

Rapid communication

Molecular cloning and phylogenetic analysis of a cDNA encoding the cat (*Felis domesticus*) Ig epsilon constant region

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Abstract

A feline splenic cDNA library was screened with a ³²P-labelled cDNA probe encoding the canine IgE epsilon heavy chain subunit. A cDNA sequence of 1614 nucleotides encoding the complete feline IgE heavy chain, as well as a portion of a variable region, was identified. A search of the GenBank database revealed an identity of 82% at the nucleotide level and 76% at the amino acid level between the feline epsilon heavy chain sequence and the canine homologue. In a separate study, feline genomic DNA, isolated from whole feline embryo cells, was subjected to PCR amplification using primers based on known partial genomic DNA sequences for the feline Cε gene. Following removal of an intron from the 683 bp PCR product, the coding sequence yielded an ORF of 506 bp. The DNA sequence of this PCR clone differed by a single nucleotide from the cDNA clone. This difference is silent, and therefore the proteins encoded by the two sequences are identical over the regions cloned and sequenced. Phylogenetic analysis of the constant regions of nine immunoglobulin epsilon genes revealed that the feline cDNA is most similar to the canine homologue. © 2000 Elsevier Science B.V. All rights reserved.

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The production of immunoglobulin epsilon (IgE) is associated with allergic responses to a variety of environmental allergens, and is associated with helminth infections (Jarrett and Miller, 1982; Sutton and Gould, 1993; Bacharier and Geha, 1999).

Cats may suffer from a variety of skin diseases including miliary dermatitis, symmetrical alopecia and eosinophilic granuloma complex. Such diseases may be related to allergic reactions to parasites (including fleas, mosquitoes and the ear mite *Otodectes*), food allergens or inhaled allergens (atopic disease). The inability to accurately measure IgE levels in cats has made the differential diagnosis of these disease states difficult. Until recently, the measurement of serum IgE concentrations in cats has not been possible. Several monoclonal and polyclonal anti-feline IgE reagents have been produced using traditional protein purification methods. It has been reported, however; that these reagents have either shown cross reactivity with other antibody isotypes (IgM, IgA) or are not widely available (DeBoer et al., 1994).

Several studies have demonstrated that cats infested with parasites have a heat-labile serum antibody that has characteristics consistent with an IgE isotype (Weisbroth et al., 1974; Powell et al., 1980; Baldwin et al., 1993; DeBoer et al., 1993; Foster et al., 1995; Gilbert and Halliwell, 1998). The presence of such an antibody has been demonstrated by passive cutaneous anaphylaxis (PCA), reverse cutaneous anaphylaxis (RCA) reactions, and by Prausnitz-Küstner (PK) tests. The sera used in these studies were derived from cats infested with the ear mite *Otodectes cynotis*, or infected with *Brugia pahangi*, *Toxocara canis* or *Toxocara cati*. In addition sera were derived from cats immunized with dinitrophenylated ascaris antigen. However, the small quantities of IgE antibody present in serum have made the isolation and purification of IgE by conventional chromatographic techniques very difficult. An alternative approach to the isolation of mammalian immunoglobulins comes from the use of molecular biology techniques. The four domains which constitute the IgE heavy chain constant region are encoded by the four exons of the C ϵ gene (Brüggemann, 1987). The B cells, under the influence of appropriate stimuli, produce mature IgE mRNA from the arrangement of C ϵ germ line transcripts (ϵ GLT) with a VDJ exon (Bacharier and Geha, 1999). In preliminary studies of the cat C ϵ gene, sequences encoding a portion of the feline C ϵ gene were identified by nested PCR (Foster, 1995). Genomic DNA was isolated from the peripheral blood cells of a cat infected with the nematode *Brugia pahangi*, and degenerate primers were designed based on the known DNA sequences for sheep, human and mouse C ϵ genes (Steen et al., 1984; Sakoyama et al., 1987; Engwerda et al., 1992). Using this approach DNA sequences encoding the second and third domains, as well as a portion of the first domain of the feline C ϵ gene, were generated.

The development of anti-IgE reagents to measure feline IgE will be greatly facilitated by the cloning and heterologous expression of sequences encoding the constant region of the feline C ϵ gene. Expression of this recombinant protein will provide sufficient quantities of material for raising specific antisera against feline IgE. In this study, we report the independent cloning of cDNA sequences encoding the complete feline C ϵ constant region, as well as of a PCR clone generated from genomic DNA encoding a portion of the C ϵ constant region.

Total RNA was extracted from splenic tissue, isolated from a domestic short-haired cat, using an acid-guanidinium-phenol-chloroform method as described by Chomczynski and

Sacchi (1987). Poly A⁺ selected RNA was isolated using oligo-dT cellulose chromatography (mRNA Purification Kit, Pharmacia Biotech, (Newark, NJ), according to the method recommended by the manufacturer. Approximately 5 µg of poly A⁺ RNA was used to produce a 1–4 kb size-selected feline splenic cDNA library in lambda-Uni-ZAPTM XR vector (Stratagene, La Jolla, CA) using Stratagene's ZAP-cDNA Synthesis Kit protocol.

Using a modification of the plaque hybridization protocols (Benton and Davis, 1977), the splenic cDNA library was screened, using duplicate plaque lifts, with a ³²P-labelled cDNA encoding the canine Ig epsilon heavy chain subunit. Approximately 1.0×10^6 plaques were screened under the following conditions. Plaques were denatured in the presence of 0.5 N NaOH, 1.5 M NaCl, and neutralized in a solution containing 1.5 M NaCl and 0.5 M Tris-HCl (pH 8.0). Following neutralization, the filters were rinsed briefly in 2x SSC (1x SSC = 0.15 M NaCl, 0.15 M NaC₆H₅O₇·2H₂O) and subjected to UV crosslinking using a Stratagene UV Stratalinker 1800. The filters were then blocked in hybridization buffer containing 5x SSC, 5x Denhardtts, 0.5% SDS, and 100 mg/ml ssDNA for 3 h at 52°C. Approximately 4.0×10^6 cpm of probe, prepared by random priming using the MegaprimeTM Labelling System (Amersham Life Science, Buckinghamshire, England), was added for each filter. Hybridization was carried out over night at 52°C in a shaking water bath. Following hybridization, the filters were washed in 2x SSC, 0.5% SDS at room temperature for 15 min, followed by two washes, for 10 min each, in 0.2x SSC, 0.1% SDS at 55°C. The filters were rinsed in 2x SSC, air dried and subjected to autoradiography. Positive plaques were picked and subjected to two rounds of purification. Plaque purified clones of the feline nucleic acid molecules encoding the IgE heavy chain were converted into double-stranded recombinant molecules using the ExAssistTM helper phage and SOLR(tm) *E. coli* according to the in vivo excision protocol described in the ZAP-cDNA Synthesis Kit (Stratagene, LaJolla, CA). Double-stranded plasmid DNA was prepared using the Quantum Prep Plasmid Midiprep Kit (available from Bio-Rad, Hercules, CA), according to the manufacturer's protocol.

Plasmids containing feline IgE heavy chain cDNAs were sequenced by the Sanger dideoxy chain termination method according to Sanger et al. (1977), using the PRISMTM Ready Dye Terminator Cycle Sequencing Kit with AmpliTaq[®] DNA Polymerase, FS (Perkin-Elmer Corporation, Norwalk, CT). PCR extensions were done in the GeneAmpTM Gel Filtration Cartridge (Advanced Genetic Technologies, Gaithersburg, MD) following their standard protocol. Samples were resuspended according to ABI protocols and were run on a Perkin-Elmer ABI-PRISMTM 377 Automated DNA Sequencer (Norwalk, CT) at Heska Corporation (Fort Collins, CO). DNA sequence analysis, including the alignment of sequences, and the determination of open reading frames, was performed using the MacVectorTM program (IBI, New Haven, CT). Protein sequence analysis, including the determination of molecular weight and isoelectric point (pI) was performed using the GCGTM program (Genetics Computer Group, Madison, WI).

Feline genomic DNA was isolated from whole feline embryo cells using the Turbo-GenTM DNA isolation kit (Invitrogen, The Netherlands) following the manufacturer's recommendations. Genomic DNA was subjected to PCR using the primers IgE-PCR/For

1	GCATATATTAGTAGTGGAGGTAACACAGACTACGCAGACTCCGTGAAGGGCCGATTCTCC	
	A Y I S S G G N T D Y A D S V K G R F S	20
61	ATCTCCAGAGACAACGCCAAGAACACGCTGTATCTGCAGATGACCAGCCTCAAGACCGAG	
	I S R D N A K N T L Y L Q M T S L K T E	40
121	GACACGGCCACATATTACTGTGCAAGAGGGACTGGTGTAAATACCGGACTACTGGGGCCAG	
	D T A T Y Y C A R G T G V I P D Y W G Q	60
		*
181	GGAGCCCTGGTGACGGTGTCTCAACCTCCATCCAGGCCCCCTCGTCTTCCCCTTGGCC	
	G A L V T V S S T S I Q A P L V F P L A	80
241	ACCTGTGTCAAAGGCACCATCGCCACTGCCCCGTCCGTGACACTGGGCTGCCTGGTCACG	
	T C C K G T I A T A P S V T L G C L V T	100
301	GGCTACTTCCCGATGCCGGTGACTGTGACCTGGGATGCAAGGTCCCTGAACAAAAGCGTC	
	G Y F P M P V T V T W D A R S L N K S V	120
361	GTGACCTTCCCCGCCACCCTCCAAGAGAACTCTGGCCTCTACACCACCACCAGCCACGTG	
	V T L P A T L Q E N S G L Y T T T S H V	140
421	ACCGTCTCGGGCGAGTGGGCCAAACAGAAGTTCACCTGCAGTGTGGCTCACGCGGAGTCC	
	T V S G E W A K Q K F T C S V A H A E S	160
481	CCCACCATCAACAAGACCGTCAGTGC GTGTACCATGAACTTCATTCCCCCACC GTGAAG	
	P T I N K T V S A C T M N F I P P T V K	180
541	CTCTTCCACTCCTCCTGTAACCCCTCGGTGACACCGGTAGCACCATCCAGCTCCTGTGC	
	L F H S S C N P L G D T G S T I Q L L C	200
	(T)	
601	CTCATCTCCGGCTACGTCCCAGGTGACATGGAGGTACCTGGCTGGTGGATGGGCAGAAG	
	L I S G Y V P G D M E V T W L V D G Q K	220
661	GCCACGAACATATTCCCATACACTGCCCCGGCAAGCAGGAGGGCAAGGTGACCTCCACC	
	A T N I F P Y T A P G K Q E G K V T S T	240
721	CACAGCGAGCTCAACATCACGCAGGGTGAGTGGGTGTCCCAAAGACCTACACTTGCCAG	
	H S E L N I T Q G E W V S Q K T Y T C Q	260
781	GTCACCTATCAAGGCTTACCTTTGAGGACCACGCTCGCAAGTGACAGAGTCTGACCCC	
	V T Y Q G F T F E D H A R K C T E S D P	280
841	CGAGGTGTGAGCACCTACCTGAGCCCCGCCAGCCCTCTTGACCTGTACGTCCACAAGTCG	
	R G V S T Y L S P P S P L D L Y V H K S	300

Fig. 1. Nucleotide sequence and translation of feline Ig epsilon constant region cDNA. The nucleotide and amino acid sequences are numbered on the left and right sides, respectively. Sequences encoding a portion of the variable region begin at nucleotide 1 and include sequences up to, approximately, nucleotide 216. Sequences encoding the constant region begin at, approximately, nucleotide 217, which is marked by an asterisk. A consensus polyadenylation signal is underlined. The bold DNA sequences indicate the Cε PCR product, which is identical to the cDNA identified, except for a single conserved base change, which is noted above the sequence.

901	CCCAAGATCACCTGCCTGGTGGTGGACCTGGCCAACACAGACGGCATGATCCTGACCTGG	320
	P K I T C L V V D L A N T D G M I L T W	
961	TGCGGGAGAATGGGGAGTCTGTGCACCCAGACCCGATGGTCAAGAAGACTCAGTACAAC	340
	S R E N G E S V H P D P M V K K T Q Y N	
1021	GGGACAATCACCGTCACCTTCCACCCTGCCTGTGGATGCCACTGACTGGGTTGAGGGGGAG	360
	G T I T V T S T L P V D A T D W V E G E	
1081	ACCTACCAATGCAAGGTGACCCATCCAGACCTGCCCAAGGACATCGTGCGCTCCATTGCC	380
	T Y Q C K V T H P D L P K D I V R S I A	
1141	AAAGCCCCGCGCCGCGTTTCCCCCGGAGGTGTACGTGTTCTCTGCCGCCGAGGGGGAG	400
	K A P G R R F P P E V Y V F L P P E G E	
1201	CCGAAGACCAAGGACAAAGTCACTCTCACGTGCCTGATCCAGAACTTCTTTCCCCCGGAC	420
	P K T K D K V T L T C L I Q N F F P P D	
1261	ATCTCGGTGCAATGGCTTCACAACGACAGCCCTGTTCCGACAGAACAGCAGGCCACCACG	440
	I S V Q W L H N D S P V R T E Q Q A T T	
1321	TGGCCCCACAAGGCCACCGGCCCCAGCCAGCCTTCTTTGTCTTCAGTCGCCTTGAGGTC	460
	W P H K A T G P S P A F F V F S R L E V	
1381	AGCCGGGCAGATTGGGAGCAGAGGGATGTGTTACCTGCCAAGTGGTGCACGAGGCGCTG	480
	S R A D W E Q R D V F T C Q V V H E A L	
1441	CCTGGCTTTAGGACGCTCAAGAAATCCGTGTCCAAAAACCTGGTAAATGATGCCACCC	496
	P G F R T L K K S V S K N P G K *	
1501	CTCCCCCAGAGCTCCATCCTGCTGGGGCGGGGAGGGGCGGCCGGACCTGCCGGTCTG	
1561	TTGTTGTCAATAAACTGCAGTGCCTGCCTCAGAAAAAAAAAAAAAAAAAAAAA	

Fig. 1. (Continued).

and IgE-PCR/Rev in conjunction with the proof reading enzyme *rTth* DNA polymerase XL (Perkin-Elmer Corporation, Norwalk, CT) according to the manufacturer's instructions. PCR was performed in a TouchdownTM thermal cycler (Hybaid, Middlesex, UK) for 35 cycles consisting of 30 s at 94°C, 60 s at 50°C and 60 s at 72°C. The forward primer contained a *Bam*HI restriction site and the reverse primer contained a *Hind*III restriction site to facilitate subcloning of the PCR product. PCR primers were designed using MacVectorTM version 6.5 and were based on primers used to obtain the partial DNA sequences for both feline IgE (Foster, 1995) and bovine IgE (Engwerda et al., 1992).

The resulting PCR product of 683 base pairs was gel purified using a Hybaid recovery DNA purification kit II (Hybaid, Middlesex, UK) and digested with the restriction enzymes *Bam*HI and *Hind*III to yield 'sticky ends' for cloning. The digested PCR product was ligated to *Bam*HI/*Hind*III digested pQE30 (Qiagen Inc., Valencia, CA) and transformed into XL1-Blue *E. coli* cells (Stratagene, La Jolla, CA). *E. coli* containing the plasmid/PCR product construct were selected by plasmid mini-prep (Qiagen Inc.,

Valencia, CA) and agarose gel analysis. Removal of the intron between exons 2 and 3, by PCR, was carried out in the following manner. The 5' and 3' exon containing ends of the cloned product were amplified for 30 cycles using the IgE-PCR/For, IgE R2 primer pair and the IgE F2, IgE-PCR/Rev primer pair, respectively. The resultant PCR products of 285 and 353 bp were gel purified. Since the design of primers IgE F2 and IgE R2 includes a complementary region, the 5' and 3' PCR products were mixed and subjected to five cycles of PCR in the absence of primers. This allowed the two halves of the coding region to anneal and be amplified. PCR was performed using this template and primers IgE-PCR/For and IgE-PCR/Rev to yield a product of 506 bp, which was cloned into pQE30 as described above. Automated DNA sequencing was performed to confirm the IgE nucleotide sequence and ensure the intron had been removed.

IgE-PCR/For	5'-GATCGGATCCCTCGGTGACACCGGTAGCACC-3'
IgE-PCR/Rev	5'-TCGATCAAGCTTGGGGGCTTTGGCAATGGAGCG-3'
IgE F2	5'-ACCACGCTCGCAAGTGCACAGAGTCTGACCCCCGAG-GTGTG-3'
IgE R2	5'-CACACCTCGGGGGTCAGACTCTGTGCACTTGCGAGCGTG-GTCC-3'

The nucleotide sequences (GenBank accession numbers AF118820 and AF162134) of the two products are shown in Fig. 1. Comparison of the partial DNA sequence, generated by PCR, with the 1614 bp cDNA sequence encoding the entire feline IgE heavy chain constant region, showed them to be greater than 99% identical. The single base difference in the sequence is silent, and therefore, the proteins encoded by the two sequences are identical over the regions shown.

The cDNA sequence of the 1614 base pair clone contains sequences encoding a complete epsilon heavy chain constant region as well as a portion of an epsilon heavy chain variable region (nucleotides 1–216). The translation stop codon (TGA) is located at positions 1489–1491 and a putative polyadenylation signal (5'-AATAAA-3') is located in a region spanning nucleotides 1569 through 1574.

Translation of this cDNA, containing both the variable and constant region sequences, yields a protein of 496 amino acids that has an estimated molecular weight of about 54.4 kDa and an estimated pI of approximately 6.84. The constant region, alone, is composed of approximately 423 amino acids. A search of the GenBank database using the BLAST program revealed that the highest scoring match at the amino acid level was canine IgE (Accession number AAA56797), which was approximately 76% identical to the feline sequence. At the nucleotide level, the search was performed using the entire Ig epsilon constant region cDNA, which was 82% identical with canine IgE cDNA (Accession number L36872).

Fig. 2 shows a phylogenetic tree constructed from the constant regions of nine immunoglobulin epsilon genes. Trees were constructed using 1000 sets of bootstrapped data and, as can be seen from the dendrogram, feline and canine IgE are always grouped together, indicating that their cDNA sequences are closely related. Furthermore, the IgE sequences for cow and sheep are closely related, but dissimilar to those of the cat and dog.

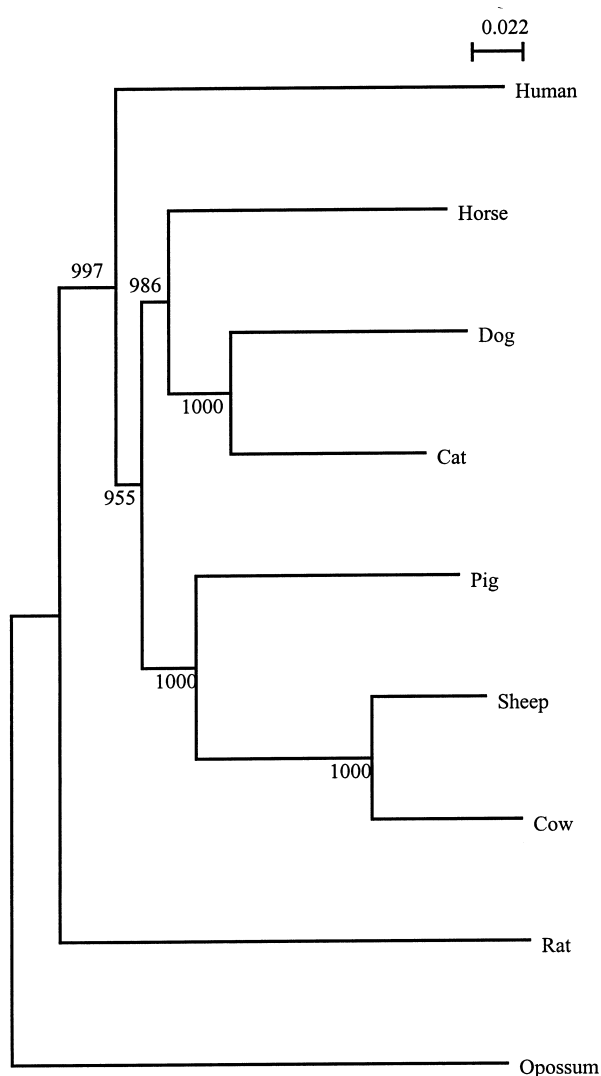


Fig. 2. Phylogeny of IgE heavy chain sequences. Sequences were aligned using CLUSTAL W (ver 1.7, European Molecular Biology Laboratory) (Thompson et al., 1994) and trees constructed from 1000 sets of bootstrapped data by the Neighbor-Joining method after Saitou and Nei (1987). Numbers at each node indicate the number of times the groups to the right of that node occurred among 1000 trees. The bar represents the mean number of differences per site.

Initial molecular studies of human IgE included the cloning of the human IgE (C ϵ) gene using a human J_H probe to screen a genomic library from a human IgE-producing myeloma cell-line (Flanagan and Rabbitts, 1982; Nishida et al., 1982; Ueda et al., 1982). Subsequently, a cDNA library constructed from the same cell-line yielded a C ϵ clone whose DNA sequence was determined (Kenten et al., 1982; Seno et al., 1983).

Molecular biology techniques have also permitted the isolation and characterization of rat, murine, canine, bovine and ovine C ϵ sequences. The rat IgE myelomas IR2 and IR162 have been used to determine the sequence of all four constant domains of the rat C ϵ chain (Hellman et al., 1982a, b; Steen et al., 1984). The sequence of mouse genomic DNA and cDNA for the C ϵ gene has been established and compared with the human C ϵ gene sequence (Ishida et al., 1982; Liu et al., 1982). Patel et al. (1995) reported the isolation of canine IgE from a genomic bacteriophage library prepared from liver tissue using a human IgE probe. The complete epsilon chain constant region cDNA was cloned and sequenced, and these data were used to generate a probe for the PCR clone reported in these studies.

The human and rodent studies of the C ϵ gene were based on IgE-producing myeloma cell lines that were probed with appropriate gene probes similar to the C ϵ gene. Detection of C ϵ genes in other species such as sheep, horse and cat was thwarted by the lack of suitable myelomas and gene probes until the advent of the PCR technique. PCR has been employed to clone both a fragment as well as a full length coding sequence of ovine IgE DNA (Engwerda et al., 1992; Kooyman et al., 1997). This was carried out using oligonucleotide primers corresponding to the epsilon heavy chain based on the sequences for rat, mouse, human, chimpanzee and orangutan (Liu et al., 1982; Max et al., 1982; Hellman et al., 1982b; Sakoyama et al., 1987). Two studies have reported partial cDNA sequences for the equine C ϵ gene using PCR analysis (Marti et al., 1995; Navarro et al., 1995). The DNA sequence for the equine C ϵ gene was identified from a genomic DNA library using both human and murine DNA probes (Wagner et al., 1997). In addition, DNA fragments encoding canine epsilon chain cDNAs have been cloned using PCR (Griot-Wenk et al., 1998). Similarly the sequence of cDNA for the bovine C ϵ gene has been established from peripheral blood lymphocytes using a PCR technique (Mousavi et al., 1997). The entire constant regions of cDNA encoding IgE from the marsupial *Monodelphis domestica* (Grey short-tailed opossum) and for the domestic pig have been determined using both PCR and hybridization screening of cDNA libraries (Vernersson et al., 1997; Aveskogh and Hellman, 1998). The totality of these studies provides useful data for the comparison of IgE sequences from several mammalian species. Furthermore, the cloning and expression of the cDNA encoding the feline IgE heavy chain constant region offers the possibility of the preparation of IgE-specific reagents to study and better understand the role of IgE in feline allergic diseases.

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