Molecular Cloning of Feline Interleukin-6 cDNA

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ABSTRACT. We cloned and sequenced feline interleukin-6 (IL-6) cDNA using the polymerase chain reaction (PCR) technique to obtain information on regulation of the IL-6 gene. The feline IL-6 cDNA cloned in this study was 752 bp long and included all the amino acid coding region. At the amino acid level, the overall identities of the feline IL-6 to the human and murine counterparts were 58% and 39%, respectively. After transfection with the expression vector containing the cDNA, CRFK cells produced biologically active proteins that showed hybridoma growth promoting activity.—**KEY WORDS:** cDNA interleukin-6, feline, polymerase chain reaction.

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IL-6 has been identified as an important cytokine involved in regulation of inflammatory and immune responses to infection or injury [16]. It has many biological functions such as stimulation of immunoglobulin (Ig) synthesis [9,21], regulation of acute phase protein synthesis by hepatocytes [5], stimulation of T lymphocyte growth and differentiation [19,26] and stimulation of hemopoietic stem cells [13]. It is produced by various types of lymphoid and non-lymphoid cells on various types of stimulation, such as by T cells stimulated with mitogens [11], B cells stimulated with IL-4 or PMA [12], monocytes stimulated with LPS [11, 20], and fibroblasts stimulated with poly(rI).poly(rC), IL-1, platelet-derived growth factor, TNF, FCS, or LPS [17, 27, 29]. IL-6 has also been shown to be involved in the immunopathology of various diseases in humans. It is an autocrine growth factor for multiple myeloma [15]. The unregulated, constitutive production of IL-6 may induce polyclonal B cell activation leading to hyper-gammaglobulinemia and autoantibody production in certain tumor-bearing patients [8]. The unregulated expression of IL-6 mRNA may also be involved in the pathogenesis of rheumatoid arthritis [7] and Castleman's disease [30].

Moreover, there are reports that human immunodeficiency virus (HIV) induces IL-6 production by monocytes [22] and B cells [3] *in vitro* and that HIV-infected individuals show elevated levels of plasma IL-6 and IL-6 production by monocytes [2, 4, 10]. We recently found that overproduction of IL-6 was associated with the pathogenesis of feline infectious peritonitis, which is associated with remarkable hyper-gammaglobulinemia [6], and that the overproduction of IL-6 is also seen in feline immunodeficiency virus (FIV)-infected cats [24].

Because of the potential pathologic role of IL-6 in feline viral diseases, it is important to investigate the mechanism regulating IL-6 expression. In this paper, as a first step in understanding the regulation of the IL-6 expression in a feline immune system, we describe the cloning and sequencing of feline IL-6 cDNA.

MATERIALS AND METHODS

Preparation of cDNA: A normal Japanese domestic cat kept for experimental purposes was anesthetized and injected intravenously with 1 mg/kg of LPS (Serotype 055:B5; Sigma Chemical Co., St. Louis, MO). Three hours later, the mandibular lymph nodes were removed and immediately frozen in liquid nitrogen and mRNA was extracted from 1 g of frozen tissue with a Fast Track mRNA Isolation kit (Invitrogen Co., San Diego, CA). Reverse transcription of 1 μ g of mRNA was performed with a cDNA Synthesis kit (Pharmacia, Uppsala, Sweden) and the cDNA was dissolved in 150 μ l of TE buffer.

Polymerase chain reaction (PCR) amplification: PCR primers were based on the sequences conserved between human and murine IL-6 [9, 28]. The primer sequences were 5'-AAGCTCTATCTCCCTCCAG-3' (primer AS) and 5'-AAAAATAATTAAAATAGTGTCC-3' (primer FR), complementary to the sequences upstream and downstream of the coding sequence of IL-6, respectively. By using these primers, an 810-base pair (bp) fragment including the whole amino acid coding region is expected to be amplified. The cDNA was amplified by PCR in a volume of 100 μ l, using primers AS and FR (0.4 μ M each), Taq polymerase (1.5 units), and the reagents and protocol recommended by the manufacturer (Perkin-Elmer/Cetus, Norwalk, CT). Amplification was achieved by 30 cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min) and polymerization (72°C, 1–5 min, gradually extended). Then 10 μ l of this PCR product was again amplified by the same procedure. The resulting product was electrophoresed in 2% low melting point agarose gel and the amplified DNA band of approximately 800 bp was extracted from the gel. The gel-purified 800-bp DNA treated with the DNA polymerase I (Klenow fragment) (Toyobo, Osaka, Japan) was cloned into the dephosphorylated *Sma* I site of Bluescript SK (+) (Stratagene, La Jolla, CA). *Escherichia coli*, DH5 α cells (BRL, Gaithersburg, MD) were transformed with the ligation

mixture and plated onto 2X TY agar plates containing 50 $\mu\text{g/ml}$ of ampicillin, 36 $\mu\text{g/ml}$ of 5-bromo-4-chloro-3-indolyl β -D-galactoside and 40 $\mu\text{g/ml}$ of isopropyl β -D-thiogalactoside. Plasmid DNA was prepared from 7 independent white colonies by either the boiling method or with a QIAGEN plasmid kit (QIAGEN, Studio City, CA). The DNA inserts of these 7 clones were further screened by PCR using another primer pair. This primer pair (sense primer, 1S, 5'-GCCTTCGTCCAGTTGCCTTC-3'; antisense primer 8R, 5'-CTGCAGCCACTGGTCTGTG-3') has been confirmed to amplify a 480-bp DNA fragment inside the 810-bp AS-FR fragment.

Nucleotide sequence analysis: Two independent clones, AF-11 and AF-13 containing feline IL-6 cDNA were sequenced. Both strands of the inserts in the plasmids were sequenced by the dideoxy chain termination method using Sequenase Ver. 2.0 (United States Biochemicals, Cleveland, OH).

Expression of IL-6 cDNA: A *Pst*I-*Kpn*I fragment of clone AF-13 was inserted into a *Pst*I-*Kpn*I site of the expression-cloning vector, pcDL-SRa296[25]. Samples of 15 μg of either pcDL-SRa296 containing feline IL-6 cDNA (pcDL-SRa296-fIL-6) or the original pcDL-SRa296 were transfected into CRFK cells by use of Lipofectin Reagent (BRL, Gaithersburg, MD). These transfectants were cultured in RPMI 1640 containing 10% FCS and 50 μg of gentamycin, and the culture supernatants were harvested for assay of IL-6 activity.

IL-6 assay: IL-6 concentrations in the culture supernatants were determined using the IL-6-responsive rat-mouse hybridoma cell clone, B3B1, as described previously [23]. The proliferation of B3B1 cells was not stimulated by other cytokines such as IL-1, IL-2, IL-3, or G-CSF. Human rIL-6 was used to construct a standard curve. The activity of human rIL-6 was estimated to be 5×10^6 units/mg [1]. The number of growing cells was determined by hexosaminidase assay as described by Landegren [18].

RESULTS AND DISCUSSION

Isolation of a cDNA clone of feline IL-6: Using LPS-stimulated feline lymph node cDNA as a template, we performed PCR amplification with AS and FR primers for isolation of a cDNA clone of feline IL-6. Electrophoresis of the PCR products of agarose gel gave 3 distinct bands of about 800 bp. These 3 bands were extracted from the gel and cloned into Bluescript SK(+) plasmid vector. Seven independent clones derived from the bands of about 800 bp were screened for the presence of IL-6 cDNA by PCR amplification with an inner primer pair, 1S and 8R, which could amplify a band of approximately 500 bp. The result showed that the expected band was amplified in 2 clones, AF-11 and AF-13.

Nucleotide sequence of feline IL-6 cDNA: The nucleotide sequences of the AF-11 and AF-13 inserts were determined on both DNA strands. The nucleotide sequences of these two clones were identical. So, we used the AF-13 clone for further experiments. The cDNA insert of

the AF-13 clone contains a single large open reading frame. In this frame, the initiator ATG is followed by 207 codons before the termination triplet TAA. The nucleotide and deduced amino acid sequences are shown in Fig. 1. The sequenced region of the cDNA corresponds to nucleotides 24-787 from the 5'-end of the mature form human IL-6 mRNA [9]. The deduced amino acid sequence of the cDNA indicated that feline IL-6 would consist of 208 amino acid residues before removal of a signal peptide and the calculated molecular weight of this encoded protein is 20,515.2. The NH_2 -terminal region of the mature feline IL-6 protein is not known, so we can not decide the exact boundary of the signal peptide of feline IL-6.

Figure 2 shows the alignment of the deduced amino acid sequence of feline IL-6 compared with those of the human and murine IL-6 sequences. At the amino acid level, the overall identities of the feline IL-6 with the human and murine counterparts are 58% and 39%, respectively. In regions that are highly conserved between human and murine IL-6 [28], the sequence of feline IL-6 is also well conserved. In particular, 4 cysteine residues at positions 68, 74, 97, and 107 of feline IL-6 are found at the same positions in human and murine IL-6 (Fig.2). This result suggests that the cysteine-rich middle region of the mature protein may be important for IL-6 activity, although

Fig. 1. Nucleotide sequence and deduced amino acid sequence of the feline IL-6 cDNA. Numbers above and below the sequence show positions of nucleotides and amino acids, respectively. These numbers refer to the 5' end of the coding region. Underlined sequences indicate the regions of oligonucleotide primers (AS and FR) used in the PCR. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases with the following accession number D13227.

Hours after transfection	IL-6 (units/ml) - Filled Circles	IL-6 (units/ml) - Open Circles
0	~0.5	~0.5
6	~3.5	~0.5
24	~15.5	~0.5
48	~11.5	~0.5

Fig. 3. IL-6 activity in the supernatants of CRFK cells transfected with IL-6 cDNA. CRFK cells were transfected with feline IL-6 cDNA cloned in the pcDL-SRa296 expression vector (●) or with pcDL-SRa296 alone (○). The supernatants of the cultures of transfected cells were collected and IL-6 activity was determined by B3B1 cell proliferation assay. Relative IL-6 activity was indicated as that of human rIL-6 used for the standard curve.

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REFERENCES

1. Asagoe, Y., Yasukawa, K., Saito, T., Maruo, N., Miyata, K., Kono, T., Miyake, T., Kato, T., Kakidani, H., and Mitani, M. 1988. Human B-cell stimulatory factor-2 expressed in *Escherichia coli*. *Biotechnology* 6: 806-809.
2. Birm, D. L., Redfield, R. R., Tencer, K., Fowler, A. Burke, D. S., and Tosato, G. 1990. Induction of interleukin-6 during human immunodeficiency virus infection. *Blood* 76: 2303-2310.
3. Boue, F., Wallon, C., Goujard, C., Barresinoussi, F., Galanaud, P., and Delfraissy, J. F. 1992. HIV induces IL-6 production by human B lymphocytes. Role of IL-4. *J. Immunol.* 148: 3761-3767.
4. Breen, E. C., Rezai, A. R., Nakajima, K., Beall, G. N., Mitsuyasu, R. T., Hirano, T., Kishimoto, T., and Martinez-maza, O. 1990. Infection with HIV is associated with elevated IL-6 levels and production. *J. Immunol.* 144: 480-484.
5. Gaudie, J., Richards, C., Harnish, D., Lansdorp, P., and Baumann, H. 1987. Interferon β_2 /B-cell stimulatory factor type 2 shares identity with monocyte-derived hepatocyte-stimulating factor and regulates the major acute phase protein response in liver cells. *Proc. Natl. Acad. Sci. U.S.A.* 84: 7251-7255.
6. Goitsuka, R., Ohashi, T., Ono, K., Yasukawa, K., Koishibara, Y., Fukui, H., Ohsugi, Y., and Hasegawa, A. 1990. IL-6 activity in feline infectious peritonitis. *J. Immunol.*

Fig. 2. Comparison of amino acid sequences of feline IL-6 with those of human and murine IL-6. Numbers above sequence are amino acid positions in feline IL-6, starting at the putative N-terminal methionine residue. Dots indicate identities with the feline IL-6 sequence and dashes indicate gaps induced for alignment. Asterisks indicate cysteine residues conserved in the sequences. The overall identities of the feline IL-6 amino acid sequence with those of the human and murine counterparts are 58% and 39%, respectively.

Jambou *et al.* [14] reported that a cysteine-free recombinant IL-6 that was chemically synthesized on the basis of the human IL-6 cDNA sequence was also biologically active.

Expression of feline IL-6 in CRFK cells: To determine whether molecularly cloned feline IL-6 cDNA encodes biologically active protein, we recloned the feline IL-6 cDNA into a transient mammalian expression vector pcDL-SRa296 to yield pcDL-SRa296-fIL-6. CRFK cells were transfected with this pcDL-SRa296-fIL-6 by the Lipofectin method, and the culture supernatants were tested for activity to induce proliferation of IL-6 responsive hybridoma cells. As shown in Fig. 3, after transfection with pcDL-SRa296-fIL-6, the culture supernatants showed significant IL-6 activity and the maximal production of IL-6 was observed at 24 hours after transfection. On the other hand, the supernatants of CRFK cells transfected with control vectors showed no IL-6 activity. This result indicates that AF-13 clone encodes biologically active feline IL-6.

The cDNA and the expression vector of feline IL-6 obtained in this study will be very useful for better understanding of the involvement of IL-6 in feline immune-mediated diseases.

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- 144: 2599-2603.
7. Hirano, T., Matsuda, T., Turner, M., Miyasaka, N., Buchan, G., Tang, B., Sato, K., Shimizu, M., Maini, R., Feldmann, M., and Kishimoto, T. 1988. Excessive production of interleukin 6/B cell stimulatory factor-2 in rheumatoid arthritis. *Eur. J. Immunol.* 18: 1797-1801.
8. Hirano, T., Taga, T., Yasukawa, K., Nakajima, K., Nakano, N., Takatsuki, F., Shimizu, M., Murashita, A., Tsunasawa, S., Sakiyama, F., and Kishimoto, T. 1987. Human B cell differentiation factor defined by an anti-peptide antibody and its possible role in autoantibody production. *Proc. Natl. Sci. U.S.A.* 84: 228-231.
9. Hirano, T., Yasukawa, K., Harada, H., Taga, T., Watanabe, Y., Matsuda, T., Nakajima, S., Koyama, K., Iwamatsu, A., Tsunasawa, S., Sakiyama, F., Matsui, H., Takehara, Y., Taniguchi, T., and Kishimoto, T. 1986. Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. *Nature (Lond.)* 324: 73-76.
10. Honda, M., Kitamura, K., Mizutani, Y., Oishi, M., Arai, M., Okura, T., Igarashi, K., Yasukawa, K., Hirano, T., Kishimoto, T., and Tokunaga, T. 1990. Quantitative analysis of serum IL-6 and its correlation with increased levels of serum IL-2R in HIV-induced diseases. *J. Immunol.* 145: 4059-4064.
11. Horii, Y., Meraguchi, A., Suematsu, S., Matsuda, T., Yoshizaki, K., Hirano, T., and Kishimoto, T. 1988. Regulation of BSF-2/IL-6 production by human mononuclear cells: Macrophage-dependent synthesis of BSF-2/IL-6 by T cells. *J. Immunol.* 141: 1529-1535.
12. Hutchins, D., Cohen, B. B., and Steel, C. M. 1990. Production and regulation of interleukin 6 in human B lymphoid cells. *Eur. J. Immunol.* 20: 961-968.
13. Ikebuchi, K., Wong, G. G., Clark, S. C., Ihle, J. N., Hirai, Y., and Ogawa, M. 1987. Interleukin-6 enhancement of interleukin-3-dependent proliferation of multipotential hemopoietic progenitors. *Proc. Natl. Acad. Sci. U.S.A.* 84: 9035-9039.
14. Jambou, R. C., Snouwaert, J. N., Bishop, G. A., Stebbins, J. R., Frelinger, J. A., and Fowlkes, D. M. 1988. High-level expression of a bioengineered, cysteine-free hepatocyte-stimulating factor (interleukin 6) like protein. *Proc. Natl. Acad. Sci. U.S.A.* 85: 9426-9430.
15. Kawano, M., Hirano, T., Matsuda, T., Horii, Y., Iwata, K., Asaoku, H., Tang, O., Tanabe, B., Tanaka, H., Kuramoto, A., and Kishimoto, T. 1988. Autocrine generation and requirement of BSF-2/IL-6 for human multiple myelomas. *Nature (Lond.)* 332: 83-85.
16. Kishimoto, T. 1989. The biology of interleukin-6. *Blood* 74: 1-10.
17. Kohase, M., May, L. T., Tamm, I., Vilcek, J., and Sehgal, P. B. 1987. A cytokine network in human diploid fibroblasts. Interactions of β -interferons, tumor necrosis factor, platelet-derived factor and interleukin-1. *Mol. Cell. Biol.* 7: 273-280.
18. Landegren, U. 1984. Measurement of cell numbers by means of the endogenous enzyme hexosaminidase. Applications to detection of lymphokines and cell surface antigens. *J. Immunol. Methods* 67: 379-380.
19. Lotz, M., Jirik, F., Kabouridis, P., Tsoukas, C., Hirano, T., Kishimoto, T., and Carson, D. A. 1988. B cell stimulating factor 2/interleukin 6 is a costimulant for human thymocytes and T lymphocytes. *J. Exp. Med.* 167: 1253-1258.
20. May, L. T., Ghrayeb, J., Santhanam, U., Tatter, S. B., Sthoege, Z., Helfgott, D. C., Chiorazzi, N., Grieninger, G., and Sehgal, P. B. 1988. Synthesis and secretion of multiple forms of β_2 -interferon/ B-cell differentiation factor 2/hepatocyte-stimulating factor by human fibroblasts and monocytes. *J. Biol. Chem.* 263: 7760-7766.
21. Muraguchi, A., Hirano, T., Tang, B., Matsuda, T., Horii, Y., Nakajima, K., and Kishimoto, T. 1988. The essential role of B cell stimulatory factor 2 (BSF-2/IL-6) for the terminal differentiation of B cells. *J. Exp. Med.* 167: 332-344.
22. Nakajima, K., Martinez-Maza, O., Hirano, T., Breen, E. C., Nishanian, P. G., Salazar-Gonzalez, J. L., and Kishimoto, T. 1989. Induction of IL-6 (B cell stimulatory factor 2/IFN- β_2) production by HIV. *J. Immunol.* 142: 531-536.
23. Ohashi, T., Goitsuka, R., Ono, K., and Hasegawa, A. 1989. Feline hybridoma growth factor/interleukin-6 activity. *J. Leukocyte Biol.* 46: 501-507.
24. Ohashi, T., Goitsuka, R., Watari, T., Tsujimoto, H., and Hasegawa, A. 1992. Elevation of feline interleukin 6-like activity in feline immunodeficiency virus infection. *Clin. Immunol. Immunopathol.* 65: 207-211.
25. Takebe, Y., Seiki, M., Fujisawa, J., Hoy, P., Yokota, K., Arai, K., Yoshida, M., and Arai, N. 1988. SR α promoter, an efficient and versatile mammalian cDNA expression system composed of the simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat. *Mol. Cell. Biol.* 8: 466-472.
26. Uyttenhove, C., Coulie, P. G., and Van Snick, J. 1988. T cell growth and differentiation induced by interleukin-HP1/IL-6, the murine hybridoma/plasmacytoma growth factor. *J. Exp. Med.* 167: 1417-1427.
27. Van Damme, J., Cayphas, S., Opdenakker, G., Billiau, A., and Van Snick, J. 1987. Interleukin 1 and poly(rI). poly(rC) induce production of a hybridoma growth factor by human fibroblasts. *Eur. J. Immunol.* 17: 1-7.
28. Van Snick, J., Cayphas, S., Szikora, J. P., Renauld, J. C., Van Roost, E., Boon, T., and Simpson, R. J. 1988. cDNA cloning of murine interleukin-HP1: homology with human interleukin 6. *Eur. J. Immunol.* 18: 193-197.
29. Walther, Z., May, L. T., and Sehgal, P. B. 1988. Transcriptional regulation of the interferon- β_2 /B cell differentiation factor BSF-2/hepatocyte-stimulating factor gene in human fibroblasts by other cytokines. *J. Immunol.* 140: 974-977.
30. Yoshizaki, K., Matsuda, T., Nishimoto, N., Kuritani, T., Taeho, L., Aozasa, K., T. Nakanhata, K., Kawai, H., Tagoh, H., Komori, T., Kishimoto, S., Hirano, T., and Kishimoto, T. 1989. Pathogenic significance of interleukin-6 (IL-6/BSF-2) in Castleman's disease. *Blood* 74: 1360-1367.