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**Complete cDNA sequences and phylogenetic analyses of the Th1 and Th2
cytokines of the bactrian camel (*Camelus bactrianus*)**

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Running head: PHYLOGENETIC ANALYSIS OF CAMEL CYTOKINES

ABSTRACT. The complementary DNAs of the Th1 (IL-2, IL-12p35, and IFN- γ) and Th2 (IL-4, IL-10 and IL-13) cytokine genes of the bactrian camel (*Camelus bactrianus*) were cloned, sequenced, and analyzed. IL-2, IL-4, IL-10, IL-12p35, IL-13, and IFN- γ were found to have 465, 402, 537, 669, 411, and 501 bp length open reading frames with 154, 133, 178, 222, 136, and 166 amino acid encodings, respectively. The homology ranged from 58.8% to 100% between the nucleotide sequences of the camel cytokine genes and the published sequences of other mammalian genes, including the llama, pig, cow, horse, human, and mouse. The cDNA had highest homology with orders *Artiodactyla* (pigs and cattle) and *Perissodactyla* (horses), especially to the recently cloned llama sequences.

KEY WORDS: camel cytokines, cDNA sequence, phylogenetic analysis, Th1, Th2.

Camels are members of the *Camelidae* family, *Tylopoda* suborder, *Artiodactyla* order, Mammalian class [2, 23]. Members of the camel family are among the principal large mammalian herbivores of arid habitats, and they make a crucial contribution to man's existence and survival in desert environments. The animals of the *Camelidae* family are extremely important in the Puna of the Andes and Gobi desert and play a major role in the lives of people.

The knowledge of camel immunology has advanced rapidly in the past several years. Studies on humoral immune response of camels and llamas had been dealt with to include findings on functional antibodies that formed with two heavy chains and no light chains [6, 10, 20, 26]. The camel is a comparatively hardy animal and is less susceptible to many of the diseases that affect other livestock species in the same areas, such as trypanosomiasis and brucellosis [1, 14]. Camels have also been diagnosed with foot and mouth disease, but no naturally occurring cases seem to occur [25]. Although some cases of disease have been reported, the resistance or susceptibility of the camel has not been elucidated. This makes it study of the immune system of *Camelidae* necessary.

The outcome of disease can be affected by cytokine responses, which play crucial roles in animal and human disease. In recent years, the central role of cytokines in the immune response has been widely studied. The T helper 1 (Th1) and Th2 cytokines are patterns of cytokines secreted by two different subpopulations of CD4+ T cells that determine the outcome of the antigenic response toward humoral or cell-mediated immunity (CMI) [7, 18]. In the recent past, a large variety of cytokines have been cloned for most important veterinary species, including the llama (*Lama glama*). We have previously reported molecular cloning and phylogenetic analysis of IL-6 and TNF- α from this species [16]. However, camel cytokines have been studied to a little extent in the present time, and most of them are not well known.

In the present experiment, phylogenetic analysis was performed on the full-length nucleotide sequences of the known Th1 (IL-2, IL-12p35, and IFN- γ) and Th2 (IL-4, IL-10, and IL-13) cytokine genes using the CLUSTALX program. Thus, the goal of the study reported here was cloning and sequence analysis of camel Th1 and Th2 cytokines and their comparison with those of other mammalian species including order *Artiodactyla*, *Primates*, *Perissodactyla*, *Carnivora* and *Rodentia*.

MATERIALS AND METHODS

Animals: Five healthy female bactrian camels (aged 4-9 years) were selected from a small herd in Erdene, Tuv Province, Mongolia, that was maintained by the Immunological Research Center, Institute of Veterinary Medicine, Ulaanbaatar, Mongolia.

Preparation of Camel PBMCs, RNA isolation, and RT-PCR: In order to clone the Th1 and Th2 cytokines of the camels, blood samples were collected by jugular venipuncture and peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation using Percoll (Amersham-Pharmacia, Sweden). The sampling of preparation was performed according to the protocols approved by the institutional committee for the use and care of animals. PBMCs were cultured in RPMI 1640 (Gibco BRL, USA) medium containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 ng/ml streptomycin. Cells (1×10^7 /ml) were grown in 24-well plates and stimulated with Concanavalin A (Con A, 5 μ g/ml) for 12 hr. Total RNA was isolated from Con A-stimulated PBMCs using TRIzol reagent (Invitrogen, USA). An aliquot of the total RNA (5 μ g) was reverse-transcribed using RAV2 reverse transcriptase (20 U/ μ l, TAKARA, Japan) and oligo-dT primer (0.5 mg/ml) in a total volume of 40 μ l reaction mixture according to the manufacturer's instructions. IL-2, IL-4, IL-10, IL-12p35, IL-13, and IFN- γ cDNAs were amplified from the mRNAs of the camel PBMCs by PCR using the primers used on bovine cytokine sequences published online [15, 17].

PCR was carried out in a total volume of 20 μ l reaction buffer containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.25 mM MgCl₂, 0.2 mM dNTPs, 5U of Taq polymerase (Takara), 10 pmol each of the primers, and 2 μ l of the cDNA sample. The cycling conditions for PCR were 35 cycles of 45s at 94°C, 45s at the annealing temperature depending on the cytokine to be amplified, and 1 min at 72°C. This was followed by a final extension for 5 min at 72°C. The resultant PCR products were separated on 2% agarose gels containing ethidium bromide (10 mg/ml) and were visualized under a UV light.

Cloning and sequencing of camel Th1 and Th2 cytokine cDNAs: The amplified bands corresponding to cytokine cDNAs were excised from the gels and purified using a GeneClean kit (Bio 101, USA). The purified cDNA fragments were ligated into pGEM-T Easy Vector (Promega, USA), and the resultant recombinant plasmids were transformed into a competent *E. coli* strain, DH-5 α . In each experiment, 10 plasmid clones containing cytokine cDNAs were sequenced using a BigDye Terminator Cycle Sequence kit (Applied Biosystems,

Warrington, United Kingdom) and an automated DNA sequencer (PRISM 310 Genetic Analyzer, Applied Biosystems). The resulting sequences were identified by a BLAST search of the NCBI (National Center for Biotechnology Information) databases for homologous sequences.

Sequence and phylogenetic analysis: Sequence data analyses were performed using the BLAST search of the NCBI. The amino acid sequences of camel IL-2, IL-4, IL-10, IL-12p35, IL-13, and IFN- γ were deduced using genetic information processing software, (GENETYX_MAC Ver.10.1.2, Software Development Co, Ltd., Japan). The sequence data herein have been submitted to Genbank and assigned Accession No. AB246671 for camel IL-2 cDNA, AB246673 for camel IL-4 cDNA, AB246674 for camel IL-10 cDNA, AB246672 for camel IL-12p35 cDNA, AB107658 for camel IL-13 cDNA, and AB107657 for camel IFN- γ cDNA.

Phylogenetic analysis was performed using the CLUSTALX program [22]. The transition/transversion rates were calculated using the PUZZLE 4.0.2 program [21]. Bootstrapping values were calculated using the modules SEQBOOT (random number seed: 123; 100 replicates), DNADIST (distance estimation: maximum likelihood; analysis of 100 data sets), NEIGHBOR (neighbor joining and UPGMA method; random number seed: 99; analysis of 100 data sets), and CONSENSE from the PHYLIP package (version 3.573) [9]. The phylogenetic trees were computed using the DNADIST and NEIGHBOR modules with the same parameters as above, and TREEVIEW version 1.6.0 [19] was used for visualization of the trees.

RESULTS

Cloning and sequence analysis of camel Th1 (IL-2, IL-12p35, and IFN- γ) cytokines: To address the goals of this paper, we cloned and sequenced bactrian camel (*Camelus bactrianus*) IL-2, IL-12p35, and IFN- γ . The cDNAs of camel IL-2, IL-12p35, and IFN- γ were found to be 465, 669, and 501 bp in length, with open reading frames (ORF) encoding 154, 222, or 166 amino acids, respectively [Fig. 1. (A), (B), and (C)]. A high level of sequence conservation of the cloned cDNAs confirmed their identity. In most cases, most of the coding region of the cDNAs were obtained. The sequences were compared to the published llama, pig, cow, horse, human, and mouse sequences (Table 1). The deduced amino acid sequences of the cloned cytokine genes and their alignment with the llama, cow, pig, and horse sequences are shown in Fig. 1. (A), (B), and (C). The cloned camel Th1 cytokine gene sequences showed the greatest homology to the published llama sequences, and the homology to llama of the

camel sequences was significantly greater than the homology to the pig and cow sequences (Table 1).

Four cysteine (Cys) residues at positions 9, 78, 126, and 146 were conserved in all the analyzed mammalian species IL-2 sequences [(Fig. 1. (A), marked with a black dot]. Potential N-linked glycosylation site (as predicted by either NXT or NXS motifs) were found at residue position 111 in the IL-2 sequence (Fig. 1. (A), marked with an open bar). Therefore, eight Cys residues and one potential N-linked glycosylation site were conserved in all the analyzed mammalian species IL-12p35 sequences. In addition, one possible N-linked glycosylation site at position 174 was present in the llama and camel IL-12p35 sequences analyzed in this study [Fig. 1. (B)]. Finally, one Cys residue at position 13 and two potential N-linked glycosylation sites were found at residue positions 39 and 106 in all the analyzed IFN- γ sequences [Fig. 1. (C)].

Cloning and sequence analysis of camel Th2 (IL-4, IL-10, and IL-13) cytokines:

Full-length cDNAs of bactrian camel (*Camelus bactrianus*) IL-4, IL-10, and IL-13 were cloned and sequenced. The sequences of IL-4, IL-10, and IL-13 had 402, 537, and 411 bp nucleotides with ORF encodings of 133, 178, and 136 amino acids, respectively [Fig. 2. (A), (B) and (C)]. The identities of the nucleotide and deduced amino acid sequences of the camel and several mammalian species for IL-4, IL-10, and IL-13 as shown in Table 1. Most of the potential N-linked glycosylation sites and Cys residues were conserved among most of the species [Fig. 2. (A), (B) and (C)]; marked with an open bar and a black dot].

The complete amino acid sequence of camel IL-4 and its comparison to llama, cattle, pig, and horse IL-4 are shown in Fig. 2. (A). The camel IL-4 deduced amino acid sequence includes four possible N-linked glycosylation sites (positions 62, 96, 102, and 108) and six Cys residues (positions 13, 17, 48, 70, 105, and 133) [Fig. 2. (A)]. Camel IL-10 had four Cys residues (positions 30, 80, 126, and 132), with only one N-linked glycosylation site (position 134) at the same position as that of the llama, cow, pig and horse. The deduced amino-acid sequence of camel IL-13 bore six N-linked glycosylation sites (positions 38, 49, 57, 72, 75, and 131) and four Cys residues (positions 13, 48, 64, and 90). The identity (%) results were further confirmed by phylogenetic analysis (Fig. 3.).

Phylogenetic analysis of camel Th1 and Th2 cytokines: In order to examine the phylogenetic relationships of camel Th1 and Th2 cytokines, we aligned mammalian those cDNA sequences with the respective sequences available in GenBank. As shown in Fig. 3, the mammalian species described in this study could be classified into the five major groups

of *Rodentia* (mouse and rats), *Primates* (human), *Perissodactyla* (horse), *Artiodactyla* (cow, sheep, pig, red deer, llama, and camel), and *Carnivora* (dog and cat). All the camel cytokine sequences reported here are most closely related to the llama, pig, cow, and horse sequences (Table 1). The homology results were further confirmed by phylogenetic analysis (Fig. 3.).

Our phylogenetic analyses indicated that camel cytokine genes are closely related to those of the order *Artiodactyla*, which includes the llama, pig, red deer, cow, and sheep. In the phylogenetic analysis, camel IL-2, IL-4, IL-12p35, IFN- γ , and IL-13 were clustered with the llama, pig, cow, sheep, and red deer (*Artiodactyla*) with a high bootstrap value.

DISCUSSION

In this study, the camel Th1 (IL-2, IL-12p35, and IFN- γ) and Th2 (IL-4, IL-10, and IL-13) cytokines were cloned and sequenced for the first time. In general, the order *Artiodactyla* comprises three main suborders, *Suiformes* (pig, hog, and peccaries), *Tylopoda* (camel, llama, alpaca, vicuna, and guanaco) and *Ruminantia* (cow, sheep, red deer, antelope, buffalo, etc.), that are defined at the molecular level [2]. The camel Th1 and Th2 cytokine cDNAs showed a high degree of homology with respect to the cDNAs from the llama, pig, cow, horse, and other mammalian species, although there was relatively lower homology to the cDNA from the mouse. Therefore, these results revealed a closer phylogenetic relationship with the camel, llama, pig, cow and horse than with the mouse or rat. Interestingly, the nucleotide and amino acid sequences of camel (llama) IL-10 were closely related to those of the horse (*Perissodactyla*), human (*Primates*), cow (suborder *Ruminantia*) [11], and pig (suborder *Suiformes*) [3] [Table 1 and Fig. 3. (B)]. In addition, phylogenetic analysis of IL-2, IL-4, IL-12p35, IFN- γ , and IL-13 from the camels, and other species indicated that *Suiformes* and *Tylopoda* are closely related to the order *Artiodactyla*. These results were similar to those obtained from phylogenetic analysis of llama Th1 and Th2 cytokines, including IL-2, IL-4, IL-10, IL-12p35/p40, IL-13, and IFN- γ [15, 17]. The alignments of the deduced amino acid sequences of the Th1 and Th2 cytokines for several mammalian species, including the camel, were similar in structure to the cDNAs described for other species [4, 5, 8, 12, 13, 24]. In particular, most of the presumed Cys residues and N-linked glycosylation sites were found to be located in the same positions in all species. This result suggests that this region is highly conserved in the cytokine protein and may therefore play an important role in determining its tertiary structure and functional integrity. It also suggests that highly conserved amino acid residues are likely regulated in a similar manner and have an equally important role in the immune system of *Camelidae*.

Examination of the role of this cytokines in camel is essential for the understanding

of *Camelidae* protective immune responses and disease development. An understanding of biological properties of camel Th1 and Th2 cytokines will be important to study the CMI and humoral immune responses of camel, which has unique properties in heavy chain antibody. This cloning, sequencing, and phylogenetic analyses of the camel Th1 and Th2 cytokines will be useful data for immunological study and formulation of disease control strategies. Future studies are needed to take advantage of these cytokines for enhancement of innate immune response in camels.

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Figure legends

Fig. 1. Alignment of the deduced amino acid sequences of the cloned camel Th1 cytokine cDNAs with those of the llama, cow, pig, and horse homologs. (A) IL-2. (B) IL-12p35. (C) IFN- γ . Conserved cysteine residues are indicated by a black dot (●). The potential N-linked glycosylation sites are indicated by a open bar (□). The GenBank accession numbers used in the sequence comparison are listed in Table 1, footnote *a*.

Fig. 2. Alignment of the deduced amino acid sequences of the cloned camel Th2 cytokine cDNAs with those of the llama, cow, pig, and horse homologs. (A) IL-4. (B) IL-10. (C) IL-13. Conserved cysteine residues are indicated by a black dot (●). The potential N-linked glycosylation sites are indicated by a open bar (□). The GenBank accession numbers used in the sequence comparison are listed in Table 1, footnote *a*.

Fig. 3. Phylogenetic relationship based on nucleic acid sequences of IL-2 (A) and IL-10 (B) from several mammalian species including the camel. Each cytokine was aligned with its corresponding sequence from various species available in Genbank. Phylogenetic relationships were constructed by the distance neighbor-joining method and rooted with *Artiodactyla*. In addition to the llama, pig, cow, horse, human, and mouse IL-2 and IL-10 nucleotide sequences (Table 1, footnote *a*), those from the red deer (U14682 and U11767), sheep (M55641 and U11421), cat (L19402 and AF060520), rat (M22899 and L02926), and dog (U28141 and U33843) were also used.

Table 1. Sequence identities between the llama, pig, cow, horse, human, and mouse cytokines

Cytokine	% Nucleotide (% amino acid) identity ^a					
	Llama	Pig	Cow	Horse	Human	Mouse
IL-2	99.1 (98.7)	88.8 (83.8)	84.4 (73.5)	81.3 (68.2)	81.2 (75.2)	66.7 (51.2)
IL-12p35	98.5 (98.2)	91.8 (89.2)	90.4 (88.3)	87.6 (80.6)	87.0 (82.0)	70.6 (59.0)
IFN- γ	97.8 (96.4)	87.5 (80.7)	88.8 (86.7)	86.0 (84.1)	79.6 (66.9)	64.2 (45.5)
IL-4	99.3 (99.2)	87.3 (81.2)	85.5 (77.8)	79.9 (71.7)	71.1 (59.6)	58.8 (41.5)
IL-10	99.6 (98.9)	86.3 (82.6)	88.1 (84.8)	92.7 (91.0)	88.5 (84.8)	81.2 (77.0)
IL-13	100 (100)	83.2 (72.9)	84.7 (75.8)	-	80.7 (69.7)	69.3 (53.7)

^a The sequence identities shown are for the portion of the sequences corresponding to the camel cDNA reported here. Nucleotide and deduced amino acid sequences corresponding to the primer sequences were excluded from the analyses of sequence identities. The GenBank accession numbers used in the sequence comparison were as follows: IL-2, AB107651, X56750, M12791, L06009, U25676, and K02292; IL-12p35, AB107653, L35765, U14416, Y11130, AF180562, and M86672; IFN- γ , AB107652, S63967, M29867, D285020, X13274, and K00083; IL-4, AB107648, L12991, M77120, AF305617, M13982, and M25892; IL-10, AB107649, L20001, U00799, U38200, M57627, and M37897; IL-13, AB107658, AF385626, NM_174089, L06801, and NM_008355; respectively.

原著 免疫学

フタコブラクダ (*Camelus bactrianus*) Th1 および Th2 型サイトカイン遺伝子の同定

Odbileg, R.1)・Purevtseren, B.2)・Batsukh, Z.2)・今内 覚 1)・大橋和彦 1)・小沼 操 1)

1)北海道大学大学院獣医学研究科動物疾病制御学講座感染症学教室

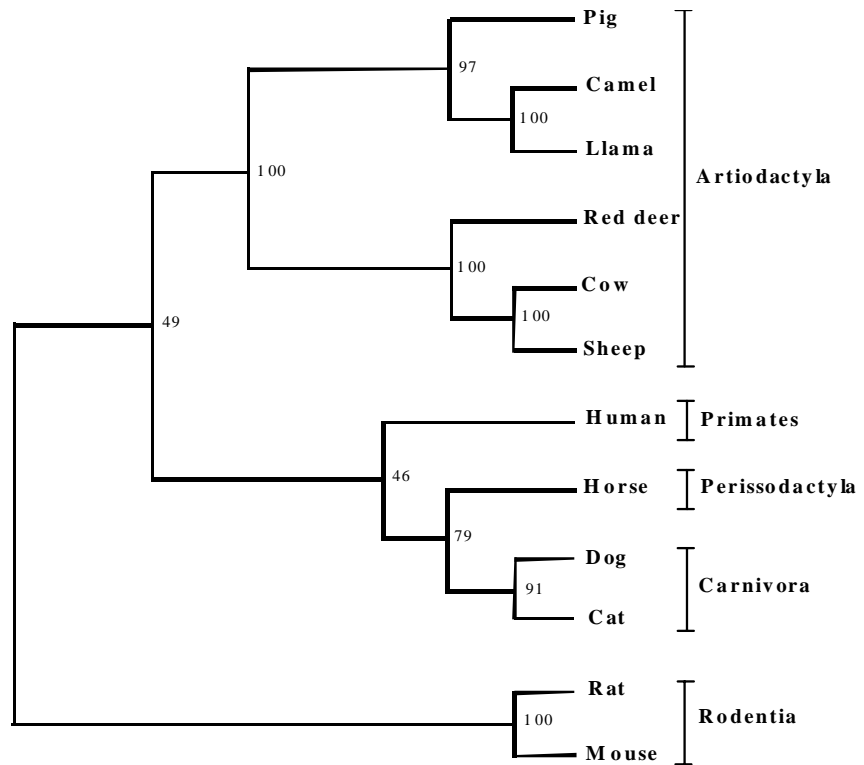
2) Immunological Research Center, Institute of Veterinary Medicine, Mongolia

和文要約

ラクダの Th1 および Th2 サイトカイン遺伝子は同定されておらず、感染症などに対する免疫応答の詳細も明らかでない。そこで、フタコブラクダ (*Camelus bactrianus*) の Th1 および Th2 サイトカイン遺伝子の同定を目的に、既知ウシサイトカイン遺伝子配列よりプライマーを作成し逆転写ポリメラーゼ連鎖反応(RT-PCR)法およびクローニングシークエンス法によりインターロイキン(IL)-2, IL-4, IL-10, IL-12p35, IL-13 およびインターフェロン(IFN)- γ の各遺伝子配列を同定した。各サイトカイン遺伝子の予想されるアミノ酸数は 154,133,178,222,136 および 166 であった。塩基配列を他の哺乳類(ラマ,ブタ,ウシ,ウマ,ヒトおよびマウス)と比較した結果,今回同定されたフタコブラクダのサイトカイン遺伝子は 58.8-100%間の相同性を示した。また,系統樹解析より本サイトカイン遺伝子は,ウシ,ブタ,ウマ,とくにラマのサイトカイン遺伝子と近縁であった。

448/600 文字

(A) IL-2



(B) IL-10

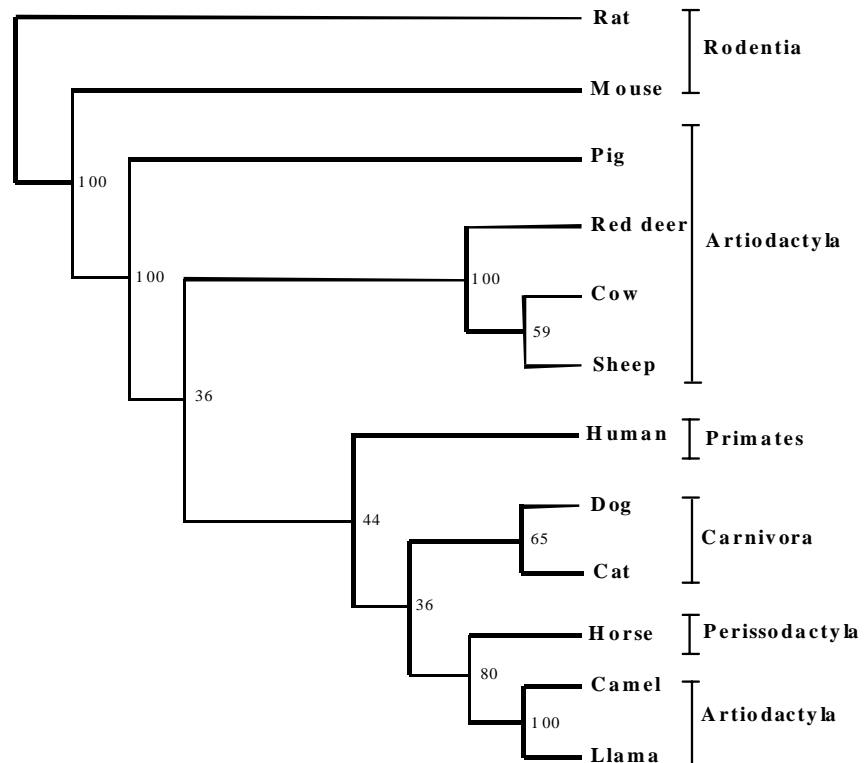


Fig. 3.