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Expression of Human Interferon-α8 Synthetic Gene under P_{BAD} Promoter

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Abstract—Recombinant human interferon-α8 (rhIFN-α8) was obtained by synthesizing a codon-optimized gene in a two-step polymerase chain reaction (PCR) and expressing it in *Escherichia coli*. The gene encoding human IFN-α8 shows a high content of rare codons. These were replaced based on *E. coli* codon usage and balancing TA-GC ratio contents of the entire gene. The two-step PCR was performed using long (45-60 nucleotides) overlapped primers and two Taq polymerases (*pfu* clone and GC-rich system) and resulted in a DNA band of 504 base pairs (bp) corresponding to the calculated size of the IFN-α8 coding sequence; the *pfu* clone failed to amplify the gene in the correct size without unspecific bands. The full gene was cloned into the pBAD-TOPO expression vector. After cloning, the gene was reoriented by *Nco*I restriction digestion and religation. The ligated pBAD-TOPO-IFN-α8 (pBAD-IFNα8) plasmid carried the IFN-α8 gene under transcriptional control of the L-arabinose-inducible P_{BAD} promoter. IFN-α8 expression was optimized with respect to L-arabinose concentration, temperature, and time of induction in shake flask cultures to maximize the yield of soluble IFN-α8. The produced IFN-α8 was characterized by polyacrylamide gel electrophoresis and immunoassays. After purification on DEAE-Sepharose, the yield was 100 mg/liter. The antiviral and anticancer activities of the IFN-α8 were evaluated in comparison with IFN-α2a, and the results are discussed.

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Interferons (IFNs) are potent antiviral and antiproliferative proteins secreted by cells in response to viruses and other agents [1]. IFNs are grouped into three separate classes (types I (IFN-α/β, etc.), II (IFN-γ), or III (IFN-λ)) based on their sequence, receptor specificity, chromosomal location, physicochemical properties, and structure. Human IFN-α forms a polypeptide family of closely related cytokines that comprises at least 13 functional human IFN-α genes with 23 subtypes. The human IFN-α8 subtype has shown the most potent antiviral activity when compared with other natural human IFN-α subtypes [2]. In addition, this protein has been obtained from yeast cells with high biological activity [3-5].

The *Escherichia coli* expression system is the most powerful tool for the production of valuable therapeutic proteins. When produced under the appropriate conditions, these proteins are fully active and can efficiently mimic the function of their original counterpart [6]. It has been demonstrated that, at high rate of expression, the presence of clusters of rare codons, which are encoded by rare tRNAs, may become a rate-limiting factor in protein synthesis. Therefore, high-level expression from one particular heterologous gene in *E. coli* generally requires the optimization of codon usage [7].

The L-arabinose promoter (*ara*BAD or P_{BAD}) has recently become commercialized; this system uses the safe and inexpensive sugar "L-arabinose" as an inducer. When a gene is cloned downstream of the P_{BAD} promoter, its expression is controlled by the *AraC* activator. Expression is induced to high levels on media containing L-arabinose. Moreover, expression is tightly shut off on media containing glucose but lacking arabinose. However, the levels of expression of P_{BAD}-controlled genes may not be zero in the repressed state [8]. The *araC*-P_{BAD} promoter system allows high-level expression in a dose-dependent manner, tightly regulated protein expression, and low basal level.

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In the present work, a synthetic human IFN-α8 gene was assembled using seventeen long (45-60-mers) overlapped primers and amplified by PCR using a pair of gene-specific primers. Rare codons were replaced with abundant ones based on the favored codon usage of *E. coli*. The PCR product was cloned into a cloning vector, 5-min TOPO-TA in *E. coli* XL2-Blue MRF′, then into the pBAD-TOPO expression vector in *E. coli* BL21. Recombinant IFN-α8 was expressed in *E. coli* carrying the pBAD-IFNα8 construct and confirmed for its identity by polyacrylamide gel electrophoresis and immunoassays. The expressed protein was purified and then tested for anticancer effect and antiviral activity to evaluate its biological activity.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media. *Escherichia coli* strains XL2-Blue MRF′ and BL21-CodonPlus (DE3)-RIPL (Stratagene, Germany) were used for gene cloning and expression, respectively. Five-minute TOPO-TA plasmid was employed for gene cloning, and pBAD-TOPO plasmid for gene expression (Invitrogen, USA). Luria–Bertani (LB) broth and agar containing X-gal (20 µg/ml) and ampicillin or kanamycin (100 µg/ml) were used for bacterial growth and selection of transformants. M9 minimal medium containing glycerol as the carbon source was used for protein expression, and expression was induced at different L-arabinose concentrations.

Design of primers. To obtain the human IFN-α8 gene by PCR, specific primers were designed using the PRIMER3 software (http://workbench.sdsc.edu). The primers were designed according to the following criteria: form unique overlaps of approximately 10-30 bp in length; give melting point compatible with annealing of other primers; minimize intermolecular base pairing; and oligonucleotides to be between 45-60 nucleotides in length. Replacement of rare codons with abundant ones in the IFN-α8 DNA sequence (Arginine codons AGA and AGG corresponding to amino acids 12, 13, 22, 23, 33, 121, 126, 145, 150, 161 were replaced with CGT; isoleucine codon ATA corresponding to amino acids 16, 24, 54, 61, 87, 107, 117, 127, 148, 156 was replaced with ATT; glycine codon GGA corresponding to amino acids 10, 105 was replaced with GGC; leucine codon CTA corresponding to amino acids 3, 9, 15, 17, 18, 30, 57, 67, 77, 81, 82, 89, 93, 96, 111, 118, 129, 131, 154, 158, 162 was replaced with CTG; and proline codon CCC corresponding to amino acids 4, 26, 39, 110 was replaced with CCG) was considered for high-level expression in *E. coli*. The primers were synthesized at Eurofins MWG Operon (Germany) and were purified using HPLC to be gene-synthesis grade. Seventeen long (45-60-mers) overlapped primers for gene assembly and two gene-specific primers with the restriction sites of *Nco*I/*Sal*I on the forward and reverse primers, respectively, for amplification were designed.

Taq polymerases. Two Taq polymerases were used to assemble and amplify the IFN-α8 gene: *pfu* clone (proofreading and expand high fidelity; Stratagene) and GC-Rich PCR system (Roche Molecular Biochemicals, Germany). This is a unique blend of thermostable Taq DNA polymerase and *Tgo* DNA polymerase, a thermostable enzyme with proofreading (3′-5′ exonuclease) activity [9]. This polymerase mixture results in more blunt-ended PCR fragments compared to Taq DNA polymerase alone, but the majority product has single A overhangs.

Gene synthesis. The coding region (without the signal peptide sequence) of the IFN-α8 sequence was synthesized by a two-step PCR. All the PCR reactions were carried out using a Techne TC-3000 DNA thermal cycler (Bibby Scientific Limited, UK). The primers were added to a final concentration of 10 pM/each in the assembling step, while the amplification step was run with 1 µl of the assembled products and 20 pM of the outermost primers of each strand or with short primers complementary to both strands. Each reaction was carried out in a final volume of 25 µl containing 1X *pfu* buffer, 0.5 mM dNTPs, 1X GC-Rich PCR reaction buffer, 1.5 M GC-Rich resolution solution, 2.5 mM $MgCl_2$, *pfu* clone 1U and GC-Rich Enzyme Mix 1U.

Each reaction was taken through 30 cycles at 94°C for 45 sec, 53°C for 2 min, 72°C for 2 min, followed by a final extension at 72°C for 10 min. For the GC-rich system the company-recommended profile (1× of 95°C for 3 min; 10× of 95°C for 30 sec, 53°C for 30 sec, 72°C for 45 sec; 25× of 95°C for 30 sec, 53°C for 30 sec, 72°C for 45 sec; 1× of 72°C for 7 min) was followed.

Cloning of the PCR product. The PCR product was cloned into 5-min TOPO-TA cloning vector following the company's instruction manual. About 3 µl of the cloning reaction was transformed into *E. coli* XL2-Blue MRF′ competent cells; then the cells were plated onto LB plates containing X-gal (20 µg/ml) and ampicillin or kanamycin (100 µg/ml). Minipreps were prepared using a Qiagen kit (Qiagen, Germany) from overnight culture of white colonies and then *Eco*RI-enzyme digested.

Cloning of IFN-α8 gene into pBAD-TOPO expression vector. The amplified product was T/A cloned into the pBAD-TOPO expression vector, digested using *Nco*I enzyme, and religated to yield the pBAD-IFNα8 construct. The ligated pBAD-IFNα8 plasmid was transformed into *E. coli* BL21 competent cells, and then the cells were plated onto LB plates containing ampicillin (100 µg/ml). Minipreps were prepared using a Qiagen kit from overnight culture of picked single colonies and then digested *with Nco*I/*Sal*I enzymes. Correct clones were subjected to nucleotide sequencing using the dye-terminator sequencing method (at our central laboratory).

Expression of recombinant IFN-α8. The transformants were grown overnight in M9 minimal medium containing 100 μg/ml ampicillin in a shaker incubator at 37°C and 200 rpm. The following day, 2 ml of the overnight culture were added to 100 ml M9 minimal medium containing 100 μg/ml ampicillin and grown in a shaker incubator at 37°C until OD_{600} reached 0.6-0.8, then the culture was induced. The P_{BAD} promoter was used to drive the synthesis of recombinant IFN-α8 by cultivating the host cells in media containing 0.2% w/v L-arabinose for 6 h at 37°C.

Protein analysis by polyacrylamide gel electrophoresis. Protein samples collected before and after induction were separated in 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [10] using an Invitrogen Novex Mini-Cell. Gels were stained with Coomassie brilliant blue R250 (Sigma, USA).

Protein analysis by immunoassays. The immunochemical analysis of the expressed IFN-α8 protein was based on ELISA, dot-blot assay, and western blot analysis. A Costar ELISA plate (Cambridge, USA) was coated with 50 μl of whole cell lysate at 5 μg/ml for 24 h at 4°C. The plate was then washed five times with 0.12 M NaCl, 0.04 M sodium phosphate, pH 7.2, buffer (PBS). The plate was then blocked using 100 μl of blocking buffer (2% w/v BSA or gelatin in PBS), incubated for 1 h at 37°C, and then washed five times with PBS. Then 50 μl of monoclonal anti-human IFN-α antibody (PBL InterferonSource, USA) diluted 1 : 2000 in 2% w/v (BSA or gelatin)-PBS was added to each well. After 1 h of incubation at 37°C, the plate was washed five times with PBS, and 50 μl of alkaline phosphatase-conjugated anti-mouse IgG (BIO-RAD, USA) diluted 1 : 1000 with 2% w/v (BSA or gelatin)-PBS was added, followed by incubation for 1 h at 37°C. *p*-Nitrophenyl phosphate (*p*-NPP) was added for color development, and optical density measured at 405 nm on an ELISA reader (MicroPlate Reader; BIO-RAD) [11]. For dot blotting, 1 μl aliquots of whole cell lysate were arrayed onto polyvinylidene difluoride (PVDF) membrane (PolyScreen; NEN Life Science Products, USA) as previously described [12, 13]. The prepared membranes were air dried, blocked using 2% w/v gelatin in PBS for 1 h, and then incubated with monoclonal anti-human IFN-α antibody diluted 1 : 2000 in 2% w/v gelatin-PBS for 1 h. Membranes were then washed three times using PBS, the secondary antibody, and anti-mouse IgG-alkaline phosphatase conjugate was applied at 1 : 1000 dilution in 2% w/v gelatin-PBS for 1 h. After washing three times, blots were developed with bromochloroindolyl phosphate/nitro blue tetrazolium (BCIP/NBT). For western blotting, the samples were resolved by SDS-PAGE and the proteins were electrophoretically transferred to a nitrocellulose membrane (Costar, USA). The membrane was then treated typically as described for dot blotting.

Optimization of expression. To optimize the yield of IFN-α8 production in shake flask cultures, expression in *E. coli* BL21 cells carrying the pBAD-IFNα8 construct was induced at different temperatures (25, 30, and 37°C) and using different concentrations (0.0002, 0.002, 0.02, and 0.2% w/v) of the L-arabinose inducer, and samples were taken at different time points (2, 4, 6, 8, and 10 h). Accumulation of a protein band corresponding to the calculated size for IFN-α8 was detected by 12% SDS-PAGE.

Batch fermentation. A 5-liter fermenter was inoculated with 500 ml of culture of *E. coli* BL21 cells carrying the pBAD-IFNα8 construct. The cells were grown in 4 liters of highly enriched medium (12 g/liter tryptone, 24 g/liter yeast extract, 4 ml/liter glycerol, 0.17 M KH_2PO_4, 0.72 M K_2HPO_4, 2 ml/liter trace metal solution (27 g/liter $FeCl_3{\cdot}6H_2O$, 2 g/liter $ZnCl_2{\cdot}4H_2O$, 2 g/liter $CaCl_2{\cdot}6H_2O$, 2 g/liter $NaMoO_4{\cdot}2H_2O$, 1 g/liter $CaCl_2{\cdot}2H_2O$, 1 g/liter $CuSO_4{\cdot}5H_2O$, 0.5 g/liter H_3BO_3, 100 ml/liter concentrated HCl), and 2 ml/liter of thiamin) and supplemented with 50 mg/liter ampicillin. Temperature was set to 37°C, and the pH value was controlled at 7.0 by the addition of 4 M NaOH. Dissolved oxygen was kept, during the batch period, at a set point of 30% air saturation. Stirring was controlled in cascade mode depending on dissolved oxygen. The culture was induced with L-arabinose at mid-exponential phase. At 20 h post-induction (stationary phase), the cells were recovered and frozen at –20°C. This experiment was repeated twice.

Purification of expressed IFN-α8. The supernatant and the pellet (inclusion bodies) of the cell lysate were prepared from 1 liter of optimized shake flask culture. The cell pellet was gently resuspended in 20 ml washing buffer (10 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 100 mM NaCl) to form a homogenous paste. This was diluted to 200 ml with washing buffer and centrifuged at 12,000*g* for 10 min. The washed cell pellet was resuspended thoroughly in 50 ml washing buffer, containing 1 mM freshly prepared phenylmethylsulfonyl fluoride (PMSF), and lysed by sonication using a Bandelin Sonopuls Sonicator (Germany). The sonicator was programmed to provide 30 sec pulses with 15 sec pause for a total period of 15 min. The suspension was centrifuged at 12,000*g* for 30 min to remove insoluble debris. The supernatant was carefully separated to be analyzed by SDS-PAGE, and the pellet containing the inclusion bodies was stirred for 1 h at 4°C with 20 ml of 8 M urea solution (freshly prepared in washing buffer containing 1 mM PMSF) and centrifuged for 45 min at 12,000*g* at 4°C. The inclusion body pellet was then washed twice with distilled water and centrifuged as before and then subjected to SDS-PAGE analysis.

The cell-lysate supernatant was loaded at 1 ml/min onto a CL-6B DEAE-Sepharose column (Pharmacia Biotech, Sweden) previously equilibrated with 20 mM Tris-HCl, pH 8.0. The column was washed with ten bed volumes of the same buffer (flow rate 2 ml/min). The

bound proteins were eluted with a 60-ml linear gradient of 0-1 M NaCl generated using an AKTAprime plus FPLC protein separator system (GE Healthcare Life Sciences Products, UK) at flow rate 1 ml/min. Twenty fractions of 3 ml each were collected. The eluted protein in the fractions was monitored by an online UV detector. Fractions containing IFN-α8 protein were analyzed by SDS-PAGE and ELISA.

Silver staining to confirm purity of the recombinant IFN-α8. The SDS-PAGE gel was kept in fixative solution (50 ml methanol, 12 ml acetic acid, 0.5 ml formaldehyde, and 100 ml MilliQ water) for 30 min; the solution was changed twice after each treatment of 10 min. The gel was kept in 50% ethanol solution for 20 min, and this process was repeated thrice. After this, the gel was treated with 0.02% sodium thiosulfate solution (0.02 g sodium thiosulfate dissolved in 100 ml MilliQ water) for 1 min and rinsed thrice in MilliQ water for 20 sec each. Staining solution (100 ml of 0.02% w/v $AgNO_3$ and 0.5 ml formaldehyde) was added and the gel was kept on the shaker for 20 min. The gel was rinsed twice with MilliQ water for 20 sec each. Developer (6 g Na_2CO_3, 2 ml of 0.02% w/v sodium thiosulfate solution and 0.5 ml formaldehyde) was added for 30-60 sec, until protein bands became visible, and the developing reaction was stopped by adding a solution of 40% ethanol, 12% acetic acid for 10 min. The gel was washed again with MilliQ water and stored in 40% methanol.

Protein and endotoxin determination. Purified IFN-α8 protein content was determined either by directly measuring the absorbance at 280 nm or by the Lowry method [14] using bovine serum albumin as a standard protein. The endotoxin content was checked [15] to avoid its mitogenic effects on the cell-culture system. All IFN-α8 batches used were free of endotoxin (data not shown).

Cell culture. Myeloma cell line P3X63Ag-8 (ATCC, USA) was cultured in RPMI-1640 medium (Lonza, Belgium) supplemented with 2% v/v L-glutamine, 20% v/v fetal bovine serum (FBS), and 1% v/v penicillin-streptomycin. The cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2/95% air in 25 cm^2 flasks (Greiner, Germany). The cells were passaged two or three times weekly.

Anticancer activity assay. Recombinant IFN-α8 was tested for anticancer activity against myeloma cell line P3X63Ag-8 and compared with commercial IFN-α2a (Shenyang Pharmaceutical Co, China) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described by Mosmann and El-Baky et al. [16, 17]. Stock samples (purified IFN-α8 and commercial IFN-α2a) were dissolved in dimethyl sulfoxide (DMSO) (Sigma) at 300 μg/ml, then diluted at 1 : 10, 1 : 20, 1 : 30, 1 : 40, 1 : 50, 1 : 60, 1 : 70, 1 : 80, 1 : 90, and 1 : 100 in RPMI-1640 medium. P3X63Ag-8 cells were plated in a 96-well tissue culture plate (Greiner) in four replicates at the density $25 \cdot 10^4$ cell/ml and cultured in RPMI-1640 medium supplemented with 20% v/v FBS, 1% v/v penicillin-streptomycin for 24 h at 37°C in 5% CO_2/95% air. The medium was refreshed with new RPMI-1640 supplemented medium, and then cells were treated with various dilutions of samples in total volume of 200 μl/well for 24 h. At 21 h, cells were centrifuged at 484*g* for 10 min and resuspended with 180 μl RPMI-1640 medium to rinse the treated samples. Twenty microliters of 5 mg/ml MTT solution (Sigma) were added to each well, and the samples were incubated at 37°C for 3 h. The formed formazan crystals were dissolved with 180 μl of DMSO. Optical density was measured at 560 nm. The percentage of cytotoxicity compared with the untreated cells as a control was determined. The plot of % cytotoxicity versus sample concentration was used to calculate the concentration lethal to 50% of the cells (LD_{50}).

Separation of human blood lymphocytes and selectivity index calculation. Peripheral blood mononuclear cells (PBMCs) were isolated as reported by El-Fakharany et al. [18]. Briefly, peripheral blood samples were diluted with five volumes of a freshly prepared red blood cell (RBC) lysis buffer (38.8 mM NH_4Cl, 2.5 mM $KHCO_3$, 1 mM EDTA, pH 8.0), incubated at room temperature for 10 min, and centrifuged at 272*g* for 5 min. The nucleated cells were precipitated and washed with PBS. Proliferation of human lymphocytes in response to IFN-α8 or commercial IFN-α2a was assessed by MTT assay. Selectivity index (SI) was calculated from the LD_{50} ratio in lymphocytes over the P3X63Ag-8 myeloma cells. The SI value indicates the selectivity of samples to the myeloma cell line.

Antiviral assay. The antiviral activity of IFN-α8 and commercial IFN-α2a was determined using a cytopathogenicity (CPE) assay against herpes simplex virus 1 (HSV-1) as described by Rashad et al. [19]. Vero cells grown to confluency in 96-well plates were infected with 100 μl of stock virus. After an adsorption period of 2 h at 37°C, the virus was removed and concentrations of the tested interferons were added (0.003, 0.03, 0.3, 3, and 30 μg/ml for recombinant IFN-α8 and 0.1, 1, 10, 100, and 1000 IU/ml for commercial IFN-α2a), then maintenance DMEM with 2% FBS was added (100 μl/well). The cultures were further incubated at 37°C for 24 h until complete CPE was observed in the infected and untreated virus control. The determination of the anti-herpes simplex virus 1 activity of the tested interferons was based on virus-induced cytopathogenicity of HSV-1-infected Vero cells measured at day 2 post virus infection by the MTT colorimetric method. Absorbance of formazan was detected at 560 nm. The results were expressed as the 50% effective concentration (EC_{50}). The 50% effective antiviral concentration (EC_{50}) was defined as the interferon concentration required for protecting 50% of the virus-infected cells against viral cytopathogenicity. The concentrations of the tested interferons which exhibited 50% cytotoxicity (LD_{50}) were also determined. The therapeutic index was calculated by dividing LD_{50} by EC_{50}.

Statistical analysis. Differences between the variants were tested using Student's *t*-test and McNemar's test [20]. *P* values of less than 0.05 were considered statistically significant.

Accession numbers. The GenBank accession number of the IFN-α8 DNA sequence synthesized in this work is X03125.

RESULTS

Construction of a synthetic IFN-α8 gene. To synthesize the IFN-α8 gene, seventeen oligonucleotides were designed based on *E. coli* codon usage. The start codon and termination codon were added to the coding sequence of the gene. The nucleotide sequence of the synthetic IFN-α8 gene (504 bp), the relative positions of the designed oligonucleotides, and the corresponding amino acid sequence are shown in Fig. 1 (see color insert). Codon optimization of the synthetic gene is illustrated in Fig. 2 (see color insert).

The gene was assembled using a pool of oligonucleotides ranging in length from 45 to 60 nucleotides. The overlap between the oligonucleotides was 10-30 bp, and the calculated T_m of overlapped oligos was 57.34 ± 2.30°C. All primers were mixed in equimolar concentrations in the same reaction tube to assemble and create short DNA duplex, thereby priming the elongation by

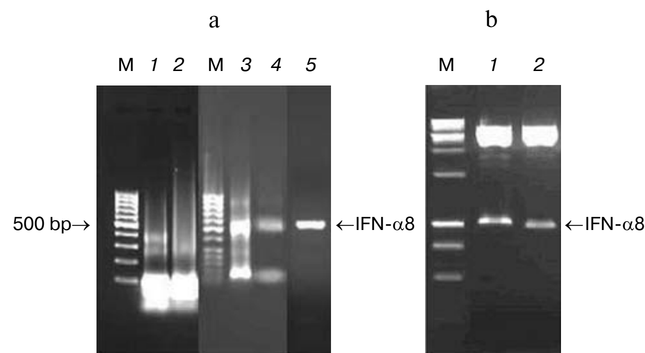

Fig. 3. a) Construction of the synthetic IFN-α8 gene. Lanes: *1*, *2*) assembling products of IFN-α8 gene using *pfu* clone enzyme and GC-rich system, respectively; *3*, *4*) amplification products of IFN-α8 gene using *pfu* clone enzyme at annealing temperatures 53 and 54°C, respectively; *5*) amplified product of IFN-α8 gene using GC-rich system at annealing temperature 53°C. b) Cloning of IFN-α8 gene into TOPO-TA and pBAD-TOPO vectors. Lane *1* shows the TOPO-TA vector fragment (3.94 kb) and the IFN-α8 band (520 bp, with 16 bp added to the actual size of the gene from the vector cloning site). Recombinant TOPO-TA was digested overnight at 37°C using *EcoR*I enzyme. Lane *2* is restriction cutting of ligated pBAD-IFNα8 plasmid with *Nco*I/*Sal*I to ensure the success of the ligation process and correct orientation of the insert inside the vector. Minipreps were digested overnight at 37°C using *Nco*I and *Sal*I enzymes to give a vector fragment of 4 kb and IFN-α8 band of 504 bp. M is the DNA-marker.

Table 1. Reactivity of monoclonal anti-human IFN-α antibody against recombinant IFN-α8 expressed in *E. coli* BL21 cells carrying the pBAD-IFNα8 construct

Sample	OD at 405 nm, mean ± SD
Commercial IFN-α2a	0.822 ± 0.005
Recombinant IFN-α8 (before induction)	0.038 ± 0.014*
Recombinant IFN-α8 (after induction)	0.450 ± 0.017*

* Monoclonal anti-human IFN-α antibody reacted significantly ($p < 0.05$) with expressed IFN-α8 protein.

DNA polymerase. These short duplexes serve as substrates for formation of longer duplexes, eventually resulting in the synthesis of the full-length gene, which was amplified using short primers flanked with restriction sites or outermost primers of each strand. Proofreading *pfu* clone failed to amplify the gene without the presence of undesirable products; the oligos were assembled (Fig. 3a, lane *1*), but many DNA bands were produced in the amplification step, one of them at the correct size of the IFN-α8 gene (504 bp) as demonstrated in Fig. 3a, lane *3*. Raising the annealing temperature to 54°C did not solve the problem of nonspecific products for the *pfu* clone (Fig. 3a, lane *4*). The GC-rich PCR system proved to be effective in amplification of the IFN-α8 gene and improved PCR performance. Figure 3a, lane *5*, shows that only one DNA band of 504 bp corresponding to the calculated size of IFN-α8 coding sequence was obtained using the GC-rich PCR system.

Gene cloning. The transformants harboring recombinant TOPO-TA were analyzed by digestion using *EcoR*I restriction enzyme, and all recombinant plasmids gave the predicted size, two bands of 3.94 kb and 520 bp (Fig. 3b, lane *1*). These data indicated that based on the size, the recombinant TOPO-TA carrying IFN-α8 gene was obtained and confirmed by sequencing results.

Construction of pBAD-IFNα8 plasmid. One transformant harboring recombinant pBAD-IFNα8 was subjected to restriction digestion and nucleotide sequencing analysis. The sequencing data indicated that the transformant carried the correct construct (data not shown). As indicated in Fig. 3b, lane *2*, the construct has the correct sized IFN-α8 gene.

Expression of IFN-α8 protein. Human IFN-α8 did not migrate on SDS-PAGE at its expected size of 20 kDa but at 27 kDa (Fig. 4). The identity of the expressed protein was confirmed further by ELISA using monoclonal anti-human IFN-α antibody (Table 1). A statistically significant difference was found between IFN-α2a or IFN-

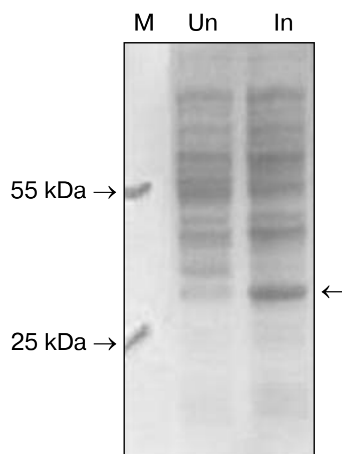

Fig. 4. SDS-PAGE analysis of recombinant IFN-α8 protein expressed in *E. coli* BL21 carrying the pBAD-IFNα8 construct. Lanes: M) protein molecular weight marker; In) total proteins obtained from induced cells; Un) total proteins obtained from uninduced cells. The arrow points to the position of the IFN-α8 product.

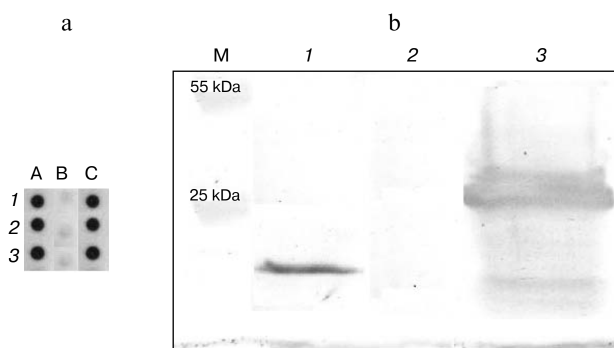

Fig. 5. Immunochemical analysis of the expressed IFN-α8 protein. a) Dot blots of recombinant IFN-α8 expressed in *E. coli* BL21 carrying the pBAD-IFNα8 construct. A1, A2, and A3 are 1 µl aliquots of commercial IFN-α2a at 5 µg/ml; B1, B2, and B3 are 1 µl aliquots of whole cell lysate before induction at 5 µg/ml; C1, C2, and C3 are 1 µl aliquots of whole cell lysate after induction at 5 µg/ml. b) Western blot of IFN-α8 expressed in *E. coli* BL21 with the specific monoclonal anti-human IFN-α antibody. Lanes: *1*) commercial IFN-α2a as positive control; *2*) whole cell lysate before induction; *3*) whole cell lysate after induction.

α8 (after induction) and the control (before induction) in the mean optical density value ($p < 0.05$). Also there was a significant difference ($p < 0.05$) in ELISA signals between commercial IFN-α2a and recombinant IFN-α8. This can be explained by the fact that commercial IFN-α2a is in purified form, while the IFN-α8 is still in the crude form at this stage. Dot blots corresponding to recombinant IFN-α8 expression in *E. coli* BL21 cells carrying the pBAD-IFNα8 construct are shown in Fig. 5a. The expressed IFN-α8 was specifically recognized by western blot analysis at the expected 27-kDa molecular weight (Fig. 5b).

Expression optimization. By varying the temperature of induction, concentration of L-arabinose, and running a time course of expression, protein expression level was optimized to ensure maximum expression of IFN-α8 protein. The results showed that while the incubation period was below 6 h, the yield of IFN-α8 was increased as the induction time prolonged; but when the incubation time exceeded 6 h, the IFN-α8 yield was not significantly raised. Reducing the temperature of the culture did not improve IFN-α8 expression, and the protein expression was maximal after 6 h at 37°C using 0.02% w/v L-arabinose. The effects of temperature, time of induction, and L-arabinose concentration on IFN-α8 production level are summarized in Table 2.

Batch fermentation. Cells were grown in batch mode in highly enriched medium in a 5-liter fermenter. The culture was induced by 0.02% w/v L-arabinose when OD_{600} reached 0.6-0.8. The volumetric product yield was 400 mg/liter, and the specific product yield was 140 mg/g DCW, this representing ~30% of the total cellular protein. The plasmid stability was checked prior to induction and found to be 100%, while recombinant human IFN-α8 expression was close to zero. These results were reproducible in the second batch. Again plasmid stability was checked, and it was observed that there was no problem of instability.

Purification of expressed IFN-α8. Both the supernatant and the pellet of the cell lysate after sonication were examined by SDS-PAGE to detect the expressed IFN-α8 protein. As shown in Fig. 6, the expressed IFN-

Table 2. Time, L-arabinose concentration, and temperature optimization of IFN-α8 expression

Optimization parameter		IFN-α8 yield, mg/liter
Induction time, h	2	66.37
	4	145.71
	6	398.44
	8	403.54
	10	426.28
Induction temperature, °C	25	239.06
	30	310.77
	37	384.23
L-Arabinose concentration, % (w/v)	0.0002	259.75
	0.002	338.92
	0.02	385.12
	0.2	392.52

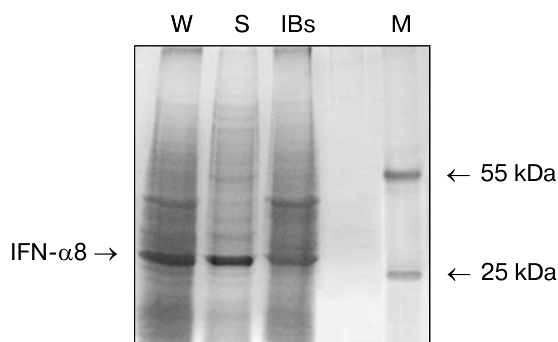

Fig. 6. SDS-PAGE analysis of IFN-α8 expressed in the soluble and insoluble inclusion form. W, whole cells; S, soluble proteins; IBs, inclusion bodies. Lane M, protein molecular weight marker.

α8 was found in the soluble form as well as the form of inclusion bodies after 6 h induction at 37°C using 0.02% w/v L-arabinose. Yields of IFN-α8 in different cell preparations are reported in Table 3. The cell-lysate supernatant was loaded onto a CL-6B DEAE-Sepharose column equilibrated with 20 mM Tris-HCl, pH 8.0. After washing, the bound proteins were eluted with a 60-ml linear gradient of 0-1 M NaCl. The elution profile of IFN-α8 is shown in Fig. 7a. Figure 7b and Table 4 show Coomassie brilliant blue stained SDS-PAGE profile and ELISA analysis of different fractions containing IFN-α8 protein. The data revealed that IFN-α8 was purified to near homogeneity utilizing single-step ion-exchange chromatography on DEAE-Sepharose. The yield was 100 mg of purified IFN-α8 from 1 liter of shake flask culture. A clear band 27-kDa in size could be observed, while no contaminating protein bands were seen on silver stained SDS-PAGE (Fig. 7c).

IFN-α8 anticancer activity. To evaluate the anticancer activity of recombinant IFN-α8, the inhibitory effect on a myeloma cell line was studied using the MTT assay. The amount of purple formazan produced by P3X63Ag-8 cells treated with recombinant IFN-α8 or commercial IFN-α2a was compared with the amount of formazan produced by untreated control cells and the effectiveness of these agents in causing death or changing metabolism of cells could be deduced through the significant decrease in the absorbance of formazan product at 560 nm after 24 h from treatment with various dilutions of samples (Fig. 8). Recombinant IFN-α8 has also shown a significantly higher antiproliferative activity ($p < 0.05$) against P3X63Ag-8 myeloma cells than IFN-α2a.

The cytotoxicity result is demonstrated as LD_{50} value that was calculated from the plot of % cytotoxicity versus sample concentration (Fig. 9). Recombinant IFN-α8 and commercial IFN-α2a showed a potent inhibitory (antiproliferative) effect on myeloma cell line after 24 h treatment (LD_{50} = 4.42 and 4.81 μg/ml, respectively). This was equivalent to 8% enhancement in activity.

Table 3. Yields of IFN-α8 expressed in *E. coli* BL21 carrying the pBAD-IFNα8 construct

Sample	IFN-α8 yield, mg/liter
Total cell protein	449.4
Total cell lysate	397.1
Soluble cell lysate	181.23* (45.63%)
Solubilized inclusion bodies	136.17 (34.29%)

* Majority of the produced protein was in the cell-lysate supernatant (about 46% of the yield in total cell lysate).

Table 4. Immunoassay signals of monoclonal anti-human IFN-α antibody against IFN-α8 in different fractions eluted from the DEAE-Sepharose column

Sample	OD at 405 nm, mean ± SD
Commercial IFN-α2a	0.923 ± 0.007
Whole cell-lysate supernatant containing IFN-α8	0.392 ± 0.008*
Fraction 14	0.096 ± 0.005
Fraction 15	0.105 ± 0.01
Fraction 16	0.114 ± 0.008
Fraction 17	0.228 ± 0.011
Fraction 18	0.684 ± 0.016*
Fraction 19	0.76 ± 0.012*

* There is a significant difference ($p < 0.05$) in ELISA signals between the whole cell-lysate supernatant containing IFN-α8 and purified fractions 18 and 19.

Table 5. EC_{50}, LD_{50}, and therapeutic index values of recombinant IFN-α8 and commercial IFN-α2a against HSV-1 by MTT assay

Interferon	EC_{50}	LD_{50}	Therapeutic index
Recombinant IFN-α8	33.16 ng/ml	12.47 μg/ml	376.05
Commercial IFN-α2a	87.06 IU/ml	994.37 IU/ml	11.42

Selectivity index calculation. The effect of recombinant IFN-α8 or commercial IFN-α2a on the proliferation of human lymphocytes was evaluated after 24 h incubation by MTT assay. The selectivity index (SI) values were calculated using the ratio SI = (LD_{50} of tested samples in normal cell (lymphocyte))/(LD_{50} of tested samples in P3X63Ag-8 myeloma cells). There was no significant difference between the selectivity of recombinant IFN-α8 to myeloma cells (SI = 2.8) and the selectivity to myeloma cells observed in the case of IFN-α2a (SI = 2.6).

Antiviral activity assay. The antiviral potency of both recombinant IFN-α8 and commercial IFN-α2a was determined in an *in vitro* assay by using Vero cells challenged with HSV-1. The EC_{50} value for recombinant IFN-α8 was 33.16 ng/ml. Protection levels obtained with

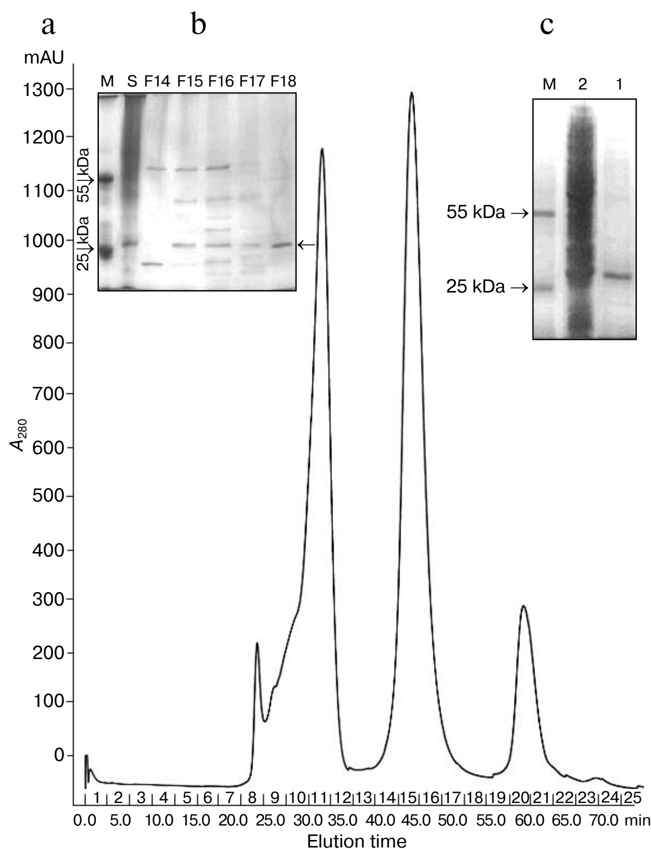

Fig. 7. FPLC-DEAE-Sepharose column purification of IFN-α8. a) FPLC-DEAE-Sepharose column chart. The chart clearly shows two peaks, fractions 10-14, 15-19. The Y-axis represents protein concentration at 280 nm, while the X-axis represents flow time. Numbers 1-25: number of collected fractions. b) Coomassie brilliant blue stained SDS-PAGE analysis of column fractions containing IFN-α8 protein. Lanes: M, protein molecular weight marker; S, whole cell-lysate supernatant containing IFN-α8. F14, F15, F16, F17, F18, and F19 corresponding to fractions 14, 15, 16, 17, 18, and 19, respectively. The arrow points to the position of IFN-α8. c) Silver stained SDS-PAGE analysis of purified IFN-α8 protein. Lane *1* shows a clear band, and no contaminating protein bands are visible. Lane *2*, whole cell-lysate supernatant containing IFN-α8. Lane M, protein molecular weight marker.

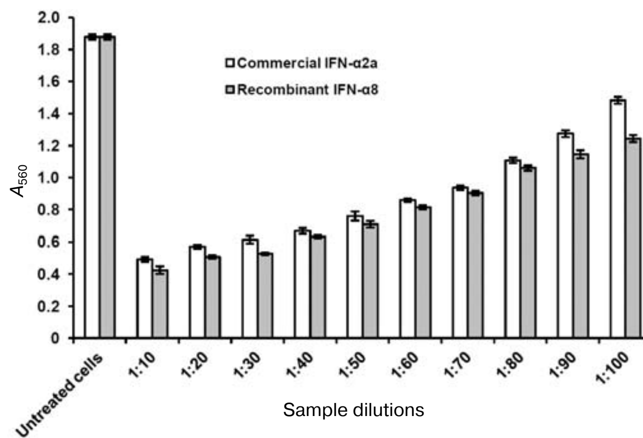

Fig. 8. MTT assay of myeloma cell line treated with different dilutions of recombinant IFN-α8 or commercial IFN-α2a compared with untreated control cells. Recombinant IFN-α8 and commercial IFN-α2a were dissolved in DMSO at 300 μg/ml, then diluted at 1 : 10, 1 : 20, 1 : 30, 1 : 40, 1 : 50, 1 : 60, 1 : 70, 1 : 80, 1 : 90, and 1 : 100 in RPMI-1640 medium. Cells were seeded in a 96-well microtiter plate at density $25 \cdot 10^4$ cell/ml and then treated with various dilutions of samples for 24 h. Twenty microliters of MTT solution (5 mg/ml) was added to each well and incubated for 3 h. The formed formazan crystals were dissolved with DMSO. Optical density was measured at 560 nm. Each column shows the mean and standard deviation of four replicates.

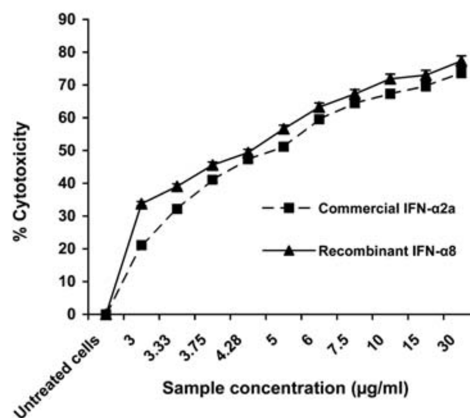

Fig. 9. Inhibitory effect (antiproliferative activity) of recombinant IFN-α8 and commercial IFN-α2a on myeloma cell line revealed by MTT assay. Recombinant IFN-α8 and commercial IFN-α2a have inhibitory effect on the myeloma cell line after 24 h treatment (LD_{50} = 4.42 and 4.81 μg/ml, respectively).

a total of 33.16 ng/ml of recombinant IFN-α8 were equivalent to the protection afforded by 87.06 IU/ml of commercial IFN-α2a. The EC_{50}, LD_{50}, and therapeutic index values for recombinant IFN-α8 and commercial IFN-α2a are presented in Table 5. Based on the definition of the IFN-α8 unit as the ability to protect 50% of cells from HSV-1-induced cytopathogenicity, it was calculated that the final purified IFN-α8 has specific antiviral activity of $2.62 \cdot 10^6$ IU/mg protein as determined by

cytopathogenicity (CPE) assay; the units were determined using commercial IFN-α2a.

DISCUSSION

IFN-α is widely used for the treatment of chronic hepatitis, Kaposi's sarcoma, and many other carcinomas. The production of IFN-α by recombinant technology for therapeutic use has been focused on IFN-α2a and IFN-α2b. However, certain cell lines are particularly responsive only to specific IFN subtypes, such as IFN-α8 but not others, like IFN-α2 [2].

The gene coding for human interferon-α8 shows a high content of low-usage arginine codons AGA/AGG resulting in low level expression in *E. coli*. In 2002, Acosta-Rivero et al. [21] attempted to improve the expression level of IFN-α8 produced in *E. coli* by PCR-based gene synthesis of the first 138 bp of the IFN-α8 gene, then amplifying the last 363 bp of the gene from human leukocyte genomic DNA as template and increasing the copy number of the *dna*Y gene (coding for $tRNA_{AGG/AGA}$). However, this strategy was not enough to increase the human IFN-α8 expression level. They explained the IFN-α8 low level expression observed; with $tRNA_{AGG/AGA}$ additional supply by the presence of other minor codons (ATA triplet, which codes for isoleucine, and CTA triplet, which encodes leucine) next to the initiation codon that cause moderate to severe inhibition of translation. Garcia et al. [22] used *E. coli* BL21-codon-plus-RIL cells to increase the IFN-α8 expression level. This strain carries additional copies of the *dna*Y, *ile*Y, and *leu*W tRNAs in an additional plasmid. The last two tRNAs decode the AUA and CUA minor codons, respectively. They reported a product yield of approximately 30 mg/liter of human IFN-α8 pure protein.

Rare codons for arginine seem to be the most difficult to express, followed by isoleucine and leucine codons, but other codons such as proline or glycine are also problematic [7], so in our strategy during codon optimization we took this into consideration. The strategy used in our work to improve IFN-α8 expression depends on removal of codon bias by complete replacement of minor codons in the synthesized gene. The recombinant product yield obtained in the present study (100 mg of purified IFN-α8 from 1 liter of shake flask culture) was higher than those obtained by Garcia et al. [22] and Platis and Foster [23], who achieved 30 and 49-62 mg/liter of human IFN-α8 pure protein, respectively. Also, both recombinant IFN-α8 proteins obtained in the previous studies were synthesized as inclusion bodies, while in the present study the expressed IFN-α8 was found in the soluble form as well as the form of inclusion bodies.

The PCR method was used to synthesize a human IFN-α8 gene of 504 bp using overlapping oligonucleotides in two steps. Rare codons were completely avoided in the designed oligonucleotides by changing to the next preferred codon as described in "Materials and Methods". All the oligonucleotides making up the gene were assembled, and then the assembling product was used as the target for PCR amplification of the gene using the two extreme end oligonucleotides as primers. This successive two-step PCR depends on the relative concentration of internal and outermost (amplifying) primers. The internal is inhibitory to the synthesis of the full-length of IFN-α8 gene. So with the internal primer concentrations lower than that of the outermost primers (in the second step), the efficient synthesis of the full-length product was obtained.

The choice of the DNA polymerase for gene synthesis was dependent on its properties. The use of enzyme mixes (Taq DNA polymerase in combination with *Tgo* DNA polymerase) in the GC-Rich system correctly amplified the full-length of the IFN-α8 gene. PCR results with proofreading *pfu* clone were impaired by the presence of nonspecific PCR products. This problem is mainly due to the degradation of primer molecules during PCR setup, caused by the otherwise advantageous $3' \rightarrow 5'$ exonuclease activity of proofreading polymerases [24].

A variety of promoters are now used for protein expression. The best choice is a promoter with little or no expression before induction and with reliable, adjustable expression. The arabinose promoter system perhaps comes closest to fulfilling these objectives [8]. Inexpensive and tightly controlled inductions were the essential features of this expression system. Recombinant IFN-α8 produced in *E. coli* carrying the pBAD-IFNα8 construct was confirmed by SDS-PAGE and immunoassays, and the system was optimized in regard to induction time and temperature and concentration of inducer. The IFN-α8 exhibited low electrophoretic mobility on SDS-PAGE due to the presence of charged amino acids Glu84 and Asp90 as previously reported by Uze et al. [25]. The synthesized IFN-α8 is biologically active as demonstrated by anticancer effect, antiviral activity, and immunoassay signals and shows higher activity than that of commercial IFN-α2a. These data coincide with the previous results of antiproliferative effects of IFN-α8 on many cell carcinomas [26, 27] and higher antiviral potency than other IFN-α subtypes [2].

The concentration of L-arabinose and the elapsed time for induction jointly affected the partition of IFN-α8 into the soluble form and inclusion bodies. Lim et al. [28] demonstrated that reaching maximum expression level for IFN-α took about 10 h using 10.7 mM L-arabinose, but the insoluble band was thick. In our results, 6 h induction using 0.02% w/v L-arabinose achieved maximum yield (Table 2), but a significant part of the product formed inclusion bodies (Fig. 6 and Table 3).

Human interferon-α8 was expressed in *Saccharomyces cerevisiae* and found to accumulate intracellularly in an insoluble form, thus needed to be extracted by vari-

ous solubilizing agents and subsequently refolded [3]. The advantage of our procedure is that majority of the expressed IFN-α8 is in the soluble active form (accounting for about 46% of the yield in total cell lysate).

In conclusion, in this study a synthetic human IFN-α8 gene with codon optimized for expression in *E. coli* was assembled and amplified using two types of Taq polymerases in two-step PCR, then cloned into the pBAD-TOPO expression vector. The recombinant IFN-α8 was produced in *E. coli* carrying the pBAD-IFNα8 construct, purified, and assayed for antiproliferative and antiviral activity in comparison to the commercially available IFN-α2a.

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