Molecular Cloning and Characterization of a cDNA Encoding Feline Interleukin-6 (43666)

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Abstract. **With the discovery that the cat is susceptible to infection by a lentivirus quite similar to the virus that causes the human acquired immune deficiency syndrome, the cat has become an important animal model with which the interactions between host and immunodeficiency virus can be studied. To facilitate investigation of the feline immune response and the response of cytokine expression to infection by feline immunodeficiency virus, our laboratory has focused on the isolation and molecular cloning of cDNA representative of feline lymphokines, cytokines, and monokines. Herein, we describe the molecular cloning and characterization of a cDNA encoding feline interleukin (IL)-6. The nucleotide sequence of feline IL-6 was found to exhibit 81%, 76%, 63%, and 61% homology with pig, human, rat, and mouse IL-6, respectively, while the predicted amino acid sequence exhibits 66%, 53%, 37%, and 30% homology with pig, human, rat, and mouse IL-6, respectively.** [P.S.E.B.M. 1993, Vol 204]

The accumulated knowledge of the feline immune system lags behind that obtained for humans and laboratory mice (1). However, progress has been made in defining the cellular immune system of cats. Since the recent discovery that the cat is susceptible to a disease similar to human acquired immune deficiency syndrome (AIDS; feline acquired immunodeficiency syndrome [FAIDS]) and that this disease can be induced by a distinct lentivirus remarkably similar to human immunodeficiency virus (HIV; feline immunodeficiency virus [FIV]), the cat has become an important animal with which to study the interactions between host and immunodeficiency virus (2–5). The discovery of feline AIDS has stimulated renewed interest in the feline immune system and the regulation of feline cytokines.

In HIV-infected humans, several cytokines have been found to be associated with potentially pathogenic effects. One of these cytokines is interleukin (IL)-6. In HIV-infected humans, elevated levels of IL-6 have been found in the serum (6) and in the cerebrospinal fluid (7). While the increased serum IL-6 may be responsible for the polyclonal B cell activation detected in HIV-1-infected patients, the elevated levels of IL-6 in the cerebrospinal fluid may be partially responsible for the neuropathology observed in these patients. It has been speculated that since IL-6 can be produced by astrocytes stimulated with tumor necrosis factor-α, and that IL-6 and tumor necrosis factor-α can act synergistically to upregulate expression and replication of HIV-1, then subtle increases in cerebrospinal fluid IL-6 are likely to stimulate HIV-1 expression and result in the neuropathology found in HIV-1-infected individuals (8).

Elevated serum levels of IL-6 can also be detected in patients predominantly infected with monocytotropic strains of HIV (9). This observation is not surprising because HIV infection of monocytes has been shown previously to induce IL-6 production in this cell population (10). Further compounding the pathology associated with AIDS, IL-6 has been found to be produced by Kaposi's sarcoma cells and to be required for optimal growth by these cells (11).

Because IL-6 is a multifunctional cytokine which plays a central role in regulating the immune response and especially because it appears to be involved with the pathogenic mechanisms associated with human

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AIDS, and therefore by extrapolation maybe involved with feline AIDS, we have pursued isolation and characterization of feline IL-6. Herein, we report the molecular cloning and analysis of a cDNA representative of feline IL-6.

Material and Methods

Animals. A specific pathogen-free, adult, female domestic short-haired cat (Harlan Sprague-Dawley, Indianapolis, IN) was utilized in this study. The cat was housed under conventional conditions in an American Association for Accreditation of Laboratory Animal Care accredited facility.

RNA Isolation. Approximately 12 ml of blood were obtained from an adult cat and defibrinated by placing 3- to 4-ml aliquots of blood into a 100-ml Erlenmeyer flask containing six small glass beads. The samples were shaken for 10 min on an orbital shaker at 250 rpm. When clots had formed around the glass beads, the remaining blood was removed, the flasks were rinsed with 2 ml of incomplete Hanks' buffer solution, and the rinse was added to the collected blood sample. The blood was diluted in 3 vol of incomplete Hanks' buffer solution and layered onto 15 ml of Lymphoprep (Nycomed Pharma AS, Oslo, Norway), and the mononuclear cells were banded by centrifugation at 1800 rpm at 4°C × 30 min. The resulting buffy coat cells were recovered, washed in 5 vol Hanks' buffer solution, and pelleted at 1800 rpm at 4°C × 10 min. To induce the expression of IL-6, approximately 1×10^8 cells were added to 70 ml of RPMI 1640 containing 10% fetal bovine serum and were incubated at 37°C in an atmosphere of 5% CO_2 for 24 hr; after 24 hr, either 20 ng/ml of phorbol myristate acetate plus 0.5 μM ionomycin or 10 μg/ml of lipopolysaccharide were added to the cultures for 4 hr, at which time the nonadherent and adherent cells were recovered separately, pelleted at 5000*g* for 10 min at 4°C, and total cell RNA was isolated using the method described by Chomczynski and Sacchi (12).

Synthesis of cDNA. Approximately 5 μg of total cell RNA obtained from the phorbol myristate acetate/ionomycin- or lipopolysaccharide-stimulated cells were used as the template for first-strand cDNA production using the cDNA cycle kit produced by Invitrogen (San Diego, CA). The RNA (5 μg in 8 μl H_2O) was mixed with 2 μl of 100 mM MeHgOH and incubated at room temperature for 5 min. To this mixture was added 2.5 μl of 0.7 M β-mercaptoethanol and 1 μl of oligodT primer. The mixture was incubated at 65°C for 5 min and then 1 μl of placental RNase inhibitor, 4 μl of 5x reverse transcriptase buffer, 1 μl of 25 mM dNTP, and 0.5 μl of reverse transcriptase were added. The mixture was incubated at 42°C for 60 min and then at 95°C for 3 min. An additional 0.5 μl of reverse transcriptase was added and the incubation was continued at 42°C for 60 min. The reaction was terminated by heating at 95°C for 5 min. The resulting first-strand cDNA was used as template in the polymerase chain reaction (PCR).

Polymerase Chain Reaction. The degenerate oligonucleotide 5′ primer GCTATGAA(G/C)T(C/T)CCTCTC and the degenerate oligonucleotide 3′ primer TTCTTC(A/G)TAGAGAACAACAT were designed by determining the regions of strongest homology existing between the human, pig, rat, and mouse IL-6 cDNA (Genbank accession nos. M14584, M80258, M26744, and J03783, respectively). The regions corresponding to the primer sequences are located at nucleotides 61–77 and 799–780 of the human IL-6 cDNA. These oligonucleotides were synthesized using Applied Biosystem's 380B DNA synthesizer (Forest City, CA). To amplify the IL-6-specific cDNA, 2 μl of the single-stranded cDNA were diluted in 49.5 μl of H_2O and placed in a 0.5-ml microfuge tube containing 10 μl of a 10X reaction buffer (500 mM KCl, 100 mM Tris-HCl [pH 8.3], 15 mM $MgCl_2$, and 0.1% [w/v] gelatin), 16 μl of a dNTP mixture containing 1.25 mM each of dATP, dCTP, dGTP, and dTTP, and 12 μl of each primer (50 ng/ml). The mixture was overlaid with 80 μl of mineral oil (Sigma Chemical Co., St. Louis, MO) to prevent evaporation. The reaction mixture was placed in a Perkin Elmer Cetus DNA thermal cycler (Cetus Corp., Emeryville, CA) and heated to 95°C for 5 min and then allowed to cool to 80°C. While at 80°C, 0.5 μl of *Thermus aquaticus* DNA polymerase (5 units/μl) was added. PCR was initiated with the following parameters: 95°C for 1 min (denaturation), 46°C for 1 min (annealing), and 72°C for 2 min (extension) for 25 to 30 cycles. The amplified DNA was analyzed by electrophoresing 10 μl of the reaction mixture through a 2% agarose gel containing 0.5 μg/ml of ethidium bromide and visualized with a UV light source.

Cloning of the IL-6 PCR Fragment. Approximately 2 μl of the IL-6-specific PCR reaction were mixed with 50 ng of the TA cloning vector (pCR) and ligated according to the manufacturer's (Invitrogen) instructions. The ligation reaction was incubated for 12 hr at 12°C and then 1 μl was used to transform INVαF' cells (Invitrogen). The cells were transformed according to the manufacturer's instructions and then plated on L-broth agar plates containing 50 μg/ml of ampicillin and coated with 25 μl of X-gal (40 mg/ml) 1 hr before plating the bacteria. After an overnight incubation at 37°C, individual white colonies were picked and used to inoculate 5 ml of L-broth containing 100 μg/ml of ampicillin. After 6 hr, 5 μl of the culture were used as substrate in an IL-6-specific PCR reaction to determine which cultures contained the proper recombinant plasmid DNA.

DNA Sequencing. Double-strand DNA sequencing was carried out by use of Sanger's dideoxy chain termination method (13) using the Sequenase version

2.0 kit produced by United States Biochemical Corp. (Cleveland, OH). Both strands of the cloned cDNA-PCR fragment were sequenced. Upon termination of the sequence reaction, the DNA samples were heated at 95°C for 5 min and then loaded onto a 0.4-mm thick 8% acrylamide-7 *M* urea gel and electrophoresed in 1x 90 m*M* Tris-HCl (pH 8.2), 45 m*M* boric acid, and 2.5 m*M* EDTA at constant power (45–55 W) for 3 to 15 hr. The gel was vacuum dried on 3MM paper (Whatman) at 80°C for 3.5 hr without prior fixation. The gel was then exposed to Kodak XAR x-ray film at room temperature for 12 to 24 hr and the resulting autoradiograph was read to reveal the DNA sequence. Compilation and computer analysis of the DNA sequence was performed with the Intelligenetics Suite software (Intelligenetics, Mountainview, CA).

Results

When reverse transcriptase-PCR was performed using the IL-6-specific degenerate primers, a 724-base pair fragment was obtained (Fig. 1). This fragment was cloned into the pCR vector and sequenced (Fig. 2). When the sequence was compared with the nucleotide sequence of the human, pig, rat, and mouse IL-6 cDNA, it was found that the cloned DNA indeed represents a cDNA derived from feline IL-6-specific mRNA.

The nucleotide sequence was found to exhibit 81%, 76%, 63%, and 61% homology with the pig, human, rat, and mouse IL-6, respectively (Table I). When the nucleotide sequence was translated into the corresponding amino acid sequence (Fig. 2), it was determined that the predicted feline IL-6 is 66%, 53%, 37%, and 30% homologous with pig, human, rat, and mouse IL-6, respectively (Table I). The amino acid sequence also suggests that feline IL-6 contains a hydrophobic secretory signal sequence which would be cleaved from the native peptide between residues 24–28.

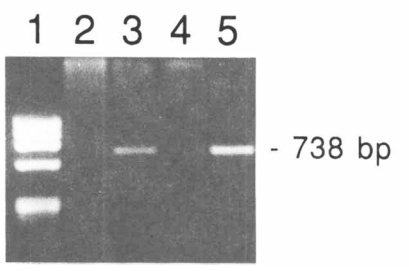

Figure 1. Detection by reverse transcriptase-PCR of mRNA encoding feline IL-6. Peripheral blood mononuclear cells were obtained and cultured in the presence or absence of lipopolysaccharide for 4 hr. The RNA from adherent or nonadherent cells in these cultures was isolated, converted to first-strand cDNA, and amplified by PCR using IL-6-specific oligonucleotide primers. Positive signals of the expected size (738 bp) were detected in the cDNA prepared from nonadherent and adherent cells stimulated with lipopolysaccharide (Lanes 3 and 5, respectively), but not from nonadherent or adherent cells cultured in the absence of lipopolysaccharide (Lanes 2 and 4, respectively). Lane 1 contains *Hae*III-digested phiX-174 DNA used as the molecular weight marker.

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GCT ATG AAC TTC CTC TCC ACA AGC GCC TTC AGT CCA CTC GCC TTC
     M   N   F   L   S   T   S   A   F   S   P   L   A   F

TCC CTG GGG CTG CTC CTG GTG GTG GCT ACT GCT TTC CCT ACC CCG
 S   L   G   L   L   L   V   V   A   T   A   F   P   T   P

GGA CCC CTG GGA GGA GAT GCC ACC TCA AAT AGA CTA CCA CTC ACC
 G   P   L   G   G   D   A   T   S   N   R   L   P   L   T

CCT GCA GAC AAA ATG GAA GAA CTC ATT AAG TAC ATC CTC GGC AAA
 P   A   D   K   M   E   E   L   I   K   Y   I   L   G   K

ATC TCT GCA CTG AAA AAG GAG ATG TGT GAC AAC TAT AAC AAA TGT
 I   S   A   L   K   K   E   M   C   D   N   Y   N   K   C

GAG GAC AGC AAG GAG GCA CTG GCA GAA AAC AAC CTG AAT CTT CCG
 E   D   S   K   E   A   L   A   E   N   N   L   N   L   P

AAA CTG GCA GAA AAA GAT GGA TGC TTC CAA TCT GGG TTC AAT CAG
 K   L   A   E   K   D   G   C   F   Q   S   G   F   N   Q

GAG ACC TGC CTG ACA AGA ATC ACT ACT GGT CTT CAG GAG TTT CAG
 E   T   C   L   T   R   I   T   T   G   L   Q   E   F   Q

ATA TAC CTG AAA TTC CTC CAG GAC AAG TAT GAG GGT GAT AAG GAA
 I   Y   L   K   F   L   Q   D   K   Y   E   G   D   K   E

AAT GCC AAG TCT GTG TAC ACC AGT ACT AAC GTC CTG CTC CAG ATG
 N   A   K   S   V   Y   T   S   T   N   V   L   L   Q   M

CTG AAG CGT AAG GGA AAG AAT CAG GAT GAG GTA ACC ATC CCT GTC
 L   K   R   K   G   K   N   Q   D   E   V   T   I   P   V

CCA ACC GTA GAA GTT GGC CTG CAG CTA AGC TGC AGT CAC AGA AGA
 P   T   V   E   V   G   L   Q   L   S   C   S   H   R   R

GTG GCT GAG GCA CAC AAC AAT CAC CTC ACC CTT CGA AGG CTA GAG
 V   A   E   A   H   N   N   H   L   T   L   R   R   L   E

GAC TTC CTT CAG TTA CGC CTC AGG GCT GTT CGG ATA ATG TAA CCT
 D   F   L   Q   L   R   L   R   A   V   R   I   M

GGG CAT CTA AGA TTG CTG TAG TTC ACG GGC ATT CCT TTC TCT GGT

CAG AAA CCT GTC CAC TGG GCA TGT AAC TGA TGT TGT TCT CTA CGA

AGA A
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Figure 2. Nucleotide and amino acid sequence of feline IL-6. The nucleotide sequence of the feline IL-6 cDNA is shown (GenBank accession no. L16914), with the predicted amino acid sequence displayed immediately under the nucleotide sequence. The start and stop codons are displayed in bold type.

Table I. Homology of the Feline IL-6 DNA and Predicted Protein with Human, Pig, Rat, and Mouse IL-6[a]

Animal	Percent homology	
	DNA	Protein
Pig	81	66
Human	76	53
Rat	63	37
Mouse	61	30

[a] The nucleotide sequence and predicted amino acid sequence deduced from the feline IL-6 cDNA was compared with the corresponding mRNA and peptide sequences from pig, human, rat, and mouse IL-6. The pig, human, rat, and mouse nucleotide sequences were obtained from GenBank accession nos. M14584, M80258, M26744, and J03783, respectively. These nucleotide sequences were translated into the corresponding amino acid sequences for comparison with feline IL-6.

The characteristic signature that IL-6 shares with the granulocyte-colony-stimulating factor (14, 15) and the myelomonocytic growth factor (16) is also observed within the predicted feline IL-6 peptide (Fig. 3). The conservation of four cysteine residues, even in regions where the surrounding residues exhibited little homol-

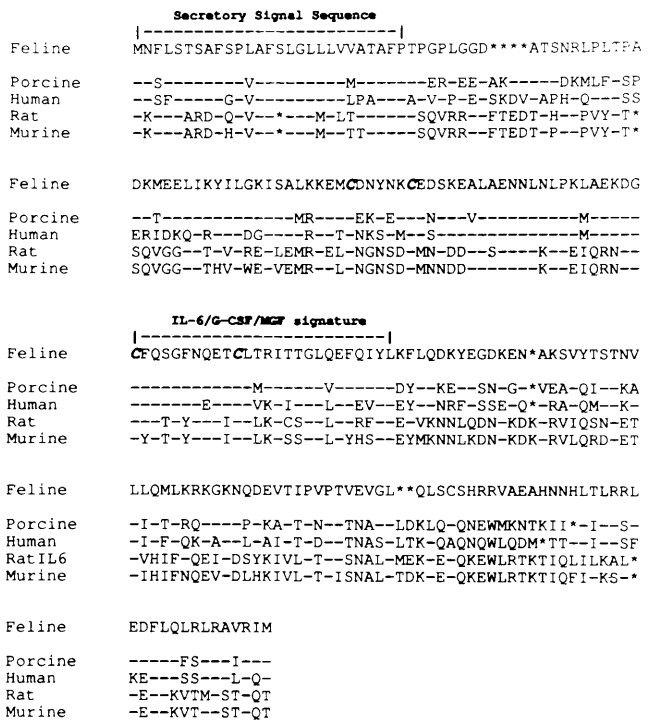

Figure 3. Homology of the predicted feline IL-6 peptide sequence with porcine, human, rat, and murine IL-6. Computer-assisted alignment of the peptide sequence of porcine, human, rat, murine, and the predicted feline IL-6 demonstrates conservation of several cysteine residues (bold italics) within the IL-6/granulocyte-colony-stimulating factor/myelomonocytic growth factor signature sequence. Also shown is the predicted location of the secretory signal sequence. Asterisks represent breaks in the sequence to optimize alignment. Dashes represent regions of homology with feline IL-6.

ogy, was detected and strongly suggests that these cysteines may be critical for creating proper secondary and tertiary structures through the establishment of disulfide bonds.

Computer-assisted analysis of the peptide encoded by the cDNA predicts that the feline IL-6 has a molecular weight of 23,212, has an isoelectric point of 8.12, and contains several potential myristylation, O-glycosylation, and phosphorylation sites (data not shown).

Discussion

Interleukin-6 is a multifunctional cytokine that is known to play a central role in regulating the immune response, in regulating hematopoiesis (17–20) and in inducing acute phase reactions (21). Since our laboratory has been examining cytokine expression and the immunopathogenic mechanisms associated with feline AIDS, we sought to clone molecularly feline IL-6. Before this report, few publications had described feline IL-6 (22, 23). In two of these publications, the laboratory of Goitsuka describes the physiochemical characteristics of feline IL-6 isolated from concanavalin A-stimulated splenocytes and from peritoneal exudate cells obtained from the feline infectious peritonitis virus-infected cats, respectively. These investigators reported that by gel filtration feline IL-6 exhibited a molecular weight of between 30,000 and 40,000, a weight much larger than that predicted from the amino acid sequence we have presented (23 kDa) and much larger than that reported for human IL-6 (21–28 kDa). This group speculated that the weight variation may be the result of species differences or that feline IL-6 may exist as a dimer. Computer-assisted analysis of the cDNA encoded IL-6 identifies a secretory signal sequence, which would be cleaved from the native peptide (reducing the size to approximately 20,000), and also identifies several potential myristylation, glycosylation, and phosphorylation sites. Therefore, it is likely that these posttranslational modifications may cause the protein to exhibit an aberrantly large molecular weight when analyzed by gel filtration. Goitsuka's group also reported that they have been able to elute feline IL-6 from an anionic exchange column (Mono Q) in fractions containing 0.2–0.3 *M* NaCl. This is in contrast to the human and mouse IL-6, which elute from a similar column in 0.1–0.2 *M* NaCl.

The cDNA for human IL-6 has been cloned from several sources and contains 212 amino acids including a hydrophobic signal sequence of 28 residues. The murine IL-6 contains 211 amino acids and includes a hydrophobic signal sequence of 24 residues. When human IL-6 is isolated to homogeneity it can be found to exhibit microheterogeneity at the N terminus (Pro in a T cell-derived protein and Ala in the osteosarcoma-derived protein). This N terminus heterogeneity may also occur in feline IL-6 if differential cleavage of the signal sequence occurs. Since four potential cleavage sites are predicted for the hydrophobic secretory sequence of feline IL-6, it is probable that differential cleavage may occur.

Both human and murine IL-6 have been demonstrated to be O-glycosylated, whereas only human IL-6 is also N-glycosylated. Computer-assisted analysis of the cDNA-encoded feline IL-6 demonstrates a lack of potential N-glycosylation sites, but reveals the presence of several potential O-glycosylation sites. Evidence that feline IL-6 is antigenically different from human IL-6 was demonstrated when antibodies directed against human IL-6 failed to neutralize feline IL-6 (22).

The molecular cloning and characterization of feline IL-6 represents a step forward in understanding the molecules of the feline immune system and their associations with health and disease. In an attempt to further expand our knowledge of the feline immune system, our laboratory has recently cloned cDNA representative of the feline IL-2 and IL-10 (Bradley *et al.*, manuscript in preparation).

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