

Cytokine profiles of peripheral blood mononuclear cells from dogs experimentally sensitized to Japanese cedar pollen

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Abstract

Japanese cedar (*Cryptomeria japonica*, CJ) pollinosis is mediated by type-I hypersensitivity and induces seasonal rhinitis and conjunctivitis in humans. Previous studies showed that dogs could be experimentally sensitized with CJ pollen. In this study, we carried out quantitative analysis of mRNA levels of various cytokines in the peripheral blood mononuclear cells (PBMC) of 12 dogs experimentally sensitized to Japanese cedar pollen. Experimental sensitization was carried out by injection of crude CJ pollen extract with aluminium hydroxide gel. The expression levels of interleukin (IL)-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-18, interferon (IFN)- γ , transforming growth factor (TGF)- β ₁, and tumor necrosis factor (TNF)- α mRNAs in the PBMC were quantified using a real-time sequence detection system. In the PBMC tested without culture, the expression levels of IL-8 and TNF- α mRNAs in experimentally sensitized dogs were significantly higher than those in control dogs. The expression level of IFN- γ mRNA in the sensitized group was significantly lower than that in the control group. When the PBMCs were cultured in the presence of CJ pollen extract, the level of IL-4 mRNA expression was markedly increased in the PBMC from the experimentally sensitized dogs. In the PBMC stimulated with the CJ pollen extract, the expression level of IL-2 mRNA in the sensitized group was also significantly higher than that in the control group. Our data indicated that a Th2 response and proliferation of PBMC occur in response to the sensitizing antigen in dogs experimentally sensitized with CJ pollen, and revealed the presence of antigen-specific Th2 cells in this canine model. In addition, the expression levels of the mRNAs encoding proinflammatory cytokines were shown to be elevated after CJ pollen sensitization, indicating the activation of monocytes and macrophages.

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1. Introduction

Japanese cedar pollinosis is a common allergic disease causing seasonal rhinitis and conjunctivitis in humans, and is known to be mediated by type-I

hypersensitivity to Japanese cedar (*Cryptomeria japonica*, CJ) pollen antigen. Similarly, we previously showed hypersensitivity to CJ pollen in dogs (Sasaki et al., 1995; Masuda et al., 2000a) showing atopic dermatitis (AD). In Japan, about approximately 10% of dogs with AD were shown to have IgE antibodies directed to CJ pollen (Hasegawa et al., 1996).

To understand the pathogenesis of CJ pollinosis and to develop a novel therapeutic approach for the

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disease, we developed a canine model experimentally sensitized to CJ pollen antigen (Yamashita et al., 2000). In this experimental sensitization model, we demonstrated an increased level of CJ pollen antigen-specific IgE antibody in the serum and positive reaction to CJ pollen antigen in the intradermal skin test (IDST).

For characterization of the immune response in allergic diseases, a number of studies on the cytokine profile have been carried out. Polarized T-cell responses were shown to lead to the release of cytokines inducing allergic responses (Mosmann and Coffman, 1989). CD4⁺ T helper cells can be divided into two major subsets termed T helper (Th) 1 and Th2, based on their pattern of secreted cytokines. Th1 cells produce interferon (IFN)- γ and interleukin (IL)-2, and promote the cell-mediated immune responses. In contrast, Th2 cells secrete IL-4 and IL-10, and provide help for humoral responses, particularly IgE synthesis. The Th2-type response is thought to play a critical role in the development of allergic diseases, and is recognized as a target for novel immunotherapies.

The aim of the present study was to characterize the immune response in dogs experimentally sensitized to CJ pollen by quantification of cytokine mRNAs in the peripheral blood mononuclear cells (PBMC). We employed a real-time sequence detection system to quantify the levels of the mRNAs of various cytokines. This system has been shown to be a rapid, reliable, and highly sensitive method for simultaneously quantifying the expression of a large number of genes (Gibson et al., 1996).

2. Materials and methods

2.1. Crude CJ pollen allergen

Crude CJ pollen allergen was prepared as described previously (Yasueda et al., 1983). In brief, Japanese cedar pollen was defatted with ether and then extracted with 0.125 M ammonium bicarbonate overnight at room temperature. After the extraction, ammonium sulfate was added to the extract with stirring to 80% saturation. After stirring overnight at 4 °C, the precipitate was collected and dissolved in phosphate buffered saline (PBS). This solution was dialyzed against PBS overnight at 4 °C. Aluminum hydroxide gel (alum)

was prepared as adjuvant by the following method: 1N NaOH was added to 5.5% aluminum potassium sulfate solution with stirring until pH of the solution reached 7.0. After stirring for 30 min at room temperature, the precipitate was collected by centrifugation and suspended in physiological saline.

2.2. Dogs

We used 18 dogs kept for experimental purposes that showed no clinical signs of allergic diseases and were negative for serum antigen-specific IgE test and IDST for CJ pollen antigen. Twelve of these dogs (seven intact males and five intact females) were used for experimental sensitization to CJ pollen antigen. These dogs were all Beagles aged 4 months. The procedures for sensitization were described previously (Yamashita et al., 2000). The dogs were injected subcutaneously with 100 μ g of crude CJ pollen allergen in 3 ml of saline-emulsified alum, and similarly injected 2 weeks after the first injection. Blood samples were collected 2 weeks after the second injection. Six other Beagles (two intact males and four intact females) were used as controls. These dogs ranged in age from 10 months to 3 years old.

2.3. PBMC separation

Heparinized peripheral blood samples were diluted with an equal volume of PBS and layered on Ficoll-Hypaque (Nycomed Pharma AS, Oslo, Norway). After centrifugation at 350 g for 40 min at room temperature, the layer containing PBMC fraction was collected and then washed three times with PBS. Approximately one-third of the PBMC preparation was immediately frozen in liquid nitrogen and stored at –80 °C for subsequent extraction of RNA samples of primary PBMC. Another two-third of the PBMC preparation was immediately suspended in Dulbecco's Modified Eagle's Medium (DMEM) containing 5% dog serum and antibiotics (penicillin 100 U/ml and streptomycin 100 μ g/ml) at a cell count of 1.25×10^6 /ml for cultivation with or without antigen stimulation.

2.4. Culturing of PBMC

For the examination of cytokine mRNA expression, the cell suspensions (1.25×10^6 /ml) allocated to each

well of a 12-well plate were incubated at 37 °C under stimulation with the crude CJ pollen extract at a concentration of 5 µg/ml as the sensitizing antigen or a crude *Dermatophagoides pteronyssinus* extract (Greer Laboratories, Lenoir, NC) at a dilution of 1:10 000 (w/v) as a non-sensitizing antigen, or without antigen stimulation. The incubation periods of PBMC with or without antigens were determined to be 24 h based on our preliminary study and studies in human PBMC (Macaubas et al., 1999; Rudin et al., 2001) for measurement of cytokine mRNA expression. The PBMC samples after cultivation for 24 h were immediately frozen in liquid nitrogen and stored at –80 °C for subsequent RNA extraction.

Lymphocyte stimulation test to CJ pollen extract was carried out as described in our previous studies (Masuda et al., 2000b; Masuda et al., 2002). Two hundred microliters of PBMC suspension (1.25×10^6 /ml) allocated to each well of a 96-well plate was incubated at 37 °C for 72 h under stimulation with a crude CJ pollen extract at a concentration of 5 µg/ml. Blastogenic responses of the antigen-stimulated and unstimulated groups were measured by assaying the incorporation of ³H-thymidine (1 µCi per well) for 18 h in triplicate cultures. The amount of incorporated radioactivity was measured using a liquid scintillation counter. The stimulation index (SI) was expressed as the ratio of the mean incorporated radioactivity in the antigen-stimulated group to that in the unstimulated group (antigen-stimulated group/unstimulated group).

2.5. RNA isolation

Total RNA was extracted by the acid guanidium–phenol–chloroform method using RNeasy B (Tel Test, Friendswood, TX). The RNA samples were dissolved in RNase-free water. The extracted RNA samples were treated with RNase-free DNase I (Gibco, Grand Island, NY) to remove the contaminating DNA and then stored at –80 °C.

2.6. Measurement of specific IgE

Specific IgE against crude CJ pollen was assayed by a fluorometric ELISA as previously described (Masuda et al., 2000c).

2.7. Intradermal skin test (IDST)

A crude extract of CJ pollen was prepared at a concentration of 10 µg/ml and then diluted with a diluent containing 0.9% sodium chloride and 0.4% phenol at a final concentration of 200 ng/ml. The diluent without allergen extract was used as a negative control. Histamine solution diluted at a concentration of 0.0275 mg/ml was used as a positive control. These solutions (0.05 ml of each per test) were carefully injected intradermally into the clipped skin of the ventro-lateral thorax, by means of a skin test syringe attached to a 26 gauge needle. The diameters of wheals were measured 15–20 min after injection. The results of the IDST were graded as follows: (+++), equal to or greater than the diameter of the positive control; (++), equal to or greater than the mean diameter of the positive and negative controls; (+), larger than the diameter of the negative control but smaller than the mean diameter of the positive and negative controls; (–), equal to or smaller than the diameter of the negative control.

2.8. TaqMan primers and probes

For each of the target genes, canine β-actin, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-18, IFN-γ, transforming growth factor (TGF)-β₁, and tumor necrosis factor (TNF)-α, a pair of oligonucleotide primers and an oligonucleotide probe were designed based on the sequences in the Genbank database using a computer software package, Primer Express software (Applied Biosystems, Foster City, CA) (Table 1). The 5' and 3' ends of the internal probe were labeled with a reporter dye FAM (6-carboxyfluorescein) and a quencher dye TAMRA (6-carboxytetramethylrhodamine), respectively. In order to achieve primer extension within a few seconds and to avoid the requirement for a separate extension step, the primers were designed to generate short PCR products (78–100 bp). In a preliminary experiment, all of the primer sets were tested for efficient amplification of each gene from a control template RNA extracted from normal canine peripheral blood mononuclear cells stimulated with phorbol myristate acetate and calcium ionophore.

Table 1
Sequences of primers and probes

Target gene	Primer probe	Sequence (5'–3')	Nucleotide position	Reference	Gene bank accession number	Length of the PCR product (bp)
β -actin	Forward primer	GACCCTGAAGTACCCCATGAG	131–152			
	Reverse primer	TTGTAGAAGGTGTGGTGCCAGAT	189–211		Z70044	81
	Probe	CGTCACCAACTGGGACGACATGGA	161–184			
IL-1 β	Forward primer	TCTCCACACAGCTCTGTAACAA	84–105			
	Reverse primer	GCAGGGCTTCTTCAGCTTCTC	143–163		Z70047	80
	Probe	CTGAGGCATTTCGTGTCAGT-CATTGTAGCTT	110–140			
IL-2	Forward primer	CATCGCACTGACGCTTGTACTT	58–79			
	Reverse primer	CCATCTGTGTCTCTGTTTCCTT	115–137	Dunham et al., 1995	D30710	80
	Probe	TCGCAACACAGTGCACCT-ATTACTTCAAGCTCT	81–112			
IL-4	Forward primer	CATCCTCACAGCGAGAAACG	117–136			
	Reverse primer	CCTTATCGCTTGTGTCTTTGGA	177–199	van der Kaaij et al., 1999	AF054833	83
	Probe	CATGGAGCTGACTGTCAAGGA-CGTCTTCA	144–172			
IL-6	Forward primer	TCCTGGTGATGGCTACTGCTT	98–118			
	Reverse primer	GACTATTTGAAGTGGCATCATCCTT	151–175	Kukielka et al., 1995	U12234	78
	Probe	CCCTACCCCGGGACCCCTGG	120–139			
IL-8	Forward primer	CAAGAGCCAGAAAGAAACCAGAAC	4–27			
	Reverse primer	AAAGCTGCCAAGAGAGCAACA	64–84	Ishikawa et al., 1993	AF048717	81
	Probe	AGGCACCTTGTGTAACA-TGACTTCCAAGC	30–59			
IL-10	Forward primer	CGCTGTCAACGATTTCTTCC	383–402			
	Reverse primer	CTGGAGCTTACTAAATGCGCTCT	438–460	Lu et al., 1995	U33843	78
	Probe	TGAGAATAAGAGCAAGGCG-GTGGAGC	406–431			
IL-18	Forward primer	CTCTCCTGTAAGAACAAACTAT-TTCCTT	328–356			
	Reverse primer	GAACACTTCTCTGAAAGAATAT-GATGTCA	399–427	Argyle et al., 1999	Y11133	100
	Probe	CAGAAAATGAGTCCCTCCGG-ATAGTATCAATGATG	358–391			
IFN- γ	Forward primer	GCGCAAGGCGATAAATGAAC	336–355			
	Reverse primer	CTGACTCCTTTTCCGCTTCT	397–417	Zucker et al., 1992	AF126247	82
	Probe	TGATGAATGATCTCTACCAAGA-TCCAACC	365–394			
TGF- β_1	Forward primer	CAGAATGGCTGTCTTTGATGTC	635–657			
	Reverse primer	AGGCGAAAGCCCTCGACTT	695–713	Manning et al., 1995	L34956	79
	Probe	CTGGAGTCGTGAGGCAGTGGCTGAG	659–683			
TNF- α	Forward primer	GAGCCGACGTGCCAATG	97–113			
	Reverse primer	CAACCCATCTGACGGCACTA	156–175	Zucker et al., 1994	Z70046	79
	Probe	CGTGGAGCTGACAGACAACCAGCTG	130–154			

2.9. Quantitation of cytokine transcripts by real-time PCR

In the real-time PCR system, the 5' $\rightarrow$ 3' exonuclease activity of *Taq* polymerase leads to release of the reporter dye from the vicinity of the quencher dye

(Heid et al., 1996; Holland et al., 1991), resulting in increased reporter fluorescence. The fluorescence intensity is correlated with the amount of target DNA and is detected using an automated fluorometer, ABI Prism 7700 Sequence Detection System (Applied Biosystems).

The samples were placed in 96-well plates. For every RNA sample, parallel reactions were performed in duplicate in separate wells for the detection of canine β -actin, IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-18, IFN- γ , TGF- β ₁, and TNF- α transcripts. Amplification conditions were identical for all reactions: 30 min at 48 °C, 10 min at 95 °C, and 45 cycles of 15 s at 95 °C and 60 s at 60 °C. Reverse transcription (RT) and PCR amplification were carried out in a single well, without modifying or moving the sample between RT and PCR. The reactions were carried out in a reaction solution supplied in the TaqMan PCR Core Reagent Kit (Applied Biosystems) in a final volume of 25 μ l containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 2.5 mM deoxynucleotide triphosphates, 0.625 U of AmpliTaq Gold, 6.25 U of MultiScribe reverse transcriptase (Applied Biosystems), 10 U of RNase inhibitor (Applied Biosystems), 0.25 μ g of template RNA, each primer at 200 nM and 100 nM probe.

Final quantification was carried out by the comparative C_T method by determining the n -fold differences relative to the calibrator RNA. Signals were regarded as positive when the fluorescence intensity exceeded $10\times$ the standard deviation of the baseline fluorescence (threshold cycle, C_T). For each sample, the C_T for the target amplicon and the calibrator were determined. Calculation of the differences between the target and internal control C_T served to normalize for differences in the amount of total nucleic acid added to each reaction and the efficiency of the RT step. For each sample, the C_T of the calibrator, β -actin, was subtracted from the C_T of the target cytokine, yielding the ΔC_T value. The amount of the target RNA, relative to the calibrator RNA, was represented as the $2^{-\Delta C_T}$ value, which expressed the quantity of the target RNA as an n -fold difference relative to β -actin RNA.

2.10. Statistics

Statistical analysis of the data was performed using a computer software package, Prism 3 for Macintosh (GraphPad, San Diego, CA). Because of the exponential range of the data, logarithmic transformation was performed for all mRNA levels before the data were analyzed. Since the cytokine/ β -actin ratios were not normally distributed even after logarithmic transformation, the data were analyzed by a non-parametric

method. The Mann–Whitney U -test was used for comparison of cytokine expression between the experimentally sensitized dogs and healthy controls. Significance was set at $P \leq 0.05$.

3. Results

3.1. IDST, *in vitro* antigen-specific IgE test, and lymphocyte stimulation test

The results of the IDST, antigen-specific IgE test, and lymphocyte stimulation test in the 12 sensitized dogs are shown in Table 2. In all of the dogs, obvious reactive wheals (+++) were formed in the IDST using a crude CJ pollen extract. The levels of IgE specific for CJ pollen allergen in all the sensitized dogs were very high, ranging from 608 to 18 217 U/ml (Mean, 4304; S.D., 5505). The results of the lymphocyte stimulation test to CJ pollen allergen (represented as SI) ranged from 1.05 to 6.07 (Mean, 2.27; S.D., 1.40). Positive values >2.0 were seen in six of the 12 dogs. The SI values in the lymphocyte stimulation test to CJ pollen extract in all of the six control dogs were smaller than 2.0.

3.2. mRNA expression of various cytokines in the primary PBMC

Spontaneous cytokine mRNA expression was examined in the primary PBMC samples frozen just

Table 2
Results of IDST, antigen-specific IgE test, and lymphocyte stimulation test for CJ pollen antigen

Dog number	IDST	Antigen-specific IgE (U/ml)	Lymphocyte stimulation test (SI)
1	+++	3428	1.12
2	+++	18217	1.59
3	+++	2072	2.49
4	+++	1089	6.07
5	+++	1910	2.70
6	+++	13289	2.45
7	+++	2514	1.05
8	+++	2998	1.32
9	+++	2332	1.81
10	+++	1420	3.30
11	+++	608	2.15
12	+++	1769	1.13

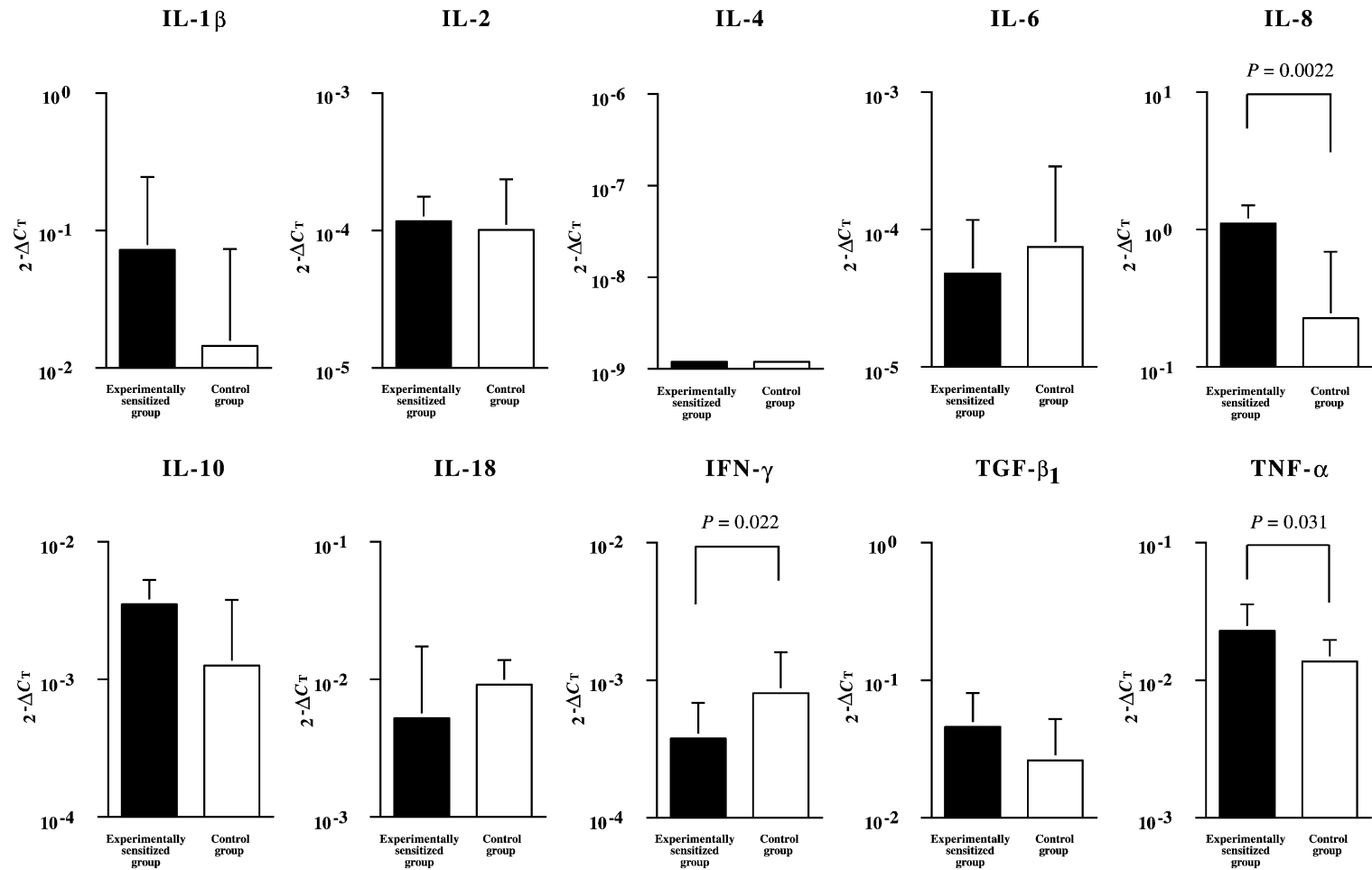


Fig. 1. Levels of mRNA expression of various cytokines in the primary PBMC from dogs experimentally sensitized to CJ pollen extract and control dogs. The amounts of mRNA are expressed relative to the amount of β -actin mRNA in each sample and are shown as the mean $\pm$ S.D. values. Black columns, experimentally sensitized group; white columns control group. Significant differences between the groups are expressed as P-values.

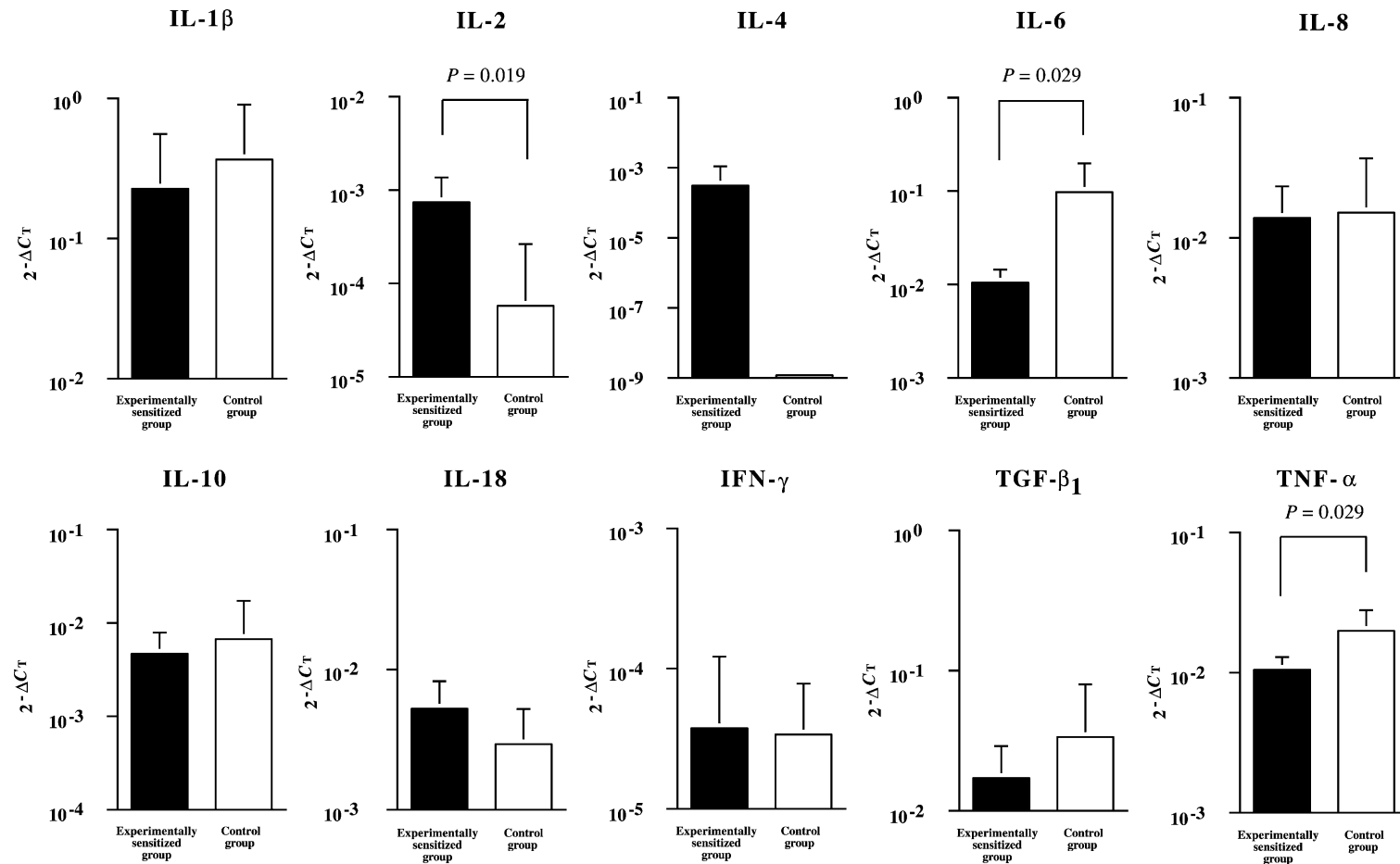


Fig. 2. Levels of mRNA expression of various cytokines in the CJ pollen antigen-stimulated PBMC from dogs experimentally sensitized to CJ pollen extract and control dogs. The amounts of mRNA are expressed relative to the amount of β -actin mRNA in each sample and are shown as the mean $\pm$ S.D. values. Black columns, experimentally sensitized group; white columns, control group. Significant differences between the groups are expressed as P-values.

after separation, and the cytokine profiles in the experimentally sensitized dogs were compared to those in the control dogs (Fig. 1). The amount of IL-4 mRNA was below the lower detection limit in all of the sensitized dogs and control dogs. IL-8 and TNF- α mRNA levels in the experimentally sensitized dogs were significantly higher ($P = 0.0022$ and 0.031 , respectively) than those in the control dogs. The IFN- γ mRNA level in the sensitized dog group was significantly lower than that in the control group ($P = 0.022$).

3.3. mRNA expression of various cytokines in PBMC stimulated with sensitizing antigen

The amounts of cytokine mRNAs in the PBMC stimulated with CJ pollen antigen for 24 h were compared between the experimentally sensitized dog group and the control dog group (Fig. 2). Since the amounts of RNA isolated from PBMC were not sufficient to measure the mRNA levels of all the cytokines, we used four to five samples from six experimentally sensitized dogs (dog numbers 9, 10, 11, and 12) and four samples from control dogs. The results in lymphocyte stimulation test in the six dogs used for the measurement of cytokine mRNA were not significantly different from those in other six sensitized dogs.

After stimulation with the crude CJ pollen antigen, the level of IL-2 mRNA in the PBMC from the experimentally sensitized dogs was significantly higher than that in the PBMC from the control dogs ($P = 0.019$). Meanwhile, the levels of IL-6 and TNF- α mRNA in the PBMC from the sensitized dogs were significantly lower than those in the PBMC from the control dogs ($P = 0.029$, both). The amount of IL-4 mRNA in the PBMC from the control dogs was below the lower detection limit in all of the four samples. However, the amount of IL-4 mRNA in the PBMC from the experimentally sensitized dogs was found to be markedly increased after stimulation with the crude CJ pollen extract, whereas it was below the lower detection limit after stimulation with a crude *D. pteronyssinus* extract as an unrelated antigen.

4. Discussion

In this study, we characterized the cytokine profiles of PBMC from dogs experimentally sensitized to CJ

pollen. Although the dogs experimentally sensitized with CJ pollen extract were shown to have CJ pollen antigen-specific IgE in the sera and give a positive reaction to crude CJ pollen antigen in IDST, they did not show clinical signs consistent with CJ pollinosis. However, in our preliminary study, these experimentally sensitized dogs showed bronchial constriction after airway exposure to CJ pollen antigen. Moreover, in the present study, the PBMC from the experimentally sensitized dogs showed markedly increased expression of IL-4 after stimulation with the CJ pollen extract in culture. A study on human CJ pollinosis showed that the serum level of IL-4 protein in the pollinosis patients was higher than that in control individuals even in the off-season of CJ pollination (Urushibata et al., 1996; Masamoto et al., 1999). These findings suggested that the present model system for experimental CJ pollen sensitization in the dog may reflect some of the immunological reactions observed in spontaneous CJ pollinosis in humans.

In the present study, the results of the IDST and antigen-specific IgE test in the 12 sensitized dogs were negative before the experimental sensitization and became positive after the sensitization. These observations were similar to those of our previous study (Yamashita et al., 2000). Although only six of the 12 sensitized dogs showed positive response on the lymphocyte stimulation test, the results of the IDST and antigen-specific IgE test clearly showed that the 12 dogs were “sensitized” to CJ pollen antigen after the experimental sensitization.

In this study, we found the increased expression of IL-8 and TNF- α mRNAs in the primary PBMC from the sensitized dogs compared to the control dogs. Although it was not significant, spontaneous IL-1 β mRNA expression showed a tendency to be increased in the experimentally sensitized dogs ($P = 0.051$). These three cytokines belong to the proinflammatory cytokines produced primarily by monocytes and macrophages. In humans, significantly increased IL-8 production was seen in the nasal mucosa of patients with allergic rhinitis after allergen provocation (Klein-Jan et al., 1999). Moreover, a study on human AD also showed that PBMC from human AD patients produced increased levels of IL-8 and TNF- α (Bunikowski et al., 2001). IL-6 is another proinflammatory cytokine, but its expression level in the primary PBMC was not increased in the dogs experimentally sensitized with CJ

pollen extract in this study. These findings showing the increased expression of IL-1 β , IL-8 and TNF- α were conceivably due to the activation of monocytes and macrophages by the experimental immunization with alum-absorbed CJ pollen extract in this canine model. The levels of IL-6 and TNF- α mRNAs in the PBMC from the sensitized dogs were lower than those in the PBMC from the control dogs after stimulation with the CJ pollen extract in culture. The suppression of IL-6 and TNF- α expression might be an effect of IL-4 secreted after stimulation with CJ pollen extract, since IL-4 is known to have an suppressive effect on these proinflammatory cytokines (Donnelly et al., 1991; Hart et al., 1989; Wertheim et al., 1993; Hsu et al., 1992; Standiford et al., 1990; te Velde et al., 1990; Morita et al., 2001).

The spontaneous expression level of IFN- γ in the primary PBMC was significantly decreased in the experimentally sensitized dogs. This observation is consistent with the findings showing Th2 polarization in human pollinosis (Masamoto et al., 1999). Similar results showing decreased IFN- γ mRNA expression in the absence of IL-4 mRNA expression in primary PBMC were reported in human AD (Katagiri et al., 1997). After stimulation with CJ pollen extract, the expression level of IFN- γ mRNA in the PBMC from the sensitized dogs was similar to that in the PBMC from the control dogs. A number of reports on human AD have demonstrated decreased IFN- γ production after antigen or mitogen stimulation (Motoki et al., 1997; Jujo et al., 1992; Poulsen et al., 1995), but these reports were based on the level of protein production. Moreover, some reports showed increased IFN- γ mRNA expression together with reduced IFN- γ secretion in PBMC of patients with AD, suggesting a post-transcriptional defect of IFN- γ production (Tang et al., 1994; Koning et al., 1997). Other reports showed that IFN- γ mRNA expression (Nakai et al., 2000) or IFN- γ production (Tsuda et al., 1998) in PBMC after stimulation did not differ between patients with pollinosis and controls, as also shown in patients with bronchial asthma (Kimura et al., 1999). Since decreased IFN- γ mRNA expression after antigen stimulation might be specific for AD and not be generally found in pollinosis, the findings observed in this canine model might be similar to those in human pollinosis. Further studies to compare the mRNA expression with the protein production are needed to make a complete interpretation.

IL-4 is mainly synthesized by activated lymphocytes. It has immunoregulatory and anti-inflammatory activities and acts as an isotype switch factor for the induction of IgE synthesis (Lebman and Coffman, 1988). IL-4 mRNA expression of PBMC was found to be increased in human pollinosis after stimulation with major allergens (Nakai et al., 2000; Kakinoki et al., 2000), and a similar result was shown in the present study. IL-4 protein was also detected in the culture supernatants of PBMC from humans with pollinosis when the PBMC were cultured in the presence of CJ pollen antigen (Kimura et al., 2001). The increased expression of IL-4 mRNA in the present study was shown to be induced by CJ pollen extract but not by an unrelated allergen. This finding indicated the presence of antigen-specific Th2 cells in the PBMC of the dogs experimentally sensitized with CJ pollen extract.

IL-2 is known to have a role in the process of B cell proliferation and differentiation. In the present study, expression of IL-2 mRNA in the PBMC from the sensitized dogs was increased after stimulation with the sensitizing antigen. Because both Th1 and Th2 cells proliferate in response to IL-2 (Mosmann and Coffman, 1989) and the results of the lymphocyte stimulation test showed a T cell proliferative response to CJ pollen antigen, the increased expression of IL-2 mRNA might be associated with proliferation specific to the sensitizing antigen. There have been several reports on the production of IL-2 in human AD, and the results are controversial. Some reports showed increased IL-2 production by PBMC stimulated with the sensitizing antigen (Morita et al., 1990; Agata et al., 1992). There was also a report about cytokine profiles in atopic asthmatic patients; increased production of IL-2 by PBMC from these patients stimulated with rye grass pollen was seen (Tang et al., 1996). On the other hand, some reports demonstrated decreased production of IL-2 by activated T cells in AD (Kapp et al., 1989; Yamamoto et al., 1997). The reason for these differences might be due to differences of the methods used or the patient populations examined.

In the present study, the cytokine profiles of PBMC were measured using a real-time sequence detection system in dogs experimentally sensitized to CJ pollen. Our data indicated that there was a Th2 response to the sensitizing antigen in this canine model, as indicated

by the generation of antigen-specific Th2 cells. In addition, the mRNA expression of proinflammatory cytokines was shown to be increased in the primary PBMC from the CJ pollen-sensitized dogs, possibly due to the activation of monocytes and macrophages. Examining the cytokine profiles in this canine model will provide information useful for understanding the immunopathological mechanism of CJ pollinosis. In addition, recognizing these cytokine profiles may lead to the identification of targets for therapeutic intervention for management of the disease.

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