In parallel, the reaction between the protein and serially two-fold diluted antibodies in PBS was analyzed by agar diffusion test at 37°C for 24 to 72 h.

Virus binding blocking assay

To evaluate the antiviral effect of the CHIFN-γ, virus plaque reduction assays were used. In brief, Vero cells were seeded in 24-well plates at 37°C for 48 to 72 h until the formation of cell monolayers. Then, the cells were incubated with CHIFN-γ at different concentrations at 37°C for 12 h. The cells were infected with vesicular stomatitis virus (VSV, strain Indiana) at an MOI of 1 after the cells were rinsed three times. One hour later, the cells were overlaid with methyl-cellulose (1% w/v in serum-free medium) and cultured for another 48 to 72 h prior to virus plaque reduction assay.[17]

Results and Discussion

Molecular cloning of CHIFN-γ

To clone the CHIFN-γ gene, the spleen cells isolated from a local chicken species were stimulated with Con A, and then total RNA extracted was used as template for RT-PCR. The results showed that only one specific DNA band was visualized in agarose electrophoresis (Fig. 1). The PCR product was sequenced directly to guarantee the authenticity of the amplicon. The sequencing report showed that the gene (438 bp) encoding CHIFN-γ shared 99.8% homologous identity with the reference sequence (GenBank accession no. U27465). One point mutation at position 486 (G to A) was found in a sequence alignment. It therefore can be concluded that this cytokine is rather conserved among different chicken species.

The gene and its deduced amino acid sequences are shown in Figure 2. Although CHIFN-γ is very conserved, compara-

tive sequence analysis of the IFN-γ proteins from avian and mammalian species reveals that IFN-γ of birds has 67% amino acid sequence identity with duck IFN-γ and about 20–30% sequence identity with IFN-γ of mammals.[18] Therefore, the phylogeny of IFN-γ among different species remains unclear. The sequence obtained in this study has been submitted to GenBank database and was assigned the GenBank accession no. GQ246226.

Expression of recombinant His-CHIFN-γ in E. coli

Many expression systems are available for heterologous expression. An optimal expression system can be selected only if the productivity, bioactivity, purpose, and physicochemical characteristics of the protein of interest are taken into
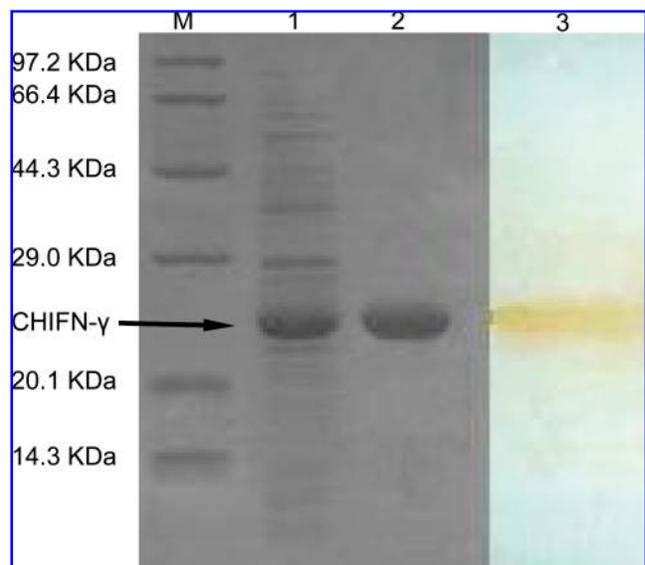

FIG. 4. Purification and Western blot analysis of CHIFN-γ. The unpurified and purified CHIFN-γ was analyzed by SDS-PAGE and the immunoreactivity of the protein to its specific monoclonal antibody was analyzed by Western blot. M, protein molecular marker; lane 1, unpurified CHIFN-γ obtained at 5 h post-IPTG induction; lane 2, purified CHIFN-γ obtained at 5 h post-IPTG induction; lane 3, identification of CHIFN-γ by monoclonal antibody by Western blot analysis.

consideration, together with the cost, convenience, and safety of the system *per se*.[19] One of the purposes of the current study is to obtain the high level expression of CHIFN-γ to facilitate further functional analysis. Therefore, the *E. coli* system was used to express the protein of interest. Both the host cells and the vector are commercially available and easily used. Our results showed that the high level expression of the CHIFN-γ was achieved in current expression system. Using bioinformatic analysis, a 28-amino acid signal peptide was identified. The hydrophobic peptide is often useless and influences the protein expression in prokaryotic expression system; therefore, we cloned a truncated gene encoding the CHIFN-γ without signal peptide into the prokaryotic expression vector pET-30a, resulting in a fusion gene that consists of N- and C-terminal histidine tags flanking the putative mature CHIFN-γ. After the recombinant plasmid, named pET-30a-CHIFN-γ, was transformed into the host cells, the expression was induced by IPTG at 37°C. SDS-PAGE indicated that CHIFN-γ was expressed as early as 1 h after IPTG induction, attaining peak levels at around 5 h and persisting for at least 7 h (Fig. 3). The molecular mass of the fused CHIFN-γ was 25 kDa as expected. At the same time, we tried to express the full-length gene encoding the CHIFN-γ in the same system; however, no expression of CHIFN-γ was detected (data not shown). The impact of the signal peptide on foreign protein expression in prokaryotic organism was confirmed in our study. Interestingly, it was reported that a recombinant chicken CHIFN-γ with C-terminal truncation has been expressed in *E. coli,* and it showed a specific activity comparable to that of the full-length molecule.[1] Therefore, which factor (vector, host cell, or expression condition) influencing the expression of CHIFN-γ needs to be investigated in the future.

### Purification of recombinant CHIFN-γ and preparation of polyclonal antiserum

By utilizing the His tag, the fused His-CHIFN-γ was purified via a Ni-NTA affinity column. Our results showed that
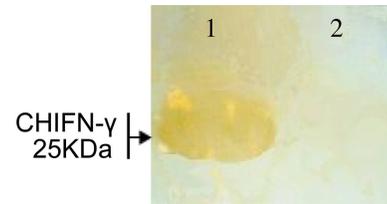
FIG. 5. Western blot analysis of the polyclonal antibody. After the proteins from bacteria harboring either plasmid encoding CHIFN-γ or empty vector were transferred on nitrocellulose membrane, conventional immunoblotting was performed using the polyclonal antibody. Lane 1, CHIFN-γ; lane 2, empty vector control.

soluble His-CHIFN-γ was easily purified, and no unrelated protein bands were detected in SDS-PAGE analysis (Fig. 4). To identify the protein, a monoclonal antibody against CHIFN-γ was used in subsequent Western blot analysis. The result indicated that the protein was recognized by the specific monoclonal antibody (Fig. 4), confirming the purity of the CHIFN-γ. In one of our previous experiments, we found that protein expressed in the form of inclusion body in *E. coli* was sometimes difficult to be purified by the affinity column compared to soluble protein and such protein could be purified using a gel purification method.[16] However, protein was denatured under gel-cutting purification condition, and the denaturation condition may influence the biological activity of the purified protein more and less.

The purified CHIFN-γ was used as an immunogen to inoculate a rabbit to achieve polyclonal antiserum. The titer of the antiserum was 1:100,000 analyzed using indirect ELISA, confirming the purity of the protein. Western blot analysis showed that the polyclonal antiserum reacted with the CHIFN-γ specifically (Fig. 5). As mentioned above, a monoclonal antibody against CHIFN-γ was able to recognize the CHIFN-γ produced in this study; therefore, the collective data
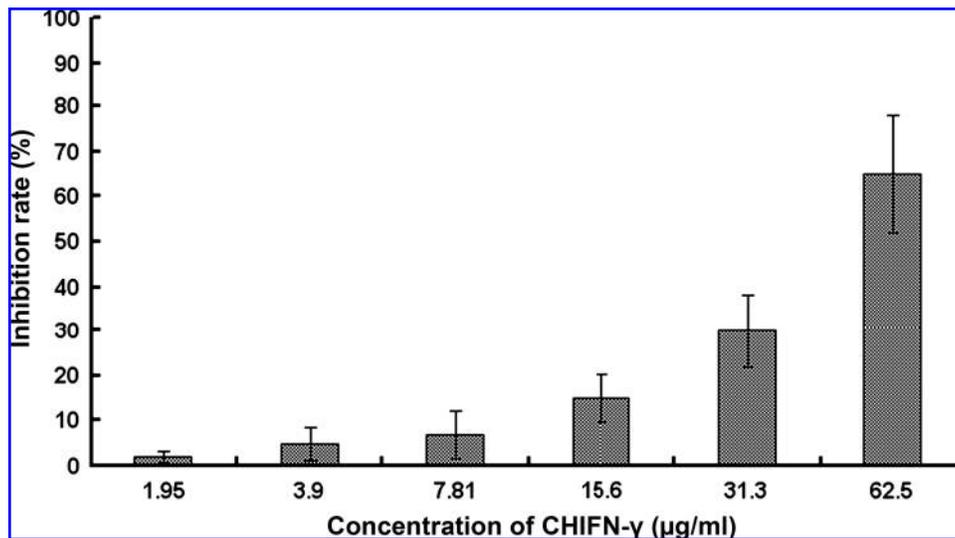
FIG. 6. Inhibitory effect of CHIFN-γ on cell infection by virus. Serially diluted CHIFN-γ was incubated with Vero cells prior to VSV infection. Infection efficiency was analyzed by plaque reduction assays. Values along the horizontal axis indicate protein concentration, and the vertical axis indicates virus infection inhibition rate.

indicate that both CHIFN-γ and the polyclonal antiserum have good immunoreactivities.

Biological activity of the CHIFN-γ

Some studies in both the mammalian and avian systems demonstrated that IFN-γ could activate macrophage cells.[20,21] It was reported that CHIFN-γ activated macrophage cell lines[2] and NCSU[22] and HD11[23] cells to generate NO.[24] In our study, we confirmed that the CHIFN-γ could stimulate the generation of NO in chicken spleen cells (data not shown).

Studies *in vitro* and *in vivo* have demonstrated that IFN-γ had activities against replication of vaccinia virus, human immunodeficiency virus, and Japanese encephalitis virus.[2] Duck IFN-γ protected duck cells from the cytopathic effects of influenza virus and VSV and Newcastle disease virus, in addition to its inhibitory effect on the replication of duck hepatitis B virus replication in primary hepatocytes.[18] In our study, we used Vero cells (a monkey kidney cell line) to analyze the effect of CHIFN-γ on virus infection. We first used fluorescence-labeled porcine pseudorabies virus (a porcine herpes virus) as a model virus to infect Vero cells; however, no inhibitory effect was observed (data not shown). Then we used VSV, a commonly used model virus, for analyzing the effect of CHIFN-γ on Vero cell infection by this virus. Our results showed that pre-treatment of cells with CHIFN-γ inhibited VSV infection in a dose-dependent manner, indicating that the protein had an effective antiviral activity (Fig. 6). However, it would be interesting to test more cell lines and virus strains to delineate the antiviral mechanism of CHIFN-γ.

In conclusion, our data indicate that the gene encoding CHIFN-γ is highly conserved in the same species. The recombinant CHIFN-γ expressed in *E. coli* is an excellent immunogen for antibody production, and it has certain biological activity.

Acknowledgments

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