

Allergen-specific immunotherapy induces Th1 shift in dogs with atopic dermatitis

Masayuki Shida, Michiyo Kadoya, Seong-Jun Park, Koji Nishifuji,
Yasuyuki Momoi, Toshiroh Iwasaki*

*Department of Veterinary Internal Medicine, Faculty of Agriculture, Tokyo University of
Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183-8509, Japan*

Received 13 May 2003; received in revised form 14 May 2004; accepted 4 June 2004

Abstract

Allergen-specific immunotherapy has been applied to canine atopic dermatitis. Despite the accumulated clinical evidence of its effect for atopic dogs, the basic immunologic mechanisms were not fully understood. In this study, the cytokine profile *ex vivo* in canine atopic dermatitis before and after allergen-specific immunotherapy was characterized using competitive reverse transcription–polymerase chain reaction (RT–PCR). Blood samples were collected from 10 dogs with atopic dermatitis and peripheral blood mononuclear cells were stimulated with house dust mite antigen. The levels of IFN- γ and IL-4 mRNA were lower in atopic dogs compared with non-atopic controls. The ratio of IFN- γ /IL-4 was low in atopic dogs indicating a cytokine profile polarized to Th2. The level of IFN- γ after immunotherapy was significantly higher than that before ($P < 0.05$) whereas that of IL-4 mRNA was not changed. Consequently, the ratio of IFN- γ /IL-4 after immunotherapy was significantly higher than that before immunotherapy ($P < 0.05$). These results indicate a Th2 cytokine bias is the dominant state in atopic dogs and allergen-specific immunotherapy causes a shift to wards a Th1 bias by enhancing IFN- γ expression.

© 2004 Elsevier B.V. All rights reserved.

Keywords: Dog; Atopic dermatitis; Allergen-specific immunotherapy; IFN- γ ; IL-4

1. Introduction

Atopic dermatitis is a common disease in dogs (Scott *et al.*, 2001) and human (Rothe and Grant-Kels, 1996). Canine atopic dermatitis has many characteristics that have counterparts in humans: familial occurrence, early age of onset, chronic pruritus, skin lesions typically located at flexor and extensor sites of extre-

mities, and presence of immediate skin test reactivity (Rhodes *et al.*, 1987a,b). Diagnosis of canine atopic dermatitis is based on history and clinical signs, exclusion of other pruritic dermatoses, and the fulfillment of the diagnostic criteria proposed by Willemse (1986). Although pathogenesis of canine atopic dermatitis was not fully understood, association with modulated cytokine balance to Th2 has been reported: overproduction of IL-4 mRNA in lesional infiltrated lymphocyte (Nuttall *et al.*, 2002), and low expression level of IFN- γ mRNA in peripheral blood mononuclear cells (Hayashiya *et al.*, 2002). These results also confirm human atopic conditions. They indicate that

* Corresponding author. Tel.: +81-42-367-5777;
fax: +81-42-367-5777.
E-mail address: bpag2180@cc.tuat.ac.jp (T. Iwasaki).

Th2 dominant state is related to the pathogenesis of canine atopic dermatitis.

Approximately 80% of dogs with atopic dermatitis develop non-seasonal clinical signs that usually require long-term therapy (Scott et al., 2001). Therapeutic options for canine atopic dermatitis include avoidance of sensitized antigen, management of secondary complicating factors such as bacterial or yeast infections, topical therapy, systemic antipruritic drugs such as antihistamine, essential fatty acid as well as glucocorticoids (Reedy et al., 1997). Allergen-specific immunotherapy is a therapeutic option that is chosen when intense pruritus is present throughout the year, when topical therapy is ineffective, or when unacceptable side effects appears by glucocorticoid therapy. In immunotherapy, sensitized antigen(s) are injected repeatedly at gradually increasing doses (Bonifazi et al., 1991; Norman, 1998). In canine atopic dermatitis, sensitized allergens can be identified either through intradermal skin testing or detecting serum allergen-specific IgE (Reedy et al., 1997). House dust mite (HDM) antigen is the most common sensitized antigen in canine atopic dermatitis, but various antigens can induce canine atopic dermatitis (Zunic, 1998). Immunotherapy has been applied for the treatment of canine atopic dermatitis for nearly 50 years. Recent studies have reported that remission of clinical signs can be achieved in ca. 60–70% of canine atopic dermatitis cases (Baker, 1967; Halliwell, 1977; Scott, 1981; Willemse et al., 1984). Despite the cumulative clinical evidence of the effect of immunotherapy in both human and dogs, basic immunologic mechanisms remain unclear. Several changes in immune reaction to allergens have been documented in human medicine (Benjaponpitak et al., 1999; Ebner et al., 1997; Jutel et al., 1995; Lu et al., 1998; Moverare et al., 2000; O'Brien et al., 1997; Pumhirun et al., 1994; Theodoropoulos and Lockey, 2000): decreased number of inflammatory cells (particularly mast cells, basophils, and eosinophils), decline of IgE and increase of IgG1 and IgG4 level, and increased production of IFN- γ and reduction of production of IL-4 in T-cell culture. Additionally, in vitro, under the stimulation of high concentration of antigen, Th cells could not differentiate but activate, and will result in unresponsiveness or anergy (Schwartz, 1996). On the other hand, modulation of cytokine balance, which may be the key to pathogenesis of canine atopic

dermatitis, has not been reported. One study has reported increased serum concentration of allergen-specific IgG in dogs (Hites et al., 1989).

This study investigated mechanisms of allergen-specific immunotherapy in atopic dogs sensitized to HDM using competitive PCR to compare Th1 and Th2 cytokine balance before and after immunotherapy.

2. Materials and methods

2.1. Atopic and control subjects

Heparinized blood samples were collected using venipuncture from 10 dogs with atopic dermatitis when they were first admitted to the University Veterinary Clinic at Tokyo University of Agriculture and Technology. Diagnosis of atopic dermatitis was based on their history, clinical signs, exclusion of other pruritic dermatoses, and fulfillment of the diagnostic criteria proposed by Willemse et al. (1984). Their ages ranged from 2 to 7 years (average 3.9 years). They were four males, one castrated male, four females and one spayed female. All atopic subjects showed positive reaction to HDM examined by intradermal skin testing (Table 1). Glucocorticoids and antihistamines were withdrawn at least 2 weeks prior to intradermal skin testing and blood collection. Intradermal skin testing and blood collection were performed on the same day. These dogs were treated with allergen-specific immunotherapy according to protocol reported by Scott et al. (2001). Remission of clinical signs was observed in all cases. Blood samples were collected before and after the dogs received immunotherapy at least for 3 months. As non-atopic control, blood samples were obtained from five healthy, adult mongrel dogs (one male and four females) maintained at our laboratory. Their ages ranged from 4 to 9 years (average 5.6 years). These dogs had neither history nor clinical signs of any skin diseases, nor had they received glucocorticoids or antihistamine. All of these dogs were negative to HDM by intradermal skin testing.

2.2. PBMC isolation and cell culture

PBMC were obtained from the heparinized blood by the density gradient centrifugation with Histopa-

que-1077 (Sigma, St. Louis, MO) (Strasser et al., 1998; Wunderli and Felsburg, 1989). Heparinized blood was diluted in 1.5 volume of PBS. Then 8 ml of the diluted blood was layered onto 3 ml of Histo-paque-1077. This overlaid solution was centrifuged at $400 \times g$ for 40 min at room temperature. Mononuclear cells at the interface were collected and washed twice with PBS at $180 \times g$ for 10 min. The isolated PBMC were suspended in lysis buffer (7.5 g/l ammonium chloride and 0.02 M Tris-HCl buffer, pH 7.2) and shaken gently for 5 min to remove residual red blood cells. Subsequently, PBMC were washed with PBS and counted in a Bürkel-Türk chamber with trypan blue stain. PBMC were suspended at 2×10^6 cells/ml in RPMI 1640 (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (MP Biomedicals, Irvine, CA), 100 µg/ml streptomycin and 100 U/ml penicillin (Gibco Invitrogen Co., Rockville, MD). The 0.5 ml of suspended cells was cultured in the presence of serially diluted HDM antigen (0, 0.01, 0.1, 1, 10, 100 µg/ml; GS Mite Mix; Greer Laboratories Inc., Lenoir, NC) for 4 h at 37 °C in a humidified atmosphere containing 5% CO₂.

2.3. RNA extraction

RNA extraction was carried out using Trizol reagent (Gibco Invitrogen Co.) according to manufacturer's instructions. Briefly, the cultured PBMC containing 2×10^6 cells were collected by centrifugation at $500 \times g$ for 5 min, lysed in 0.5 ml of Trizol reagent, and then stored at -80 °C until further processing. The samples were thawed and incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. The samples were mixed with 0.1 ml of chloroform by vortexing for 15 s, incubated for 3 min at room temperature, and centrifuged at $15,800 \times g$ for 15 min at 4 °C. Following centrifugation, upper aqueous phase was collected and 0.5 ml of isopropyl alcohol was added. Finally, the mixture was centrifuged at $15,800 \times g$ for 10 min at 4 °C to precipitate the RNA. The RNA pellet was rinsed with 75% ethanol and dissolved in 13.5 µl of diethyl pyrocarbonate-treated (DEPC) water. It was then incubated for 10 min at 56 °C. RNA concentration was determined by measuring the optical density at 260 nm with a spectrophotometer (DU 530; Beckman Coulter Inc., Fullerton, CA).

2.4. Reverse transcription-polymerase chain reaction

The RNA was reverse-transcribed to cDNA using a cDNA Cycle Kit (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instruction with a PCR Thermal Cycler MP (Takara Bio Inc., Shiga, Japan). A solution containing 0.05 µg of total RNA (total volume 11.5 µl) and 0.2 µg of oligo dT primer was mixed and incubated at 65 °C for 10 min and then at 20 °C for 2 min. It was added to the RT reaction solution containing 10 U of RNase inhibitor, 1 × RT buffer, 5 mM dNTPs, 4 mM sodium pyrophosphate, and 5 U of avian myeloblastosis virus (AMV) reverse transcriptase in a final volume of 20 µl. The sample was incubated at 42 °C for 60 min and then at 95 °C for 2 min.

2.5. Competitive polymerase chain reaction

Competitive PCR was carried out to determine mRNA expression level of IFN-γ, IL-4 and glyceraldehydes-3-phosphate dehydrogenase (G3PDH) as internal control, using the TaKaRa Taq kit (Takara Bio Inc.) with PCR Thermal Cycler MP. The competitive DNA fragment was produced using a DNA competitor construction kit (Takara Bio Inc.). The 5'- and the 3'-primer sequences were added to each end of competitor. This process was accomplished by PCR using large primers (ca. 40 mers). Competitors were designed to be larger than the native cytokine sequence (Table 2). Reaction mixtures for PCR contained template cDNA synthesized from 0.05 µg of total RNA, 1–10⁸ copies of DNA competitor, 0.2 mM of each dNTP, 0.65 U of Taq DNA polymerase, and 5 pmol of each primer 1 × PCR buffer; the volume was adjusted to 25 µl. PCR primers were purchased from Rikaken Co. Ltd., Nagoya, Japan. Table 2 shows nucleotide sequences of primers used in this. PCR conditions for G3PDH and IL-4 were denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min for 35 cycles, preceded by 2 min denaturation at 94 °C and followed by 10 min extension at 72 °C. PCR conditions for IFN-γ were denaturation at 94 °C for 1 min, annealing at 53 °C for 1 min, extension at 72 °C for 1 min for 40 cycles, preceded by 2 min denaturation at 94 °C and followed by 10 min extension at 72 °C.

2.6. Quantification of PCR products

PCR products and DNA ladder (1 kb DNA Extension Ladder; Gibco Invitrogen Co.) were electrophoresed in 2.5% agarose gel (Agarose LO3 Takara; Takara Bio Inc.) and visualized by ethidium bromide (Bio-Rad Laboratories Inc., Hercules, CA) staining. The visualized PCR products were analyzed with Bio-1D software (Vilber Lourmat, Marne la Vallee, France). Results were expressed as a ratio of optical density readings between the DNA competitor and the target DNA. At the point of equivalence, at which the ratio of competitor to target was 1:1, the amount of the target mRNA in the samples regarded as equal to the input of the competitor. The amount of cytokine mRNA was expressed relative to G3PDH mRNA.

2.7. Statistical analysis

The values of IFN- γ and IL-4 mRNA expression of five control dogs and 10 atopic dogs were analyzed by the Mann–Whitney *U*-test. Changes of cytokine mRNA expression before and after immunotherapy were analyzed by Wilcoxon's rank sum tests. Correlations between IFN- γ and IL-4 mRNA expressions were analyzed by Spearman rank correlation tests. A *P*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Competitive reverse transcription PCR (RT-PCR)

IFN- γ and IL-4 mRNA expression level in PBMC were analyzed by competitive RT-PCR assay. Representative examples of the gel analysis are shown in Fig. 1. The target IFN- γ DNA fragment and its competitor were detected at 367 and 444 bp, respectively (Fig. 1A). The target IL-4 DNA fragment and its competitor were detected at 278 and 340 bp, respectively (Fig. 1B). The target G3PDH DNA fragment and its competitor were detected at 452 and 540 bp, respectively (Fig. 1C). The IL-4 and IFN- γ mRNA levels were determined by competitive RT-PCR.

3.2. Expression of IFN- γ mRNA in PBMC stimulated with HDM antigen

The level of IFN- γ mRNA expression was determined in five control dogs and in 10 atopic dogs before and after immunotherapy. Mean expression levels of 10 atopic dogs and five control dogs are shown in Fig. 2. In control dogs, IFN- γ level increased at higher

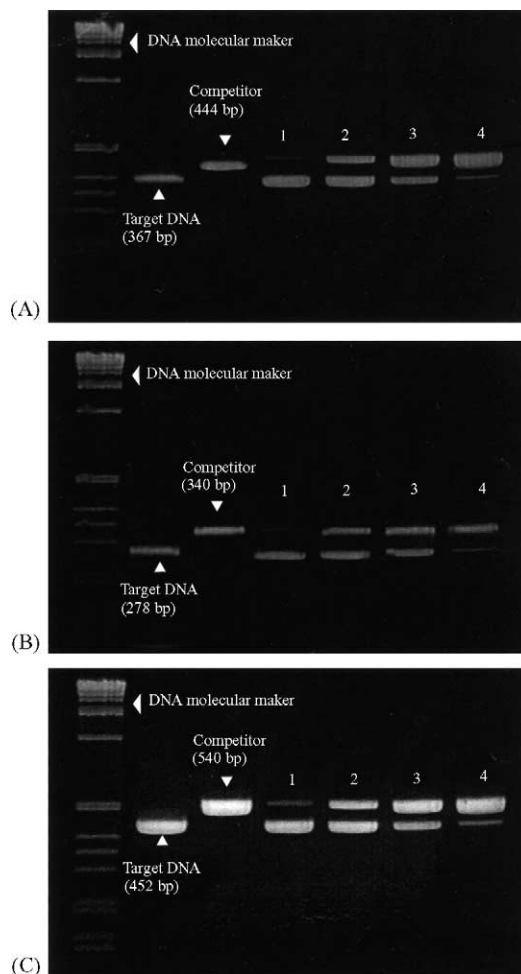


Fig. 1. Competitive RT-PCR assays for cytokine mRNA. Messenger RNA was extracted from PBMC stimulated with 1 μ g/ml of house dust mite antigen. Competitive RT-PCR assays were applied to analyze (A) IFN- γ ; (B) IL-4; and (C) G3PDH mRNA expression level in PBMC. PCR products from target DNA and competitor are indicated with their predicted size. Target DNA synthesized from 0.05 mg of total RNA was amplified with serial 10-fold dilutions of competitor DNA (lane 1, 1 copies; lane 2, 10 copies; lane 3, 102 copies; lane 4, 103 copies).

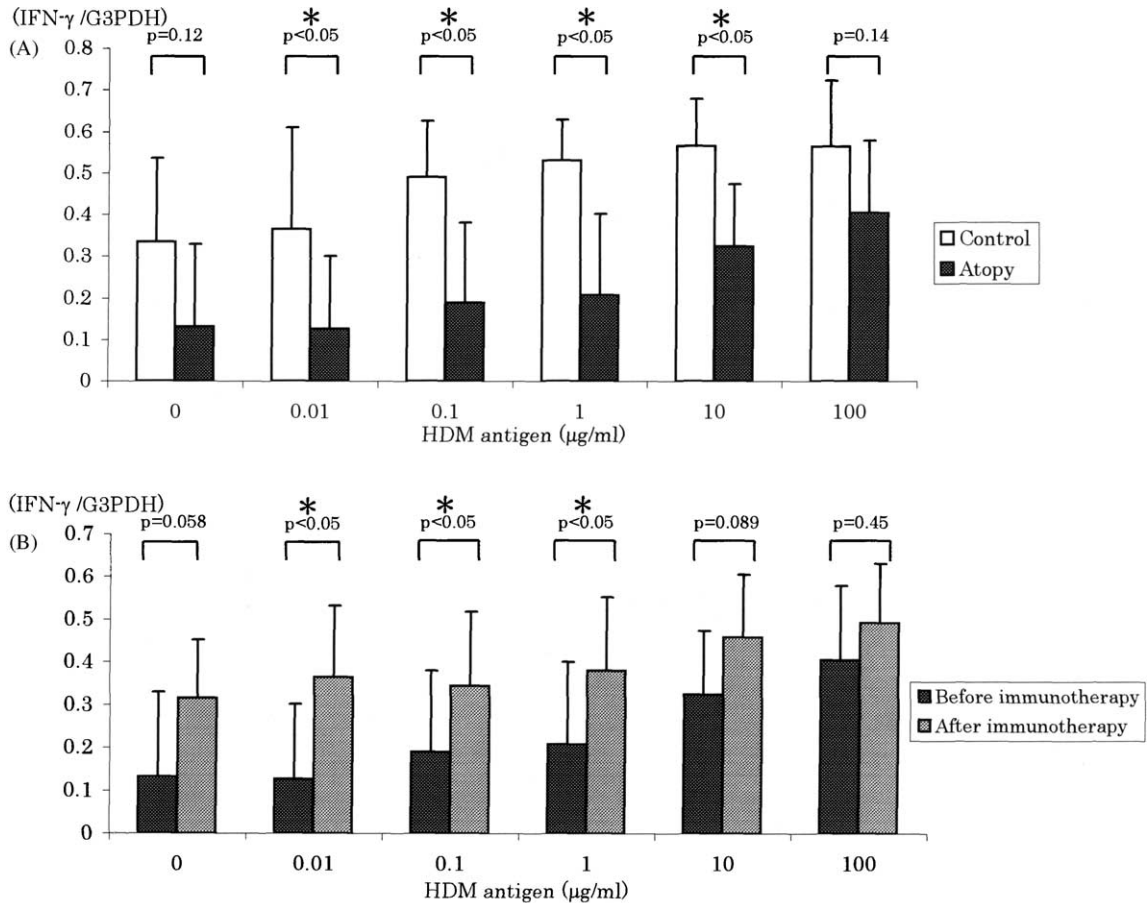


Fig. 2. Expression of IFN- γ mRNA after house dust mite stimulation in control and atopic dogs. Messenger RNA was extracted from PBMC stimulated with serially diluted house dust mite antigen. Competitive RT-PCR were applied to analyze IFN- γ mRNA expression level in PBMC from (A) controls ($n = 5$) and atopic dog (before immunotherapy, $n = 10$) and (B) atopic dog before ($n = 10$) and after immunotherapy ($n = 10$), respectively. Data is presented as mean value of each group. Error bars indicate standard deviations.

concentrations of HDM antigen. In atopic dogs before immunotherapy, expression of IFN- γ was significantly lower than in control dogs at 0.01, 0.1, 1 and 10 $\mu\text{g/ml}$ of HDM antigen concentration ($P < 0.05$, Mann-Whitney U -test). Nevertheless, the level increased gradually at higher concentration. The pattern of IFN- γ expression after immunotherapy was similar to that before immunotherapy: the level of IFN- γ expression gradually increased concomitant with the increased antigen concentration. The level of IFN- γ mRNA expression of after immunotherapy was significantly higher than before immunotherapy in 0.01, 0.1 and 1 $\mu\text{g/ml}$ of HDM antigen ($P < 0.05$, Mann-Whitney U -test).

3.3. Expression of IL-4 mRNA in PBMC stimulated with HDM antigen

The level of IL-4 mRNA expression was determined in five control dogs and in 10 atopic dogs before and after immunotherapy. Fig. 3 shows the mean expression level of 10 atopic dogs (before immunotherapy) and control dogs. Similar to IFN- γ mRNA expression, the level of IL-4 expression increased at higher concentration of HDM antigen in control dogs. The level of IL-4 mRNA of atopic dogs before immunotherapy was significantly lower than that of control dogs at 0.01, 0.1, 1, 10, 100 $\mu\text{g/ml}$ of the antigen concentration. We also compared the

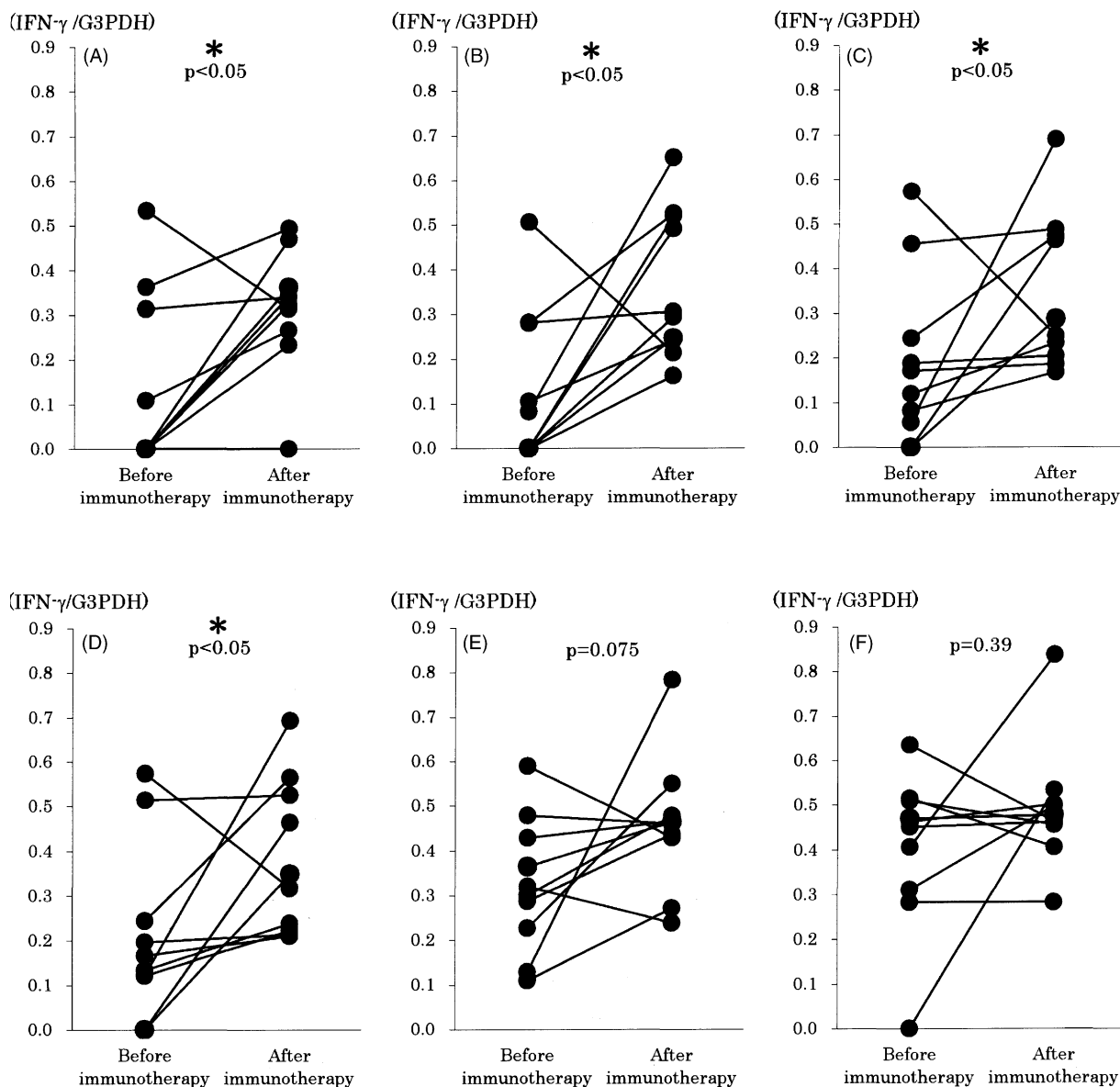


Fig. 3. The level of IFN- γ mRNA expression before and after immunotherapy in individual cases. Blood samples were obtained from 10 atopic dogs before and after immunotherapy. PBMCs were isolated and stimulated (A) without house dust mite antigen; (B) with 0.01 $\mu\text{g/ml}$; (C) 0.1 $\mu\text{g/ml}$; (D) 1 $\mu\text{g/ml}$; (E) 10 $\mu\text{g/ml}$; and (F) 100 $\mu\text{g/ml}$ of house dust mite antigen to analyze IFN- γ mRNA expression level by competitive RT-PCR.

level of IL-4 mRNA expression before and after immunotherapy in 10 atopic dogs; no significant difference was observed statistically at any concentration (Fig. 3).

3.4. The ratio of IFN- γ /IL-4 mRNA expression

To evaluate Th1 and Th2 cytokine balance, the value was expressed as the ratio of IFN- γ /IL-4 mRNA

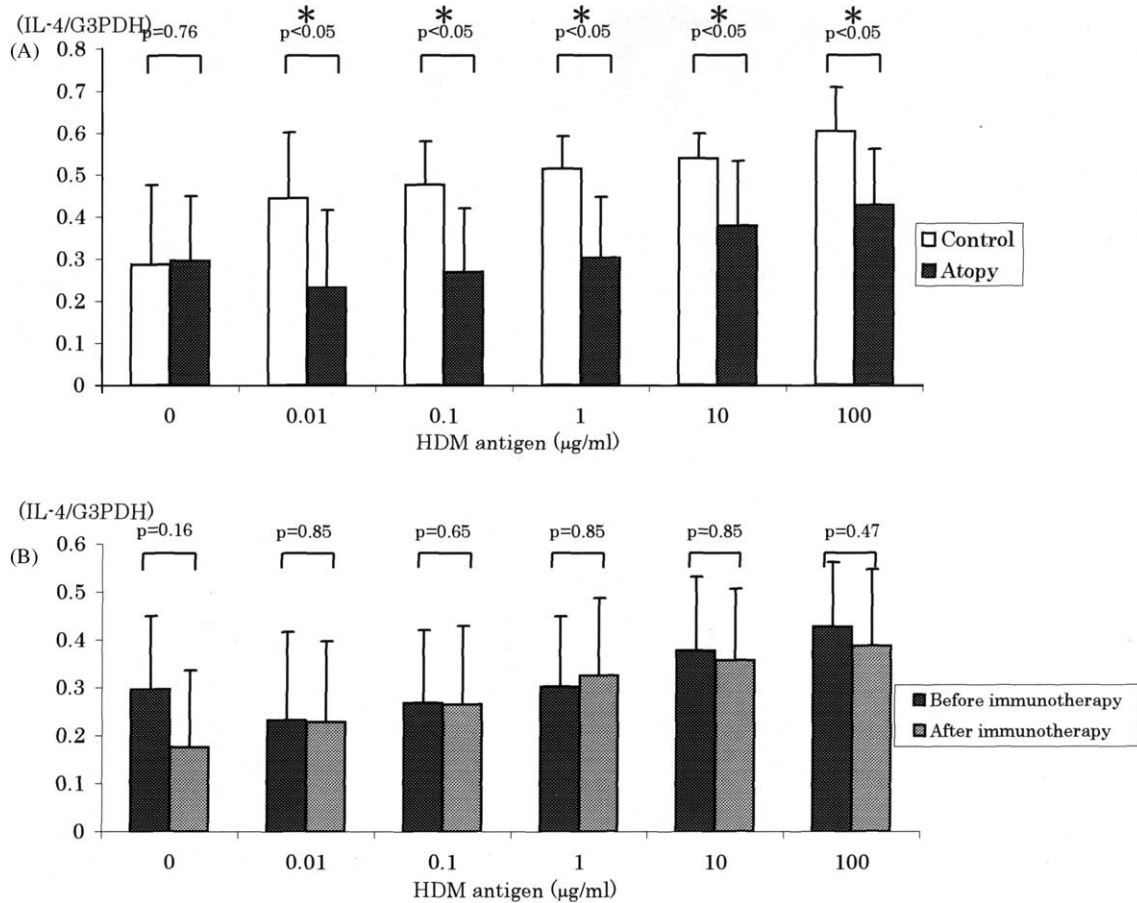


Fig. 4. Expression of IL-4 mRNA after house dust mite stimulation in control and atopic dogs. Messenger RNA was extracted from PBMC stimulated with serially diluted house dust mite antigen. Competitive RT-PCR were applied to analyze IL-4 mRNA expression level in PBMC from (A) controls ($n = 5$) and atopic dog (before immunotherapy, $n = 10$) and (B) atopic dog before ($n = 10$) and after immunotherapy ($n = 10$), respectively. Data is presented as mean value of each group. Error bars indicate standard deviations.

expression at the concentration of HDM antigen 0.1–100 µg/ml, where IL-4 mRNA was detected in all cases. Fig. 4 shows the ratio of 10 atopic dogs and of five control dogs. No statistically significant difference was seen between atopic dogs before immunotherapy and control dogs at any concentration. The ratios of 10 atopic dogs were also compared before and after immunotherapy (Fig. 4). The ratio after immunotherapy was significantly higher than that before immunotherapy at all HDM concentration ($P < 0.05$).

Increase the ratio of IFN- γ /IL-4 mRNA expression was observed in 9 of 10 cases at 0.1, 1, 10 µg/ml and 7 of 10 cases in 100 µg/ml of HDM antigen stimulation.

3.5. Correlation of IFN- γ and IL-4 mRNA expression

Fig. 5 depicts the correlation between IFN- γ and IL-4 mRNA expression in atopic dogs. Slopes of a correlation coefficient lines were almost constant after immunotherapy. In contrast, correlation coefficient lines before immunotherapy tend to approach that after immunotherapy at higher concentration. The cytokine balance shifted from Th2 to Th1 dominant state after immunotherapy. At higher concentration, however, PBMCs tended to showed Th0 status, whereby they express both IFN- γ and IL-4 mRNA (Figs. 6–8).

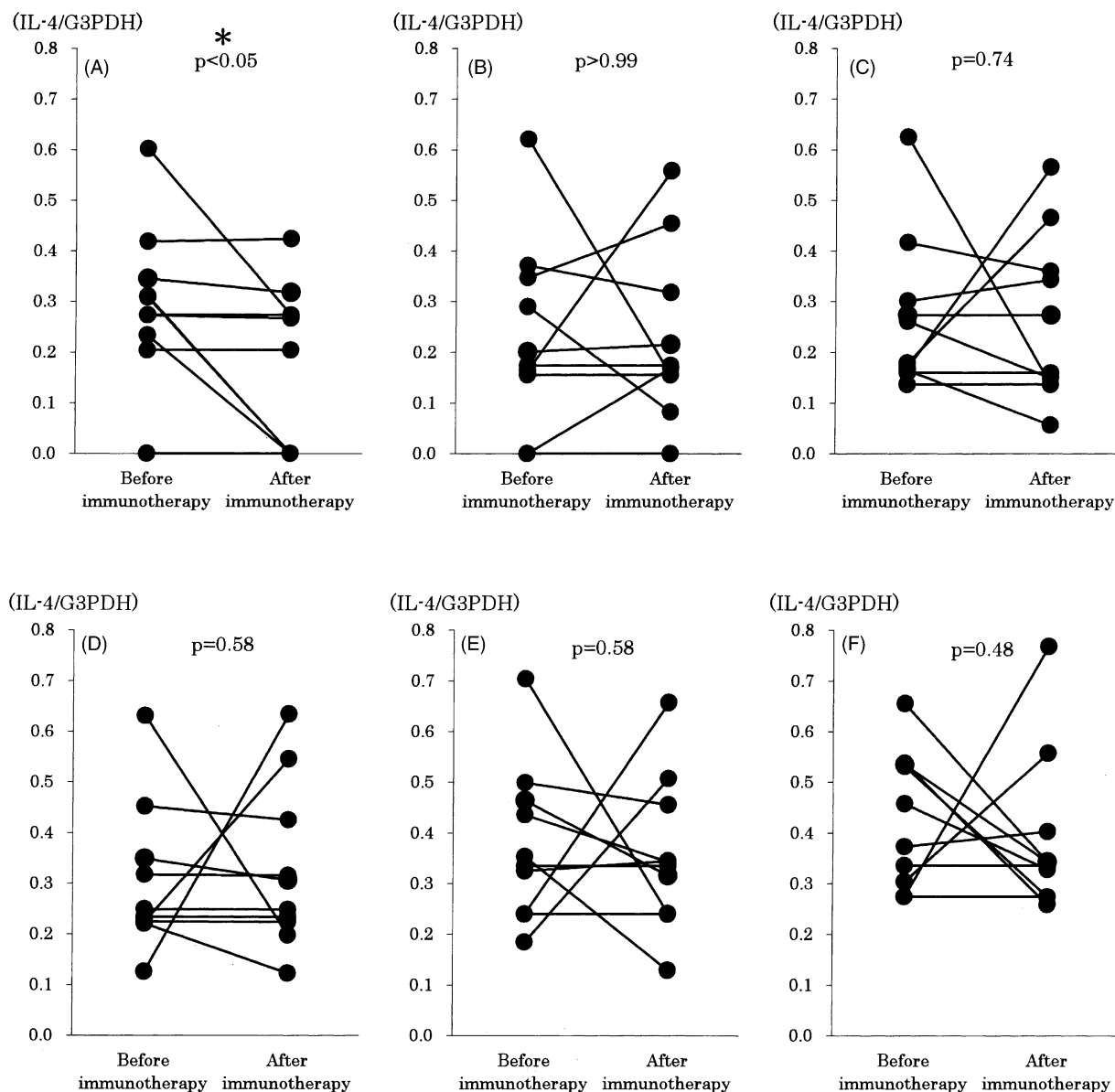


Fig. 5. The level of IL-4 mRNA expression before and after immunotherapy in individual cases. Blood samples were obtained from 10 atopic dogs before and after immunotherapy. PBMCs were isolated and stimulated (A) without house dust mite antigen; (B) with 0.01 $\mu\text{g/ml}$; (C) 0.1 $\mu\text{g/ml}$; (D) 1 $\mu\text{g/ml}$; (E) 10 $\mu\text{g/ml}$; and (F) 100 $\mu\text{g/ml}$ of house dust mite antigen to analyze IL-4 mRNA expression level by competitive RT-PCR.

Spearman rank correlation tests showed a positive correlation existed between the level of IFN- γ and IL-4 mRNA expression at high concentration of HDM antigen (10 and 100 $\mu\text{g/ml}$).

4. Discussion

This study characterized the ex vivo cytokine profile in canine atopic dermatitis before and after

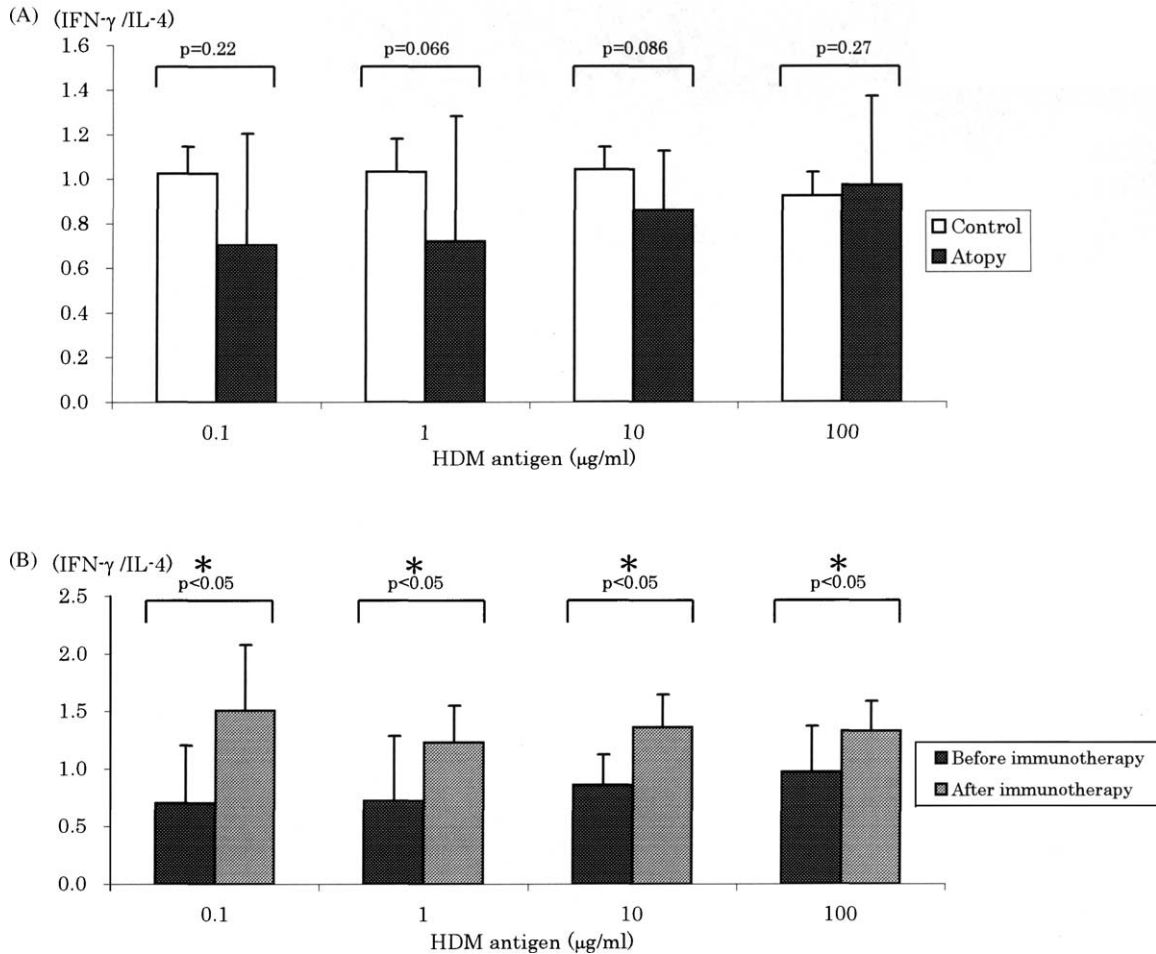


Fig. 6. The ratio of IFN- γ to IL-4 mRNA expression in control and atopic dogs. Messenger RNA was extracted from PBMC stimulated with serially diluted house dust mite antigen. The level of IFN- γ and IL-4 mRNA expression was determined by competitive RT-PCR. (A) The ratio of IFN- γ /IL-4 in control dogs ($n = 5$) and atopic dogs before immunotherapy ($n = 10$). (B) The ratio of IFN- γ /IL-4 in atopic dogs before ($n = 10$) and after immunotherapy ($n = 10$). Data is presented as mean value of each group. Error bars indicate standard deviations.

allergen-specific immunotherapy using competitive RT-PCR established for canine IFN- γ and IL-4 mRNA expression. This is the first study to examine the mechanism of immunotherapy for canine atopic dermatitis on cytokine expression before and after therapy in the same dogs.

Results revealed that IFN- γ and IL-4 mRNA level, with or without HDM stimulation, tended to be lower in dogs with atopic dermatitis compared with non-atopic control dogs. We found no mechanism of the low-level expression in atopic dogs. Notwithstanding, one possible explanation is that PBMC from atopic dogs poorly differentiated to produce cytokines.

Schwartz (1996) reported that, Th cells could not differentiate under stimulation of high concentration of antigen, but that they could activate, engendering lack of response, or anergy. In human medicine, many studies demonstrated that IL-4 production and mRNA expression were increased in PBMC from human atopic patients stimulated with antigen or mitogen (Byron et al., 1994; Esnault et al., 1996; Essayan et al., 1996; Kimura et al., 1998). The low level of IL-4 expression in dogs with atopic dermatitis, especially at low antigen concentration, contrasted to that reported in humans (Secrist et al., 1995). We infer that this difference on of IL-4 expression may reflect

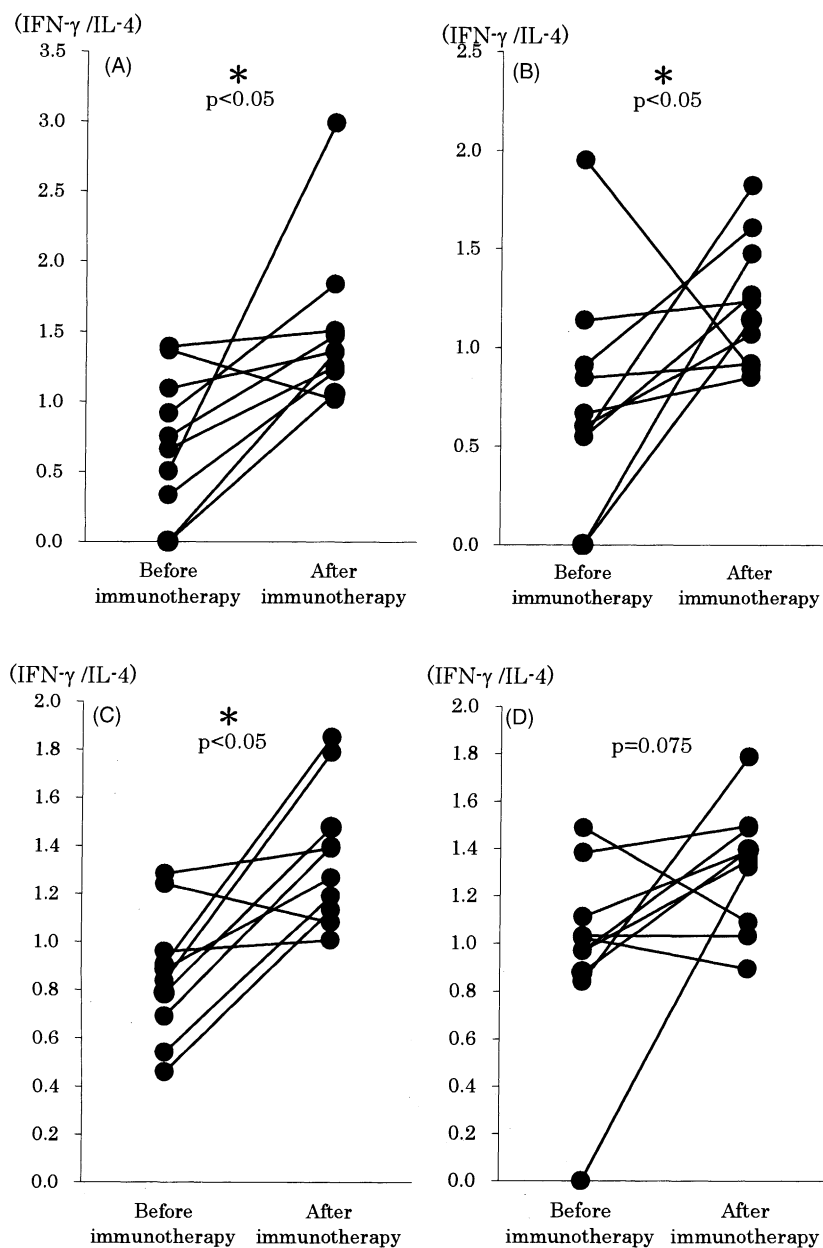


Fig. 7. The ratio of IFN- γ to IL-4 mRNA expression before and after immunotherapy in individual cases. Blood samples were obtained from 10 atopic dogs before and after immunotherapy. PBMCs were isolated and stimulated (A) with 0.1 $\mu\text{g/ml}$; (B) 1 $\mu\text{g/ml}$; (C) 10 $\mu\text{g/ml}$; and (D) 100 $\mu\text{g/ml}$ of house dust mite antigen to analyze IFN- γ mRNA expression level by competitive RT-PCR.

clinical-pathological feature of atopic dermatitis in both species. Significant eosinophilia, which closely related to IL-5 produced by Th2 cells (Lopez et al., 1988), is often observed in human patients with atopic dermatitis (Calbi et al., 1993; Kiehl et al., 2001), but it

is not common in atopic dogs (Scott et al., 2001). Total serum IgE levels in human atopic patients are 10–100 times higher than in atopic dogs (Byron et al., 1994; Esnault et al., 1996; Kikkawa et al., 1994; Olivry et al., 1996). The ratio of IFN- γ /IL-4 mRNA expression was

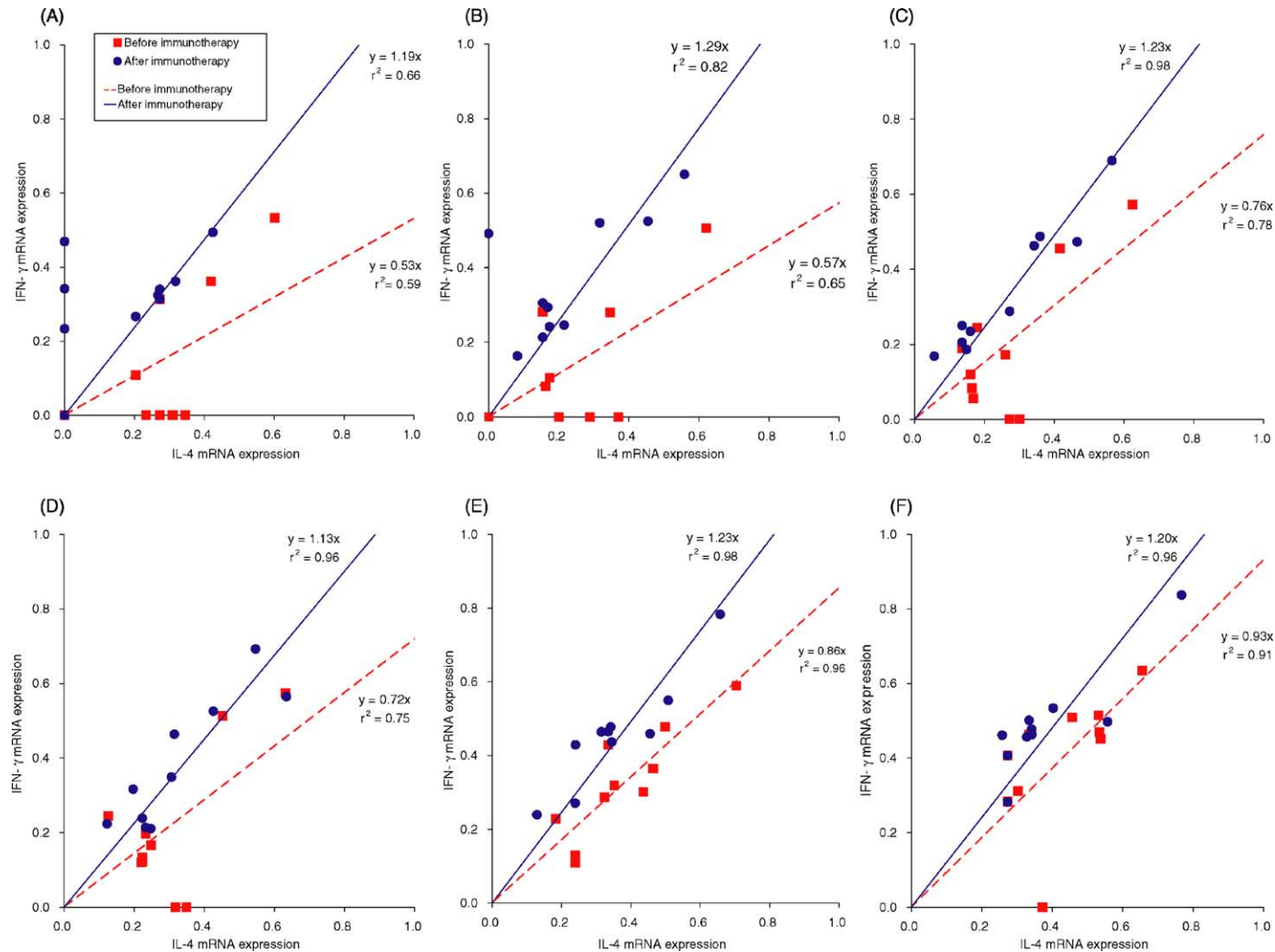


Fig. 8. Dot blot analysis of IFN- γ and IL-4 mRNA expression in PBMC from atopic dogs before and after immunotherapy stimulated with serially diluted house dust mite antigen. PBMCs were isolated from 10 atopic dogs before and after immunotherapy. The level of IFN- γ and IL-4 mRNA expression in PBMC stimulated (A) without house dust mite antigen; (B) with 0.01 $\mu\text{g/ml}$; (C) 0.1 $\mu\text{g/ml}$; (D) 1 $\mu\text{g/ml}$; (E) 10 $\mu\text{g/ml}$; and (F) 100 $\mu\text{g/ml}$ of house dust mite antigen were measured by competitive PCR. The levels of mRNA expression in individual cases before immunotherapy (closed squares) and after immunotherapy (closed circles) are indicated. Broken line and solid line were regression lines calculated from the data before and after immunotherapy, respectively.

also low, indicating that cytokine profile in atopic dogs polarized to Th2. This result concurs with previous studies on lymphocytes infiltrated in the skin lesions (Nuttall et al., 2002; Olivry et al., 1999) and non-stimulated PBMCs in atopic dogs (Hayashiya et al., 2002). Therefore, we infer that not absolute value of cytokines, but relative value (cytokine balance) is related to the pathogenesis of canine atopic dermatitis.

IFN- γ and IL-4 mRNA expression of atopic and control dogs were slightly increased with the rise of antigen stimulus concentration, which agrees with the experiment using PBMC of human atopic patients (Gabrielsson et al., 1997). HDM allergen is a ubiquitous allergen; most non-atopic dogs must be exposed to the antigen. PBMC isolated from control dogs may include lymphocytes, which respond to HDM.

This study also revealed that the IFN- γ expression increased in dogs after immunotherapy, but that the level of IL-4 did not change. As a result, the ratio of IFN- γ /IL-4 increased significantly after immunotherapy. It is suggested that immunotherapy promotes differentiation to Th1 without influence on Th2 activities and induces a shift to Th1 dominant state. In human medicine, the effect of allergen-specific immunotherapy on cytokine profiles is controversial: immunotherapy reduced IL-4 production, but had no effect on IFN- γ secretion (Benjaponpitak et al., 1999), whereas other reports showed an increase in IFN- γ production (Jutel et al., 1995; Varney et al., 1993). This discrepancy may result from the differences of experimental method. However, all studies indicated that immunotherapy caused cytokine balance modulation to Th1. Consequently, our results and previous studies of humans presumed that a shift of Th1/Th2 cytokine balance is a crucial event that brings clinical improvement for both dogs and humans.

Regression curve slopes were almost constant at any HDM concentration after immunotherapy. In contrast, regression curves before immunotherapy indicated Th2 dominant state at low HDM concentration, and a shift to Th1 in a dose dependent manner. These results indicate that atopic dogs show Th2 dominant response when they are exposed to low concentration of antigen, as in natural environment. In such cases, allergen-specific immunotherapy may alter the responsiveness to Th1 dominant reaction to the same antigen stimulation.

In conclusion, this study elucidates the cytokine profile in canine atopic dermatitis. In atopic dogs, cytokine balance is the Th2 dominant state. Allergen-specific immunotherapy induces the increasing of IFN- γ level and it produces a shift to the Th1 dominant state. In dogs, modulation to a Th1 dominant state leads improvement of clinical signs, so administration of Th1 cytokine such as IFN- γ , IL-12 and IL-18 may also be useful for treatment of atopic dermatitis. Additionally, better understanding of immunotherapy mechanisms may lead to new treatment strategies in canine atopic dermatitis.

References

- Baker, E., 1967. Management of allergic disease by hyposensitization. *J. Am. Vet. Med. Assoc.* 154, 491–494.
- Benjaponpitak, S., Oro, A., Maguire, P., Marinkovich, V., DeKruyff, R.H., Umetsu, D.T., 1999. The kinetics of change in cytokine production by CD4 T cells during conventional allergen immunotherapy. *J. Allergy Clin. Immunol.* 103, 468–475.
- Bonifazi, F., Antonicelli, L., Bilo, M.B., Pucci, S., Taylor, I.H., Wheeler, A.W., Youlten, L.J., 1991. Hyposensitization therapy of *Parietaria*-sensitive patients with a tyrosine adsorbed allergoid, Pollinex *Parietaria* (*Bencard Parietaria*). *J. Invest. Allergol. Clin. Immunol.* 1, 37–44.
- Byron, K.A., O'Brien, M.R., Varigos, G.A., Wootton, A.M., 1994. *Dermatophagoides pteronyssinus* II-induced interleukin-4 and interferon- γ expression by freshly isolated lymphocytes of atopic individuals. *Clin. Exp. Allergy* 24, 878–883.
- Calbi, M., Scarpellino, B., Giacchetti, L., 1993. Usefulness of neonatal eosinophil counts as a marker of atopy? *Pediatr. Allergy Immunol.* 4, 86–88.
- Ebner, C., Siemann, U., Bohle, B., Willheim, M., Wiedermann, U., Schenk, S., Klotz, F., Ebner, H., Kraft, D., Scheiner, O., 1997. Immunological changes during specific immunotherapy of grass pollen allergy: reduced lymphoproliferative responses to allergen and shift from TH2 to TH1 in T-cell clones specific for Phl p 1, a major grass pollen allergen. *Clin. Exp. Allergy* 27, 1007–1015.
- Esnault, S., Benbernou, N., Lavaud, F., Shin, H.C., Potron, G., Guenounou, M., 1996. Differential spontaneous expression of mRNA for IL-4, IL-10, IL-13, IL-2 and interferon-gamma (IFN- γ) in peripheral blood mononuclear cells (PBMC) from atopic patients. *Clin. Exp. Immunol.* 103, 111–118.
- Essayan, D.M., Han, W., Li, X., Xiao, H., Kliene-Tebbe, J., Huang, S., 1996. Clonal diversity of IL-4 and IL-13 expression in human allergen-specific T lymphocytes. *J. Allergy Clin. Immunol.* 98, 1035–1044.
- Gabrielsson, S., Paulie, S., Rak, S., Lagging, E., Van Hage-Hamsten, M., Harfast, B., Troye-Blomberg, M., 1997. Specific induction of interleukin-4-producing cells in response to in

- vitro allergen stimulation in atopic individuals. *Clin. Exp. Allergy* 27., 808–815.
- Halliwell, R.E.W., 1977. Hyposensitization in the treatment of atopic disease. In: Kirk, R.W. (Ed.), *Current Veterinary Therapy*, vol. IV. Saunders, Philadelphia, PA, pp. 537–541.
- Hayashiya, S., Tani, K., Morimoto, M., Hayashi, T., Hayasaki, M., Nomura, T., Une, S., Nakaichi, M., Taura, Y., 2002. Expression of T helper 1 and T helper 2 cytokine mRNAs in freshly isolated peripheral blood mononuclear cells from dogs with atopic dermatitis. *J. Vet. Med. A. Physiol. Pathol. Clin. Med.* 49, 27–31.
- Hites, M.J., Kleinbeck, M.L., Loker, J.L., Lee, K.W., 1989. Effect of immunotherapy on the serum concentrations of allergen-specific IgG antibodies in dog sera. *Vet. Immunol. Immunopathol.* 22, 39–51.
- Jutel, M., Pichler, W.J., Skrbic, D., Urwyler, A., Dahinden, C., Müller, U.R., 1995. Bee venom immunotherapy results in decrease of IL-4 and IL-5 and increase of IFN- γ secretion in specific allergen stimulated T cell cultures. *J. Immunol.* 154, 4187–4194.
- Kiehl, P., Falkenberg, K., Vogelbruch, M., Kapp, A., 2001. Tissue eosinophilia in acute and chronic atopic dermatitis: a morphometric approach using quantitative image analysis of immunostaining. *Br. J. Dermatol.* 145, 720–729.
- Kikkawa, Y., Motoki, Y., Maruyama, K., Kojima, Y., Iwatsuki, K., Kaneko, F., 1994. Interleukin (IL)-6 production by peripheral blood mononuclear cells stimulated with house dust mite antigen and serum IL-6 in patients with adult type atopic dermatitis. *Jpn. J. Dermatol.* 104, 1339–1345 (in Japanese).
- Kimura, M., Tsuruta, S., Yoshida, T., 1998. Unique profile of IL-4 and IFN- γ production by peripheral blood mononuclear cells in infants with atopic dermatitis. *J. Allergy Clin. Immunol.* 102, 238–244.
- Lopez, A.F., Sanderson, C.J., Gamble, J.R., Campbell, H.D., Young, I.G., Vadas, M.A., 1988. Recombinant human interleukin 5 is a selective activator of human eosinophil function. *J. Exp. Med.* 167, 219–224.
- Lu, F.M., Chou, C.C., Chiang, B.L., Hsieh, K.H., 1998. Immunologic changes during immunotherapy in asthmatic children: increased IL-13 and allergen-specific IgG4 antibody levels. *Ann. Allergy Asthma Immunol.* 80, 419–423.
- Moverare, R., Elfman, L., Björnsson, E., Stalenheim, G., 2000. Cytokine production by peripheral blood mononuclear cells following birch-pollen immunotherapy. *Immunol. Lett.* 73, 51–56.
- Norman, P.S., 1998. Immunotherapy: past and present. *J. Allergy Clin. Immunol.* 101, 403–404.
- Nuttall, T.J., Knight, P.A., McAleese, S.M., Lamb, J.R., Hill, P.B., 2002. T-helper 1, T-helper 2 and immunosuppressive cytokine in canine atopic dermatitis. *Vet. Immunol. Immunopathol.* 87, 379–384.
- O'Brien, R.M., Byron, K.A., Varigos, G.A., Thomas, W.R., 1997. House dust mite immunotherapy results in a decrease in Der p 2-specific IFN- γ and IL-4 expression by circulating T lymphocytes. *Clin. Exp. Allergy* 27, 46–51.
- Olivry, T., Dean, G.A., Tompkins, M.B., Dow, J.L., Moore, P.F., 1999. Toward a canine model of atopic dermatitis: amplification of cytokine-gene transcripts in the skin of atopic dogs. *Exp. Dermatol.* 8, 204–211.
- Olivry, T., Moore, P.F., Affolter, V.K., Naydan, D.K., 1996. Langerhans cell hyperplasia and IgE expression in canine atopic dermatitis. *Arch. Dermatol. Res.* 288, 579–585.
- Pumhirun, P., Tuchinda, S., Nondavanich, A., Jarujinda, S., Poommark, C., 1994. Immunological and clinical evaluation during a 12-month period of immunotherapy. *Asian Pac. J. Allergy Immunol.* 12, 111–115.
- Reedy, L.M., Miller Jr., W.H., Willemse, T., 1997. Medical management of allergic disease. In: *Allergic Skin Disease of Dog and Cat*, second ed. Saunders, Philadelphia, PA, pp. 150–172.
- Rhodes, K.H., Kerdell, F., Soter, N.A., 1987a. Comparative aspects of canine and human atopic dermatitis. *Semin. Vet. Med. Surg. (Small Anim.)* 2, 166–172.
- Rhodes, K.H., Kerdell, F., Soter, N.A., 1987b. Investigation into the immunopathogenesis of canine atopy. *Semin. Vet. Med. Surg. (Small Anim.)* 2, 199–201.
- Rothe, M.J., Grant-Kels, J.M., 1996. Atopic dermatitis: an update. *J. Am. Acad. Dermatol.* 35, 1–13.
- Schwartz, R.H., 1996. Models of T cell anergy: is there a common molecular mechanism? *J. Exp. Med.* 184, 1–8.
- Scott, D.W., 1981. Observation in canine atopy. *J. Anim. Hosp. Assoc.* 17, 91–95.
- Scott, D.W., Miller Jr., W.H., Griffin, C.E., 2001. Skin immune system and allergic skin disease. In: *Muller and Kirk's Small Animal Dermatology*, sixth ed. Saunders, Philadelphia, PA, pp. 543–666.
- Secrist, H., DeKruyff, R.H., Umetsu, D.T., 1995. Interleukin 4 production by CD4+ T cells from allergic individuals is modulated by antigen concentration and antigen-presenting cell type. *J. Exp. Med.* 181, 1081–1089.
- Strasser, A., Kalmar, E., Niedermüller, H., 1998. A simple method for the simultaneous separation of peripheral blood mononuclear and polymorphonuclear cells in the dog. *Vet. Immunol. Immunopathol.* 62, 29–35.
- Theodoropoulos, D.S., Lockey, R.F., 2000. Allergen immunotherapy: guidelines, update and recommendations of the World Health Organization. *Allergy Asthma Proc.* 21, 159–166.
- Varney, V.A., Hamid, Q.A., Gaga, M., Ying, S., Jacobson, M., Frew, A.J., Kay, A.B., Durham, S.R., 1993. Influence of grass pollen immunotherapy on cellular infiltration and cytokine mRNA expression during allergen-induced late-phase cutaneous responses. *J. Clin. Invest.* 92, 644–651.
- Willemse, T., 1986. Atopic skin disease: a review and reconsideration of diagnostic criteria. *J. Small Anim. Pract.* 27, 771–778.
- Willemse, A., Van den Brom, W.E., Rijnberk, A., 1984. Effect of hyposensitization on atopic dermatitis in dogs. *J. Am. Vet. Med. Assoc.* 184, 1277–1280.
- Wunderli, P.S., Felsburg, P.J., 1989. An improved method for the isolation of enriched canine peripheral blood mononuclear cell and peripheral blood lymphocyte preparations. *Vet. Immunol. Immunopathol.* 20, 334–335.
- Zunic, M., 1998. Comparison between IMMUNODOT tests and the intradermal skin test in atopic dogs. *Vet. Dermatol.* 9, 201–205.