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Cyclosporine A Inhibits Transcription of Cytokine Genes and Decreases The Frequencies of IL-2 Producing Cells in Feline Mononuclear Cells

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ABSTRACT. Cyclosporine A (CsA) has been widely used for suppression of transplant rejection and controlling pruritus in allergic dermatitis in humans, dogs and cats. CsA is known to suppress the expression of inflammatory cytokines, including IL-2, IL-4, IFN-γ and TNF-α in humans, dogs and experimental mice. However, little is known about the immunomodulating effect of CsA in cats. The aim of this study was to evaluate the effects of CsA on the expression of inflammatory cytokines in feline peripheral blood mononuclear cells (PBMC). Real-time PCR analyses with *Concanavalin A* (ConA)-stimulated PBMC obtained from 5 cats revealed that the expression of mRNAs for IL-2, IL-4, IFN-γ and TNF-α was inhibited by CsA in a dose-dependent manner. Moreover, an enzyme-linked immunospot (ELISPOT) assay, which is capable of detecting IL-2 secreting cells as single spots, revealed that the frequency of IL-2 secreting cells in ConA-stimulated feline PBMC was significantly reduced in the presence of CsA. These results might provide an explanation for the mechanisms of action of CsA in the suppression of transplant rejection and the control of pruritus in cats.
KEY WORDS: allergic dermatitis, cyclosporine A, cytokines, ELISPOT, feline.

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Cyclosporine A (CsA), a cyclic polypeptide metabolite extracted from the fungus, *Tolyplocadium inflatum Gams*, has been widely used both in human and veterinary medicine. Classically, CsA has been used as an immunosuppressive agent in organ transplantation in humans and cats [2, 3, 12, 36]. Recently, CsA has been reported as an effective drug for the long-term management of pruritus in human and canine atopic dermatitis (AD) [10, 11, 22, 30]. In addition, several clinical trials have revealed that CsA is also effective for controlling pruritus in feline skin diseases [21, 34].

The mechanism of action of CsA in modulating immunological processes has been characterized in humans [7, 25]. CsA permeates into target cells and binds to the cyclophilins. This cyclosporine-cyclophilin complex binds to calcineurin, preventing nuclear factor of activated T cell (NFAT) dephosphorylation and thereby inactivating the transcription factor NFAT [17, 29]. Because NFAT dephosphorylation is essential for the transcription of a number of cytokine genes, including IL-2, IL-4, IFN-γ and TNF-α [25], the prevention of NFAT dephosphorylation suppresses the transcription of these cytokines and inhibits activation of various T cells, macrophages and B cells. Notably, the suppression of IL-2 expression by CsA is thought to play an important role in T-cell-mediated immunological processes in humans [7]. Recently, an inhibitory effect of CsA on lymphocyte activation and expression of cytokine mRNA in dogs has also been reported [15, 23, 31], while the transcription of canine TNF-α was not inhibited by CsA, unlike the situation in humans and mice [1, 8, 14, 15, 19, 35]. Conversely, although efficacy of CsA in inhibiting transplant rejection and controlling pruritus in cats has been reported, there have been no reports describing the mechanisms of action of CsA on modulating immunological processes in cats. The aim of this study was to determine the effects of CsA on mRNA expression and production of cytokines in feline peripheral blood monocular cells (PBMC).

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MATERIALS AND METHODS

Isolation of PBMC: Blood samples were collected from cervical veins of 5 healthy cats under sedation with 10 μg/kg medetomidine hydrochloride (Domitor®, Nippon Zenyaku Kogyo, Fukushima, Japan.), injected intramuscularly. 10 m*l* blood samples were layered onto equal amount of Histopaque®-1077 (Sigma, St. Louis, MO), and centrifuged at 340 × g for 30 min at room temperature. After the centrifugation, PBMC were collected from the opaque interface just below the plasma layer, and washed twice in phosphate-buffered saline (PBS). Residual red blood cells were removed from PBMC with lysis buffer (7.5 g/*l* ammonium chloride and 0.02 M Tris-HCl, pH 7.2) for 5 min with gentle agitation. The PBMC were further washed once in culture medium {RPMI-1640 medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (ICN Biomedicals, Inc., Aurora, OH), 100 U/m*l* of penicillin and streptomycin, and 0.25 μg/m*l* of amphotericin B (Gibco Invitrogen Co., Rockville, MD)}. The number of viable cells in the suspension of PBMC was counted in a Bürkel-Türk chamber using the trypan blue dye exclusion assay.

Cytotoxicity assay: PBMC isolated from 3 healthy cats were suspended in culture medium at a density of 2×10^6 cells/m*l*, duplicated in a 96 well tissue culture plate (MICROTEST™ 96, FALCON, Oxnard, CA), and incubated with CsA (10, 100 or 1,000 *n*g/m*l*; Sigma-Aldrich; St. Louis, MO) in the presence of 5 μg/m*l* ConA (Nacalai Tesque, Kyoto, Japan) for 24 hr at 37°C in humidified air with 5% CO_2. After incubation, cell suspensions were treated with Celltiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI). The absorbance at 490 nm was measured with a Multiscan biochromatic plate reader (Multiskan JX: Thermo Fisher Scientific, Waltham, MA). The survival rate of PBMC after incubation was calculated using the following formula:

Survival rate = (Optical density (OD) of PBMC with CsA stimulation—OD of culture medium/OD of PBMC without CsA stimulation—OD of culture medium) × 100.

Isolation of total RNA from PBMC: PBMC isolated from 5 healthy cats were suspended in culture medium at a density of 2×10^6 cells/m*l*. 500 μl of the PBMC were incubated with CsA (50, 150, 450 or 1350 *n*g/m*l*) in the presence of 5 μg/m*l* ConA at 37°C in humidified air with 5% CO_2 for 10 hr. ConA-stimulated PBMC were stored at –80°C until RNA extraction. Total RNA was extracted from PBMC using an RNeasy® Plus Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. The extracted RNA was suspended in 30 μl of diethylphyrocarbonate (DEPC)-treated water, and used immediately for two-step real-time PCR analysis or stored at –70°C.

Two-step real-time RT-PCR assay: Real-time RT-PCR assay was performed to evaluate the effects of CsA on transcription of IL-2, IL-4, IFN-γ and TNF-α genes in ConA-stimulated PBMC obtained from 5 healthy cats. Oligonucleotide primers for the isolation of mRNA for feline IL-2, IL-4, IFN-γ, TNF-α and glyceraldehydes-3-phosphate dehidrogenase (GAPDH) were designed by the Primer Express® software (Applied Biosystems, Foster City, CA), according to previously reported nucleotide sequences of the feline genome. The nucleotide sequences of the primers used, and GenBank accession numbers of the feline genome are listed in Table 1.

First-stand cDNA synthesis and real-time PCR were performed using a SuperScript™ III Platinum® Two-Step qRT-PCR Kit with SYBR® Green (Invitrogen Corp, Carlsbad, CA). The RT reaction mixture (28 μl of total RNA, 35 μl of 2 × RT Reaction Mix and 7 μl of RT Enzyme Mix) was incubated at 25°C for 10 min, 42°C for 50 min and 85°C for 5 min. Subsequently, 3.5 μl of *Escherichia coli* RNase H were added to the RT reaction mixture and incubated at 37°C for 10 min to remove residual RNA. The first-strand cDNA was stored at –20°C until PCR amplification. To perform real-time PCR, PCR mixture (50 μl of mixture containing 4 μl of cDNA, 1 μl of ROX Reference Dye, 25 μl of Platinum® SYBR® Green qPCR SuperMix-UDG, 18 μl of DEPC-treated water and 10 μM of forward and reverse primers) in duplicate were analyzed using the 7300 Real Time PCR System (Applied Biosystems, Foster city, CA). The reaction was performed at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 10 min.

A comparative (Ct) method was applied for the quantification of each cytokine. For each sample, the Ct values for the target and endogenous reference (GAPDH) genes were determined to calculate the relative transcription of the target mRNA against GAPDH mRNA. Subsequently, ΔCT was calculated to normalize the amount of sample mRNA using the following formula:

$$\Delta CT = \text{Ct value of the target cytokine} - \text{Ct value of GAPDH}$$

All samples were examined in duplicate and the mean value of ΔCT was used for further analysis. The amount of mRNA for each cytokine was calculated by $2^{-\Delta CT}$. The mRNA expression of each cytokine was calculated using the following formula:

$$\text{Cytokine mRNA expression rate} = (2^{-\Delta CT} \text{ of sample with CsA} / 2^{-\Delta CT} \text{ of sample without CsA}) \times 100$$

Enzyme-linked immunospot (ELISPOT) assay: An ELISPOT assay was performed to determine the frequency of IL-2 secreting cells in feline PBMC using Feline IL-2 Development Module (R&D Systems, Minneapolis, MN). Briefly, a polyvinylidene difluoride (PVDF)-bottomed 96-well plate (Millipore, Beverly, MA) was pre-wet with 70% ethanol and washed three times with PBS. The plate was then coated with 100 μl/well of a 1:60 diluted Feline IL-2

Table 1. Nucleotide sequences of the cytokine primers used for real-time RT-PCR

Cytokines	Primer sequences (5'-3')	Product length	GenBank accession No.
IL-2	Forward: CGGTTGCTTTTGAATGGAGTTAA	65 bp	L19402
	Reverse: TTAAATGTGAGCATCCTGGAGAGTT		
IL-4	Forward: CGTCTTGGCAGCCCCTAAG	59 bp	U39634
	Reverse: CGGTTGTGGCTCTGCAGAA		
IFN-γ	Forward: AGGAGCATGGACACCATCAAG	67 bp	D30619
	Reverse: CCCGTTTACTGGAGCTGGTATT		
TNF-α	Forward: CCGCTTTGCCGTTTCCTA	58 bp	M92061
	Reverse: AGGGCTCTTGATGGCAGAGA		
GAPDH	Forward: AAATTCCACGGCACAGTCAAG	61 bp	AB038241
	Reverse: TGATGGGCTTTCCATTGATGA		

Capture Antibody Concentrate (R&D Systems) overnight at 4°C. The plate was washed three times with 0.05% Tween®20 in PBS (PBS-T), blocked with blocking buffer (1% BSA, 5% sucrose in PBS) at room temperature for 2 hr, and rinsed once with culture medium. PBMC isolated from 5 healthy cats were suspended in the culture medium at a density of 5×10^5 cells/m*l*. As the primary reaction, 200 μ*l*/well of the cell suspensions were incubated on the plate with/without CsA (1000 *n*g/m*l*) in the presence of 5 μg/m*l* ConA at 37°C in humidified air with 5 % CO_2 for 16 hr. The plate was also incubated with PBMC without ConA as negative controls. After the primary incubation, the plate was washed 4 times in PBS-T, and incubated with 100 μ*l*/well of 1:60 diluted Feline IL-2 Detection Antibody Concentrate (R&D Systems) overnight at 4°C. The plate was then washed three times with PBS-T, and treated with 100 μ*l*/well of 1:60 diluted streptavidin-conjugated to alkaline phosphatase (ELISpot Blue Color Module: R&D Systems) at room temperature for 2 hr. The plate was washed three more times with PBS-T, rinsed with distilled water, and incubated with 5-bromo-4-chloro-3' indolylphosphate p-toluidine salt/nitro blue tetrazololium chloride chromogen solution (ELISpot Blue Color Module: R&D Systems) for 20 min. IL-2 bound to the membrane was visualized as blue spots, and the number of spots represents the number of IL-2 secreting cells. After rinsing of the plate with distilled water and drying in air, the number of spots was counted under a dissecting microscope. The optimal dilutions of coated antibody and secondary antibody were determined using soluble recombinant human IL-2 (1 *n*g/well; R&D Systems) as positive control. All experiments were carried out in duplicate and all results represent the mean of duplicate values.

RESULTS

CsA shows no apparent cytotoxicity to feline PBMC: The cytotoxity of CsA to feline PBMC was evaluated. Incubation of the ConA-stimulated feline PBMC obtained from 3 healthy cats with 10–1,000 *n*g/m*l* of CsA did not alter the survival rate of the feline PBMC compared with that of PBMC cultured without CsA (Fig. 1).

CsA inhibits transcription of IL-2, IL-4, IFN-γ and TNF-α genes in feline PBMC: The effects of CsA on the transcription of genes for IL-2, IL-4, IFN-γ and TNF-α was investigated using real-time PCR analysis. Expression of mRNAs for IL-2, IL-4, IFN-γ and TNF-α was reduced in a dose-dependent manner when the feline PBMC were incubated with serially-diluted CsA (Fig. 2). The expression levels of mRNAs for each cytokine were significantly lower in PBMC incubated with ≥150 *n*g/m*l* of CsA than in those incubated without CsA (Tukey-Kramer test, n=5, $p<0.01$). The inhibition of mRNA expression of the cytokines seemed to reach a plateau (≤32% of mRNA in PBMC without CsA) when the PBMC were incubated with ≥450 *n*g/m*l* of CsA.

CsA decreases the frequencies of IL-2 producing cells in feline PBMC: Feline IL-2 ELISPOT assay was performed with ConA-stimulated feline PBMC cultured with/without CsA (1,000 *n*g/m*l*). Positive spots were detected in PBMC obtained from 5 cats in the presence and absence of CsA (Fig. 3A, 3B). However, the numbers of positive spots in PBMC cultured with CsA (51.8 ± 22.5) were significantly lower than in those without CsA (102.0 ± 33.8) (paired *t*-test, n=5, p=0.0156) (Fig. 3C).

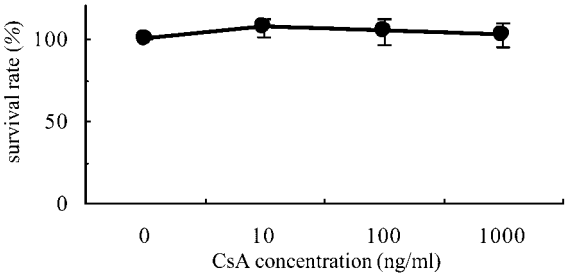

Fig. 1. CsA shows no apparent cytotoxicity in feline PBMC. PBMC obtained from 3 healthy cats were incubated with serially-diluted CsA (10–1,000 *n*g/m*l*) in the presence of 5 μg/m*l* ConA for 24 hr. Cytotoxicity assay was performed to determine the survival rate of PBMC. The dots and error bars represent mean and SD of survival rate of PBMC from 3 cats, respectively.

DISCUSSION

In this study, we found that CsA inhibited the transcription of cytokine genes in feline PBMC. The frequencies of IL-2 producing cells were significantly decreased by the presence of ≥450 *n*g/m*l* of CsA. The cytotoxic effect of CsA was negligible when feline PBMC were cultured with ≤1000 *n*g/m*l* of CsA. A previous study demonstrated that the maximum blood CsA concentration reached ≤1000 *n*g/m*l* in cats given 5 mg/kg CsA orally [18]. Considering our observations with the previous finding together, it is speculated that oral CsA suppresses the transcription of genes for cytokines, including IL-2, and thus contributes to immune suppression and control of pruritus in feline patients.

IL-2, which is produced by various types of cells including Th1 cells, plays central roles in the activation of the inflammatory process by stimulating proliferation and differentiation of T cells [13]. $CD4^+$ and $CD8^+$ T cells infiltrate lesioned skin of cats with allergic dermatitis [28, 33]. On the other hand, IL-4 produced in Th2 cells is known to differentiate B cells into plasma cells, and IgE secreted by these cells induces mast cell activation and release of chemical mediators [13]. Involvement of IL-2 and IL-4 might be important in the development of pruritus and cutaneous lesions in feline allergic skin diseases, in which infiltration of activated inflammatory T cells producing IL-4 are recognized [27]. In addition, circulating allergen-specific IgE was detected in approximately 50% of cats with AD, although these IgE were undetectable in almost all healthy cats (Kadoya M, personal communication). Our findings

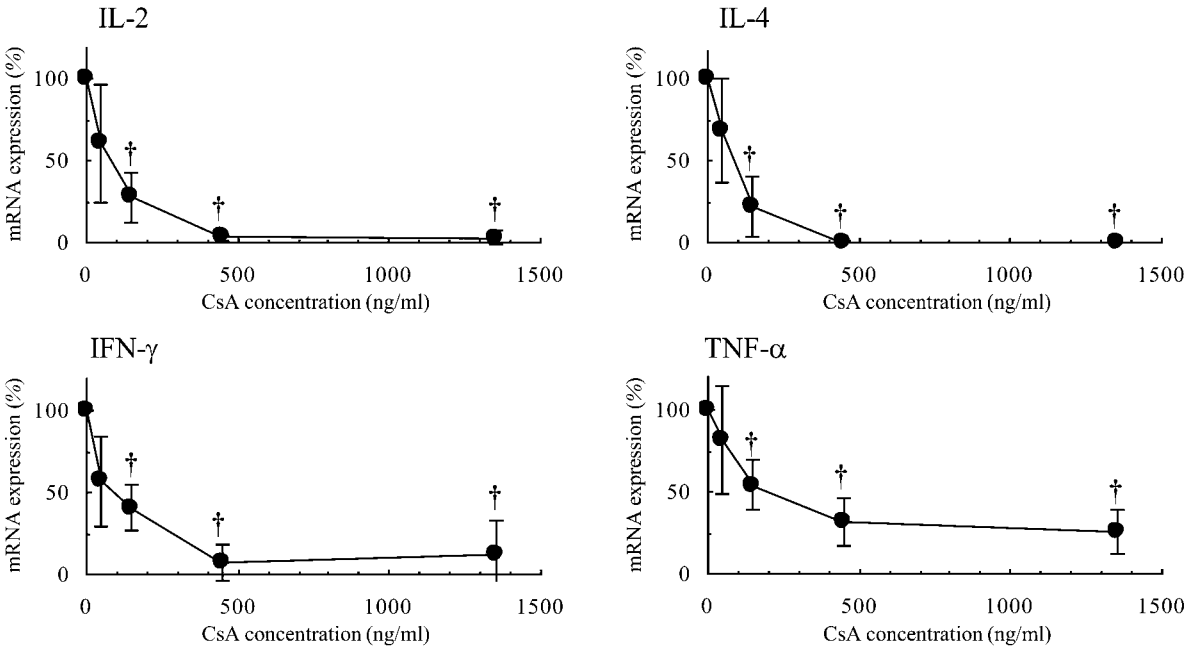

Fig. 2. Expression of mRNAs for IL-2, IL-4, IFN-γ and TNF-α in feline PBMC were suppressed by CsA. PBMC obtained from 5 healthy cats were incubated with serially-diluted CsA (10–1,350 *n*g/m*l*) in the presence of 5 *μ*g/m*l* ConA for 10 hr. Two-step real-time RT-PCR assay was performed in duplicate to evaluate the expression rates of mRNAs for IL-2, IL-4, IFN-γ and TNF-α. The dots and error bars represent mean and SD of mRNA expression rates in PBMC from 5 cats, respectively. † denotes values that are statistically different from the PBMC without CsA (Tukey-Kramer test, n=5, $p<0.01$).

raise the possibility that suppression of IL-2 and IL-4 expression contributes to control of pruritus in cats with allergic skin diseases.

IFN-γ produced in Th1 cells is known to activate macrophages and induce the release of inflammatory mediators from macrophages in humans [13]. In human AD, IFN-γ is reported to play a key role in the development of chronic skin lesions [9, 16]. TNF-α is also an important cytokine for the activation of macrophages. In humans and dogs, TNF-α is reported to play a major role in the pathogenesis of chronic inflammatory diseases and autoimmune diseases [13, 20, 24, 26]. The exact roles of IFN-γ and TNF-α in cats remain to be further elucidated.

In this study, we used an ELISPOT assay, which has recently emerged as a powerful tool to detect ongoing cytokine expression in single mononuclear cells [4–6, 13, 32]. Our results indicate that frequencies of IL-2 producing cells were significantly decreased by the presence of CsA, although CsA did not completely inhibit IL-2 production in feline PBMC at the concentration of 1,000 *n*g/m*l*. Possible explanation for the imcomplete inhibition of IL-2 production is that 1000 *n*g/m*l* of CsA is not enough to inhibit calcineurin activation completely, or that there are signaling cascades other than calcineurin-NFAT dephosphorylation to facilitate transcription of cytokine genes in feline mononuclear cells. Further studies are expected to clarify the exact role of transcription of cytokine genes in feline mononuclear cells.

In conclusion, we have demonstrated that CsA inhibits the transcription of cytokine genes for IL-2, IL-4, IFN-γ and TNF-α in feline PBMC. In addition, CsA causes a decrease in the frequency of IL-2 producing cells, which are thought to play central roles in the stimulation of both T helper cells and cytotoxic T cells. The results of this study will improve understanding of the mechanisms of action of CsA in modulating the immunological process and help to improve the clinical efficacy of suppression of transplant rejection and control of pruritus in feline patients.

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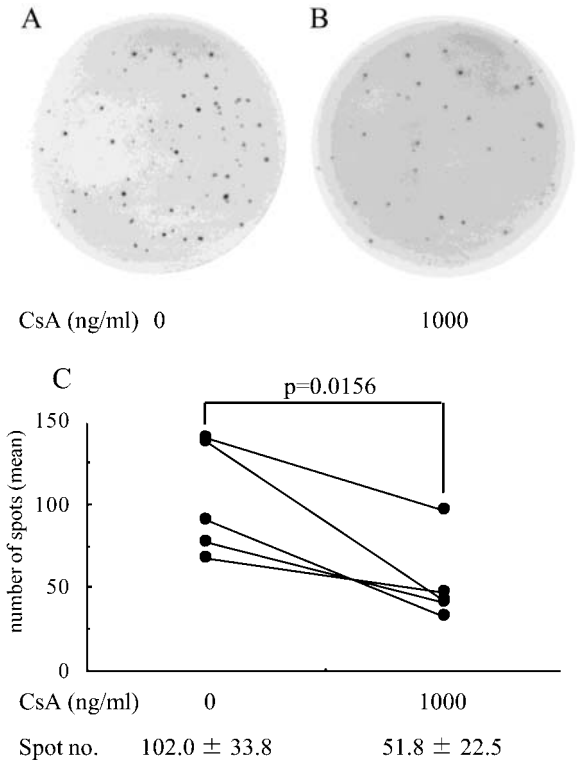

Fig. 3. (A) Detection of IL-2 secreting cells in feline PBMC by an ELISPOT assay. Positive spots represent IL-2 secreting cells. Note that the number of positive spots obtained from feline PBMC with CsA is lower than that without CsA. (B) Frequency of IL-2 secreting cells in feline PBMC was significantly reduced by the presence of CsA. PBMC obtained from 5 healthy cats were incubated with/without 1,000 *n*g/m*l* of CsA in the presence of 5 μg/m*l* ConA for 16 hr, and subjected to the ELISPOT assay. Paired *t*-test was performed to analyze the differences in frequencies of IL-2 secreting cells.

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