Determination of the cDNA sequence and mRNA expression of interleukin-1 receptor antagonist in horses

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Objective—To determine the complementary DNA (cDNA) sequence of interleukin-1 receptor antagonist (IL-1ra) in horses and compare messenger RNA (mRNA) expression of IL-1ra among horses of various breeds.

Sample Population—Blood samples from neonatal and adult horses examined for a variety of diseases.

Procedure—A polymerase chain reaction procedure was used to amplify a 220 base pair (bp) portion of the genomic DNA. The upstream and downstream regions of the cDNA sequence were determined by means of 5' and 3' rapid amplification of cDNA ends (RACE) procedures. Northern blot hybridization was used to examine steady-state mRNA expression of IL-1ra.

Results—The consensus sequence of the cDNA obtained with the 5'-RACE procedure and the sequence for the 220 bp portion of the genomic DNA represented the putative sequence for secreted IL-1ra. The predicted secreted IL-1ra amino acid sequence contained 176 residues with an in-frame stop codon; the N-terminal 25 amino acid residues resembled the signal peptide reported for human secreted IL-1ra. An approximately 1.3 kilobase pair (kb) band that represented a portion of the 3' end of the coding region and the 3' untranslated region was obtained by use of the 3' RACE procedure. Northern blot hybridization detected a 1.6 kb transcript in blood RNA from adult Arabian, Belgian, Thorougbred, and Standardbred horses.

Conclusions—Results suggest that the DNA sequence of equine secreted IL-1ra has a short (29 bp) 5' untranslated region, a 534 bp coding region, and a long (approximately 1,080 bp) untranslated region. (Am J Vet Res 2000;61:920–924)

Interleukin-1 (IL-1) plays a key role in initiating and modulating the immune response associated with inflammatory disorders, particularly sepsis and chronic inflammatory disorders.1 The IL-1 family consists of 3 distinct but structurally related proteins: IL-1α, IL-1β, and IL-1 receptor antagonist (IL-1ra). Interleukin-1α and IL-1β elicit physiologic and pathologic responses, whereas IL-1ra acts as a receptor antagonist with no known agonistic activity.1

The IL-1ra gene encodes a soluble protein synthesized by macrophages and monocytes in response to various inducers, including tumor necrosis factor-1, IgG, and lipopolysaccharides.2 Two isoforms of IL-1ra have been identified: secreted IL-1ra (sIL-1ra) and intracellular IL-1ra (icIL-1ra). Both isoforms have been cloned from human epithelial and blood cells2,3 and rabbit colonic mucosal cells4 and sequenced, whereas only the sIL-1ra isoform from mice,5 rats,6 and horses9,10 has been characterized. Analyses of the genomic structure of the human IL-1ra gene revealed that both IL-1ra isoforms originate from the same gene by alternative splicing of the first exon.3

Previous studies5,11 have examined the potential of using recombinant IL-1ra (rIL-1ra) to mediate acute and chronic inflammation.5,11 Administration of rIL-1ra and gene transfer of IL-1ra have been found to reduce the severity of inflammatory responses,6,12 suggesting that IL-1ra may be useful for treatment of inflammatory diseases. Until 1996, only the complementary DNA (cDNA) sequences for IL-1ra in humans, mice, rats, and rabbits were available. Therefore, the present study was undertaken to determine the IL-1ra sequence for horses and to compare messenger RNA (mRNA) expression of IL-1ra within and among various breeds of horses.

Materials and Methods

Extraction of nucleic acids from horse blood cells—Blood samples were collected from neonatal foals (1 to 19 days old) and adult horses (3 to 7 years old) admitted to the Tufts Large Animal Hospital because of various diseases and from healthy foals and adult horses at local breeding farms. Blood samples were centrifuged at 2,000 × g at 4 C for 10 minutes, and total DNA and RNA were extracted from the buffy coat by use of a guanidine isothiocyanate procedure.13

Determination of a portion of the genomic IL-1ra DNA sequence—A 220 base pair (bp) segment of the genomic equine IL-1ra DNA sequence was amplified by means of a polymerase chain reaction (PCR) procedure using DNA from an adult female Thorougbred with colic. Degenerate primers (sense, 5'-CAGGWZGGTTAACATCAGT-3'; antisense, 5'-CTAYTXGYCCCCGGAAGTAG-3') based on the conserved region of exons (amino acid positions 82 through 152) in IL-1ra from humans, mice, and rats were used. The amplified DNA was cloned; and DNA from 3 clones was sequenced using the M13 forward and M13 reverse primers derived from the vector sequence.
Determination of the IL-1ra cDNA sequence—To determine the cDNA sequence of the portion of the IL-1ra gene upstream of the 220 bp genomic DNA sequence, RNA from an adult female Thoroughbred with colic and from a female Thoroughbred foal with sepsis was used in a 5'-rapid amplification of cDNA ends (5'-RACE) procedure. For this procedure, cDNA was synthesized from the horse's and foal's RNA using a primer designed from the 220 bp genomic DNA sequence (5'-CTGTTTGAGCGGATGAAGGT-3'). Homopolymeric tailing of the cDNA was done using dCTP, and the cDNA was amplified by use of a PCR procedure using an antisense primer (5'-CAUCAUCAUCAUGTTCTCCTC-CTTGTTCTTGCT-3') synthesized from the 220 bp genomic DNA sequence that was 9 bp upstream of the primer used for cDNA synthesis and a universal anchor primer as the sense primer. Amplified cDNA in bands that corresponded to approximately 300 bp and to approximately 400 bp were gel-purified and cloned. Three clones were sequenced, using the SP6 and T7 primers derived from the vector. Sequence analyses were done using the GCC program.

Evaluation of mRNA expression of IL-1ra—Northern blot hybridization was used to examine steady-state mRNA expression of IL-1ra in blood samples from unrelated adult horses. Aliquots of total RNA (0.5 to 3.0 µg) from adult Thoroughbred (n = 8), Standardbred (8), Arabian (10), and Belgian (6) horses were electrophoresed in 1% agarose gels, blotted onto nitrocellulose membranes, and hybridized overnight at 42°C in a formamide-base hybridization solution containing a 32P-labeled 220 bp IL-1ra DNA probe (2 X 10^8 cpm/µg of probe DNA). Membranes were washed at 50°C, using the medium-stringency washing protocol, and exposed to x-ray film at –70°C for 5 to 15 days.

Results

The sequence of a 220 bp portion of the genomic DNA of IL-1ra in horses was determined (Fig 1; GenBank accession number AF072476). This portion of the genomic DNA represented the 220 bp upstream of the stop codon for exon 4 of human IL-1ra. The sequence of this portion of the genomic DNA was 78 to 84% homologous with the corresponding sequences for human, mouse, rat, and rabbit IL-1ra. The 5'-RACE procedures resulted in a faint smery band of approximately 400 bp and an intense smery band of approximately 300 bp (Fig 2). The cDNA sequence of the approximately 400 bp band overlapped with the first 43 bases of the 220 bp portion of the genomic DNA sequence (Fig 1), suggesting that this band represented the cDNA upstream of the 220 bp portion of the genomic DNA sequence. The consensus sequence of the cDNA obtained with the 5'-RACE procedure (GenBank accession number AF088186) and the sequence for the 220 bp portion of the genomic DNA represented the putative sequence for equine sIL-1ra. The predicted equine sIL-1ra amino acid sequence contained 176 residues with an in-frame stop codon (TAA). All 3 clones that were sequenced (1 from a foal with sepsis and 2 from an adult horse with colic) had the same in-frame stop codon, suggesting it was not a sequence artifact. The autoradiogram of the region sur-
rounding the in-frame stop codon (Fig 3) did not indicate any sequence compression attributable to secondary structure or GC content in this region of the IL-1ra sequence. This suggests that the in-frame stop codon was not a result of sequencing artifact, but a reflection of the cDNA sequence. Only a partial sequence (198 bp) was obtained from the approximately 300 bp band obtained with the 5'-RACE procedure, and this sequence completely overlapped the sequence obtained for the approximately 400 bp band.

The cDNA and putative amino acid sequences obtained for sIL-1ra were approximately 99% homologous with previously published equine sIL-1ra cDNA sequences and 80 to 82% homologous with sequences reported for humans, mice, rats, and rabbits. The cDNA sequence obtained in the present study differed from the sequence reported by Kato et al at positions 104 (T vs C), 225 (T vs A), and 546 (T vs C). The difference at position 104 did not alter the putative amino acid sequence, but the other 2 differences introduced a change from phenylalanine to leucine at position 225 and resulted in a change from lysine to the in-frame stop codon at position 546.

The cDNA sequence obtained in the present study differed from the sequence reported by Howard et al at positions 84 (T vs C), 105 (T vs C), 225 (T vs A), 336 (A vs T), 337 (T vs A), and 546 (T vs C). The differences at positions 105 and 337 did not result in alterations in the putative amino acid sequence; however, the other 4 differences resulted in a change from leucine to phenylalanine at position 84, lysine to the in-frame stop codon at position 225, phenylalanine to asparagine at position 336, and leucine to phenylalanine at position 546.

An approximately 1.3 kilobase pair (kb) band was obtained by use of the 3'-RACE procedure (Fig 4; GenBank accession number AF072535). To determine the specificity of the 3'-RACE procedure, 2 internal controls were used for the PCR procedure. The first internal control consisted of amplification of a 220 bp fragment of cDNA from aliquots of the cDNA synthesized with an oligo d(T)-adapter primer and used for the 3'-RACE procedure. Lanes 1 and 2 = Internal control that consisted of amplification of a 220 bp fragment of cDNA from the gel-purified product of the 3'-RACE procedure. Lanes 5 through 8 = Amplification of IL-1ra cDNA by use of the 3'-RACE procedure. Lane M = 100 bp Ladder. Lanes 1, 3, 5, and 6 represented samples from an adult horse with colic; lanes 2, 4, 7, and 8 represented samples from a foal with sepsis.

Northern blot hybridization revealed a single transcript of approximately 1.6 kb in RNA from adult Arabian, Belgian, Thoroughbred, and Standardbred horses (Fig 5). Subjective assessment of hybridization...
reported for human sIL-1ra, and the putative amino acid sequence obtained in the present study resembled the signal peptide residues of the putative amino acid sequence obtained including horses. The N-terminal 25 amino acid residues of the putative amino acid sequence obtained in the previous study resembled the signal peptide reported for human sIL-1ra, and the putative amino acid sequence had the N-linked glycosylation site and five conserved cysteine residues (amino acid positions 25, 91, 94, 141, and 147) found in the previously reported human, mouse, rabbit, and horse sequences. In humans and rabbits, the precursor protein undergoes a proteolytic cleavage between cysteine and arginine (amino acids 25 and 26 of the human sIL-1ra sequence), resulting in a mature secreted IL-1ra protein of 152 amino acids. In the present study, although a cysteine residue was detected at amino acid position 25, a histidine residue, instead of an arginine residue, was detected at amino acid position 26, similar to results reported by Kato et al. and Howard et al. Purification and characterization of the equine sIL-1ra protein is needed to determine whether the precursor protein undergoes proteolytic cleavage to produce a mature protein.

Detection of an in-frame stop codon in the equine sIL-1ra open reading frame in the present study was unexpected and unique. Mutations introducing premature stop codons reduce the amount of functional mRNA. Although the biological importance of this in-frame stop codon in the cDNA sequence of equine sIL-1ra is not known, we speculate that the IL-1ra mRNA in the diseased animals from which samples were obtained for the present study may have undergone posttranscriptional modification to introduce such an in-frame stop codon. Determining the genomic sequence of the IL-1ra gene encompassing the stop codon may help to elucidate whether the in-frame stop codon arose as a result of posttranscriptional modification. Such modification may result in production of a truncated biologically inactive IL-1ra protein in diseased animals, which may result in disruption of the homeostatic balance among IL-1α, IL-1β, and IL-1ra.

Use of 2 positive controls for the PCR procedure to capture the 3’-end of IL-1ra provided a way to increase the specificity of our 3'-RACE procedure. An alternative method of increasing the specificity of the 3'-RACE procedure involving changing concentrations of general and transcript-specific primers used in the PCR procedure has also been reported. Combining results for our 5’-RACE procedure, sequencing of a portion of the genomic DNA, and our 3'-RACE procedure suggested that the DNA for equine sIL-1ra has a short (29 bp) 5' untranslated region, a 534 bp coding region, and a long (approximately 1,080 bp) untranslated region similar to other members of the IL-1 family of genes. Sequences of the long 3'-untranslated regions of the IL-1 family of genes were more homologous than sequences of the coding regions, suggesting that the 3'-untranslated region probably exerts a similar regulatory mechanism for this family of genes.

Future studies should be aimed at determining whether IL-1ra mRNA expression varies depending on physiologic or health status of the animals, using a quantitative method of measuring mRNA expression.

Figure 5—Composite figure of northern blot autoradiographs illustrating expression of IL-1ra mRNA in 8 Thoroughbred, 8 Standardbred, 10 Arabian, and 6 Belgian horses. Aliquots of RNA were electrophoresed in 1% agarose gels, blotted onto nitrocellulose membranes, and hybridized with a 220 bp IL-1ra DNA probe. T = Control mouse testis RNA. The corresponding photographs of ethidium-bromide stained gels indicate amount of total RNA loaded per lane.

Discussion

In the present study, we determined the cDNA sequence and putative amino acid sequence of sIL-1ra in horses. Two reports on the cDNA sequence of IL-1ra in horses have been published since the present study was initiated. However, the present study identified some unique features that were hitherto unknown in the IL-1ra mRNA expression levels, northern blots should be hybridized with a constitutively expressed gene probe, such as 18s ribosomal RNA, as an internal standard so that the corresponding signals of IL-1ra mRNA expression can be compared.

References


