Expression of Feline Interferon- α Subtypes in *Escherichia coli*, and Their Antiviral Activity and Animal Species Specificity

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(Received 26 October 2004/Accepted 4 February 2005)

ABSTRACT. Two kinds of FeIFN- α consisting of 166 amino acids (aa) and 171 aa were expressed in *Escherichia coli*, and the purified proteins were tested for antiviral activity on homologous and heterologous animal cells. Crude FeIFN induced in feline cells revealed antiviral activity on both homologous and heterologous animal cells. In contrast, both types of recombinant FeIFN- α revealed antiviral activity only on the feline cells. All of the FeIFN- α subtypes showed high activity to vesicular stomatitis virus, and the three species of feline viruses belonging to different families.

KEY WORDS: animal species-specificity, expression, feline IFN- α .

J. Vet. Med. Sci. 67(5): 543–545, 2005

Interferon (IFN) has multiple functions, for example antiviral, anti-microbial, anti-cell proliferation functions, and immunomodulating activity [3, 8, 15]. All mammals that have been studied have genes of IFN- α and IFN- β of type I, and IFN- γ (type II) genes. The IFN- ω gene diverged from the IFN- α gene approximately 130 million years ago, and the genes of IFN- τ , which play a role in the reproduction of ruminants [6], diverged from the IFN- ω gene approximately 36 million years ago; these genes are present in a limited number of animal species [19].

IFN- β and IFN- γ are animal species-specific, but IFN- α and its divergent IFN- ω and IFN- τ genes have shown antiviral activity on heterologous animal cells [10, 11, 16, 19, 24].

cDNA encoding feline interferon (FeIFN) was cloned for the first time by Nakamura *et al.* from a feline T-cell line [17]. It is a mature protein that has 171 amino acids. It has been called FeIFN- ω , because it has five more amino acids than ordinary IFN- α [7, 22]. Multiple subtypes of cDNA encoding FeIFN- α were recently cloned from feline lymph node cells and from a feline epithelial cell line [14, 25], and it was found that compared with FeIFN- α , FeIFN- ω has 5 continuous amino acid insertion sequences at positions 117 to 121 of the mature protein. The IFN- ω (also IFN- τ) of other animals has six additional amino acids at the carboxyl end compared with IFN- α , and it has distinct antigenicity, with approximately 60% amino acid homology [26]. In contrast, FeIFN- α and FeIFN- ω have 94.8% or more amino acids homology, and FeIFN- ω is more similar to IFN- α than to the IFN- ω of other animal species. On account of this, the type of FeIFN which is longer in length has also been called IFN- α by other authors [14, 19, 25]. FeIFN- ω has been reported to inhibit the growth of canine tumor cell lines [18,

21], and to have therapeutic effects on canine parvovirus infection [7, 13]. We previously cloned 8 subtypes of FeIFN- α from a feline epithelial cell line [14]. In the present study, FeIFN- α 7 (171 aa), FeIFN- α 8 (166 aa) and FeIFN- α 10 (166 aa) were expressed, and the antiviral activities of these subtypes against four species of virus as well as the species specificity on many kinds of animal cells were studied. Our objective was to determine whether there are differences between the two kinds of FeIFN- α (the ordinary kind and the long kind) with regard to antiviral activity and species specificity, and to find out how many animal species are sensitive to FeIFN- α .

The PCR primers were designed based on the sequences of FeIFN- α 7, FeIFN- α 8, and FeIFN- α 10 [14]. The sense and anti-sense PCR primers were designed for regions +70 to +87 with the +1 position corresponding to the initiation codon of the signal peptide, and +573 to 590 of the FeIFN- α 7, FeIFN- α 8, and FeIFN- α 10 genes. The sense primer was 24-mer and consisted of the sequence 5'-**GGATCCTGTGACCTGCCTCAGACC**-3'. The anti-sense primer was 24-mer and consisted of the sequence 5'-**GTCGACTTCAGGTCTCATTCTCGC**-3'; the *Bam*HI and *Sal*I restriction sites are indicated as bold letters. The PCR conditions included a 2-min denaturation step at 94°C, followed by 30 cycles of 30 sec at 94°C, 30 sec at 60°C, and 1 min at 72°C. A final extension for 10 min at 72°C was then performed. The PCR products were ligated between the *Bam*HI and *Sal*I restriction sites of the pMAL-c2x expression vector (New England BioLabs, Inc., Beverly, MA.). The constructed plasmid was transformed into the *E. coli* TOP10 strain. The expression of recombinant proteins was performed, according to the manufacturer's protocols, using a pMAL Protein Fusion and Purification System (New England BioLabs, Inc., Beverly, MA.). The purified proteins of the subtypes of recombinant (r) FeIFN- α were analyzed by sodium dodecyl sulfate-polyacrylamide gel

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electrophoresis (SDS-PAGE) and visualized by Coomassie brilliant blue R-250 (CBB R-250; E Merck AG Co., Ltd., Darmstadt, Germany). FeIFN- ω which is sold under the name Interact (Toray Industries, Tokyo, Japan) was also used. The cells used in this study were CRFK cells (feline), fewf-4 cells (feline), MDCK cells (canine), MDBK cells (bovine), RK-13 cells (rabbit), L-929 cells (murine), and FL cells (human). Eagle's Minimum Essential Medium (Nissui Co., Ltd., Tokyo) with the addition of 10% fetal calf serum (JRH Bio. Sci., U.S.A.) was used for cell growth, and MEM with 2% FCS for cell maintenance. For the production of natural IFN, CRFK cells were stimulated with an ultraviolet light-inactivated B1 strain of the *Newcastle disease virus* (10^9 TCID₅₀/ml) for 24 hr. The culture medium was centrifuged, and the supernatant was dialyzed against the 0.2 M KCl-HCl buffer (pH 2) and then against the 0.01 M Tris-HCl buffer (pH 7.4). Antiviral activity was assayed by modification of the semi-micro dye-binding method of Armstrong [1]. The cells (2×10^4 cells/0.1 ml) were seeded in a 96-well plate (Greiner Bio One, Germany) and incubated at a temperature of 5% CO₂ in air at 37°C for two days. The medium was then removed, and the triplicated wells were exposed for 24 hr at 37°C to 0.1 ml/well of serial two-fold dilutions of FeIFN in MEM with 2% FCS. The subtypes of IFN were removed and challenged with viruses (10^4 TCID₅₀ in the case of VSV). When CPE reached 100% in viral control, the cells were stained with crystal violet. The dye was dissolved by 50 mM NaOH, and OD₅₉₅ was measured by a MICROPLATE READER (BIO-RAD). Titers were expressed in units based on a 50% reduction in the extraction of the crystal violet dye.

The antiviral activities of the subtypes of rFeIFN- α were assayed with viruses belonging to different families: *vesicular stomatitis New Jersey virus* (VSV; *Rabdoviridae*), *Feline Calicivirus* (FCV; *Caliciviridae*), *Feline infectious peritonitis virus* (FIPV; *Coronaviridae*), and *Felid herpesvirus 1* (FHV-1; *Herpesviridae*).

A 22-kDa protein of FeIFN- $\alpha 7$ with a 5-amino acid insertion and a 20-kDa protein of FeIFN- $\alpha 8$ and FeIFN- $\alpha 10$ are shown in Fig. 1.

All three subtypes of rFeIFN- α and rFeIFN- ω revealed antiviral activity to all of the four species of virus on the fewf-4 cells (Table 1). The activity of rFeIFN- $\alpha 7$ (8.5×10^6 U/mg) was higher than that of rFeIFN- $\alpha 8$ (1.1×10^6 U/mg) and rFeIFN- $\alpha 10$ (0.65×10^6 U/mg) against VSV. The subtypes of rFeIFN- α were found to be most active to VSV, and least active to FHV-1. From the mean titers of the subtypes of rFeIFN- α against the 4 viruses, the subtypes of FeIFN- α were 7.2-fold, 13.2-fold, and 20.7-fold more active to VSV than FIPV, FC, and FHV, respectively.

The antiviral activities of FeIFN and the subtypes of rFeIFN- α against VSV on the heterologous animal cells are presented in Table 2. Contrary to our expectations, no rFeIFN- α revealed antiviral activity on the heterologous animal cells. In contrast, crude FeIFN produced by CRFK cells revealed antiviral activity on RK-13 cells, MDBK cells, MDCK cells, and L-929 cells, but not on the FL cells. The

Fig. 1. SDS-PAGE analyses of purified FeIFN- $\alpha 7$, $\alpha 8$ and $\alpha 10$. The proteins were stained with Coomassie brilliant blue R-250 after electrophoresis. Lane 1, rFeIFN- $\alpha 7$; lane 2, rFeIFN- $\alpha 8$; lane 3, rFeIFN- $\alpha 10$; lane M, molecular marker.

Table 1. Antiviral activities of the subtypes of rFeIFN- α against viruses on the fewf-4 cell

FeIFN	Antiviral activity ($\times 10^6$ U/mg)			
	VSV	FCV	FIPV	FHV-1
rFeIFN- $\alpha 7$	8.50	0.33	0.66	0.16
rFeIFN- $\alpha 8$	1.10	0.02	0.06	0.04
rFeIFN- $\alpha 10$	0.65	0.16	0.03	0.08
rFeIFN- ω ^{a)}	13.10	4.60	2.30	0.82

a) Antiviral activity of rFeIFN- ω is shown as units per vial.

antiviral activities of crude FeIFN were high on MDBK and RK-13 cells, but low on MDCK and L-929 cells.

The human IFN- α monomer is composed of five α -helices (A-E). The AB loop is linked to helix E by a Cys29-Cys138 disulfide bond [9]. Based on a mutagenesis study of HuIFN- α , the functionally important residues are clustered on the AB loop, the bend in helix B, and helices C, D, and E. Arg 33 (AB loop), Arg144, and Glu 146 (helix E) have been shown to be major contributors [4]. The five-amino acid insertions at positions 117–121 of FeIFN- $\alpha 7$ and FeIFN- ω are the positions that affect the functionally important residues of helix D in the case of rHuIFN- α (Arg-120, Lys-121, Gln-124, Lys-131, and Glu-132). Antiviral activity of rFeIFN- $\alpha 7$ was 2–22-fold higher than that of rFeIFN- $\alpha 8$ or rFeIFN- $\alpha 10$ against VSV, FCV, FIPV, and FHV-1. It has also been reported that the insertion of 5aa does not affect the antiviral activity of the subtypes of FeIFN- α , expressed in Chinese hamster ovary cells, against VSV and FCV [2]. These results suggest that the insertion of 5aa in FeIFN- α s does not diminish its activity on the cells, but instead increases it.

Table 2. Antiviral activities of crude FeIFN and the subtypes of rFeIFN- α against VSV on the various animal cells

FeIFN	Antiviral activity ($\times 10^6$ U/mg) on the cells					
	fcwf-4	RK-13	MDBK	MDCK	L-929	FL
Crude FeIFN ^{a)}	0.05	0.033	0.014	0.0009	0.0007	<0.00006
rFeIFN- α 7	8.50	<0.00006	<0.00006	<0.00006	<0.00006	<0.00006
rFeIFN- α 8	1.10	<0.00006	<0.00006	<0.00006	<0.00006	<0.00006
rFeIFN- α 10	0.65	<0.00006	<0.00006	<0.00006	<0.00006	<0.00006
rFeIFN- ω ^{b)}	13.10	<0.00006	<0.00006	<0.00006	<0.00006	<0.00006

a) Antiviral activity of crude FeIFN is shown as units per ml.

b) Antiviral activity of rFeIFN- ω is shown as units per vial.

All four subtypes of rFeIFN- α 7, rFeIFN- α 8, rFeIFN- α 10, and rFeIFN- ω have high antiviral activity to FCV, FIPV, and FHV-1, which belong to a distinct family. The crude FeIFN produced by CRFK cells has antiviral activity on the cells of many animal species, except for human FL cells. In contrast, none of the subtypes of rFeIFN- α revealed antiviral activity on the heterologous animal cells. These results lead us to suspect that crude FeIFN produced by CRFK cells includes IFN or cytokine other than IFN- α , which is active on heterologous animal cells. Although FeIFN- α and crude FeIFN did not show antiviral activity on human cells in this study, recombinant human IFN- α has been shown to be effective against viruses infecting cats, both *in vivo* and *in vitro* [5, 20, 23]. rFeIFN- α has been reported to inhibit growth in both feline and canine tumor cell lines. Less inhibition was observed in the canine tumor cell lines than in the feline ones, though rFeIFN- ω did not inhibit the growth of human, monkey, or hamster cells [18, 21]. Another study has indicated that five subtypes of rFeIFN- α (rFeIFN- α 1, rFeIFN- α 2, rFeIFN- α 3, rFeIFN- α 5, and rFeIFN- α 6) are able to inhibit the growth of a feline T-cell line (FeT-J); but they did not inhibit the growth of human and murine cells [2].

Considering that the subtypes of rFeIFN- α , including rFeIFN- ω , were found to be highly species-specific with regard to antiviral activity in this study, the therapeutic effect of rFeIFN- ω on *Canine parvovirus* infection [7, 13] was caused by something other than antiviral activity.

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