FULL PAPER *Internal Medicine*

Proliferative Responses to Canine Thyroglobulin of Peripheral Blood Mononuclear Cells from Hypothyroid Dogs

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(Received 26 July 2004/Accepted 12 November 2004)

ABSTRACT. The immune responses of hypothyroid dogs to canine thyroglobulin (cTg) were evaluated for the proliferative ability of peripheral blood mononuclear cells (PBMC). PBMC from three hypothyroid dogs with high titers of thyroglobulin autoantibody (TgAA) and 3 clinically normal dogs were cultured with 5, 10, or 20 μg/m*l* of cTg for 72 hr. The proliferative responses of the cells were determined by the level of incorporated BrdU. The numbers of cells expressing Thy-1, CD4, CD8 and IgG in the PBMC were counted by the immunofluorescence method. Proliferative responses to cTg were observed in the cells from hypothyroid dogs. The number of cells expressing IgG and CD8 in the hypothyroid dogs tended to be high compared with the clinically normal dogs. The $CD4^+$ cells in cultures from hypothyroid dogs increased depending upon the amount of cTg. There was a significant ($P<0.05$) positive correlation between the number of $CD4^+$ cells and the concentration of cTg in the cultures from hypothyroid dogs. These findings suggest a possible relationship between canine hypothyroidism and cellular immunity. Loss of self tolerance to thyroid antigens in $CD4^+$ T cells may play an important role in the development of canine hypothyroidism.

KEY WORDS: canine, hypothyroidism, lymphocyte subpopulations, peripheral blood mononuclear cell, thyroglobulin.

J. Vet. Med. Sci. 67(4): 363–368, 2005

Hypothyroidism is a common endocrine disorder in dogs [6]. Thyroid hormone deficiency can cause a variety of clinical symptoms such as lethargy, obesity, skin diseases, cold intolerance, hair loss, and reproductive problems [6, 16]. Most canine hypothyroidism results in the pathological process termed lymphocytic thyroiditis. Canine lymphocytic thyroiditis is considered to be an immune-mediated disease with clinical and histological similarities to Hashimoto's thyroiditis (HT) in man [3, 15]. HT is the thyroid autoimmune disease characterized by thyroid tissue destruction and inhibition of thyroid functions. Among thyroid antigens, thyroglobulin (Tg) and thyroperoxidase have been considered to be the major antigens related to HT. As antibody synthesis in autoimmune thyroid diseases (AITD) in humans includes IgG antibodies, which are helper T cell dependent, it is likely that helper T cell activity is important to the pathogenesis. The helper effect of T cells on antibody synthesis by B cells has been shown both *in vivo* and *in vitro* [7, 14, 19].

Thyroglobulin-specific antibodies have also been found in a high proportion of clinically diagnosed hypothyroid dogs [2, 9, 11, 13]. Thyroperoxidase antibodies were not found in one study [23], but uncharacterized microsomal antibodies have been reported in others [10, 15, 25]. The predominant thyroid antigen in dogs must be considered to be thyroglobulin. However, no evidence of a relationship between hypothyroidism and cellular immunity has been reported in dogs. With the aim of elucidating the role played by T cells in canine hypothyroidism, we studied the proliferative responses of peripheral blood mononuclear cells (PBMC) to canine thyroglobulin.

MATERIALS AND METHODS

Animals: Three dogs with hypothyroidism were used (Table 1). Diagnosis of hypothyroidism was confirmed by clinical signs such as alopecia, lethargy, and weight gain as well as the following laboratory findings: hypercholesterolemia, low serum free thyroxine (FT_4) levels, high serum thyroid stimulating hormone (TSH) levels, and detectable thyroglobulin autoantibody (TgAA). The serum FT_4 levels and TSH levels were determined by a commercial diagnostic laboratory (IDEXX Laboratories, KK, Tokyo, Japan). The detection method for canine TgAA in serum is described below. Two of three dogs (HYP-2, HYP-3) were treated with thyroxine replacement therapy for over one year. There was no change in treatment during the course of the study. Three purebred beagle dogs were also studied as normal controls (Table 1). They were determined as clinically normal by physical examination and laboratory data. The present study was conducted in accordance with NIH guidelines and the regulations of our Institutional Animal Care and Use Committee, and the dogs' owners signed a consent form before enrolling their dogs.

Thyroglobulin: cTg was purified from histologic normal thyroid glands from two clinically normal, purebred beagle dogs by gel filtration on a Sepharose CL-4B column (2.5 × 75 cm, Amersham Pharmacia Biotech UK, Little Chalfont, Buckinghamshire, UK) as described in detail previously [22]. The purified cTg was characterized in our previous report [22]. The serum FT_4 and TSH levels in the beagle dogs were normal. The serum was stored at –80°C and used as normal controls.

ELISA for thyroglobulin autoantibody: Canine TgAA in the serum samples was detected by the method of Iversen *et*

Table 1. Measurement of serum free T_4, TSH and TgAA titer in hypothyroid dogs and normal dogs

	Breed	Sex	Age (year)	Weight (kg)	FT_4* (pmol/*l*)	TSH* (ng/m*l*)	TgAA* titer
Hypothyroid							
HYP-1	Beagle	M	12	14.4	4.5	2.90	>2560
HYP-2	Golden Retriever	F	6	30.6	<3.0	2.20	>2560
HYP-3	Golden Retriever	M	2	37.0	<3.0	0.54	>2560
Normal							
Normal-1	Beagle	M	2	14.0	13.2	0.09	<20
Normal-2	Beagle	M	5	14.3	13.4	0.14	<20
Normal-3	Beagle	F	6	15.4	16.0	0.63	<20

* The data in the first medical examination before any treatments. F, Female; M, Male; FT_4, Serum free thyroxine concentrations; TSH, Serum thyroid stimulating hormone concentrations; TgAA, Thyroglobulin autoantibody.

al. [13], with minor modifications [22]. Horseradish peroxidase-conjugated sheep anti-dog IgG (Serotec Ltd, Kidlington, Oxford, UK) was used. Serum samples were titrated in 2 fold dilutions from 1/20 to 1/2,560. The standard curve, negative for TgAA, was constructed from duplicate tests of pooled sera from two clinically normal dogs that were euthanatized for purification of thyroglobulin. Serum samples were judged to be positive at dilutions in which the optical density values exceeded twice the standard deviation above the negative curve. The intra-assay coefficient of variation in the present assay was less than 5%. Each sample was assayed in duplicate in the same manner.

Proliferation assay and evaluation of lymphocyte subpopulations: Canine PBMC were evaluated for proliferative responses by the modified method of Wagner *et al.* [26]. Lymphocyte subpopulations were evaluated by counting the number of cells expressing Thy-1, CD4, CD8, or IgG in the PBMC. These tests were repeated twice or three times in each dog.

Ten milliliters of heparinized peripheral blood were collected from the cephalic vein, and diluted 1:1.4 with Hank's balanced salts solution (HBSS: Sigma Chemical, St. Louis, MO, U.S.A.). Lymphocyte Separation Solution (d=1.077, Nacalai Tesque, Inc., Kyoto, Japan) was used for separation of PBMC from whole blood. The mixture, 8 volumes of diluted blood in 3 volumes of Lymphocyte Separation Solution, was centrifuged at 400 × g for 30 min at room temperature. The PBMC at the interface were aspirated and washed twice with 5 m*l* of HBSS by centrifugation at 840 × g for 5 min at 4°C. After washing, the isolated cells were suspended in RPMI 1640 (SIGMA) supplemented with 10% fetal calf serum, 100 U penicillin/m*l* and 100 μg streptomycin/m*l*. The number of isolated cells was quantified microscopically. Viability was determined by the trypan blue dye exclusion test.

One hundred microliters of each cell suspension with 5×10^5 cells/m*l* was added to wells of 96-well flat bottom tissue microculture plates (Iwaki, Tokyo, Japan). As an antigen, canine Tg diluted with culture medium at final concentrations of 5, 10, or 20 μg/m*l* was used. Concanavalin A (Con A; Wako Pure Chemical Industries, Ltd, Osaka, Japan) was also diluted with culture medium at the final concentration of 4 μg/m*l* as a mitogen. These antigens were sterilized by filtration with 0.22 μm pore size membrane filter before adding to the cultures. One hundred microliters of the different concentrations of canine Tg, Con A, or culture medium were added to the wells for a final volume of 200 μl. Each culture condition for each sample was tested in quadruplicate in the same culture. Two of the four wells were used for the proliferation assay, the rest of the wells were used for immunofluorescence. The cultures were incubated at 37°C with 5% CO_2 for 72 hr.

The BrdU assay procedure was carried out according to the original protocol from the manufacturer (Cell Proliferation ELISA BrdU, colorimetric; Roche Diagnostics, Mannheim, Germany). The reaction was quantified by measuring the optical density at a wavelength of 450 nm and a reference wavelength of 595 nm. The stimulation index (SI) value (mean optical density values of antigen stimulated cultures/mean optical density values of medium-only cultures) was calculated for each culture treatment.

Immunofluorescence was used for evaluation of the lymphocyte subpopulations in PBMC. After incubation, the cells in each culture condition were recovered from each well of the microculture plates and washed twice with HBSS by centrifugation at 470 × g for 10 min at 4°C. The cells were resuspended in HBSS and placed on a silanized slide (Dako Cytomation Co., Ltd., Glostrup, Denmark). The cell smears were dried and stored at –80°C for the immunofluorescence assay. The smears were exposed to mouse anti-dog Thy-1, rat anti-dog CD4, rat anti-dog CD8 (Serotec) or FITC labeled goat anti-dog IgG (ICN/Cappel, Aurora, Ohio, U.S.A.) diluted at 1:50 with HBSS for 1 hr at 37°C. After three times washing with HBSS, the smears were subsequently exposed to FITC labeled goat anti-mouse IgG (Sigma) or FITC labeled goat anti-rat IgG (Leinco Technologies, Inc., Missouri, U.S.A.) diluted at 1:50 with HBSS for 1 hr at 37°C except the slides for IgG detection. After three times washing with HBSS, the smears were counterstained with 0.1% Evans blue. The fluorescent signals were captured with a digital microscope camera (PDMC Ie: Polaroid Co., Waltham, MA, U.S.A.). The number of cells expressing Thy-1, CD4, CD8, and IgG was

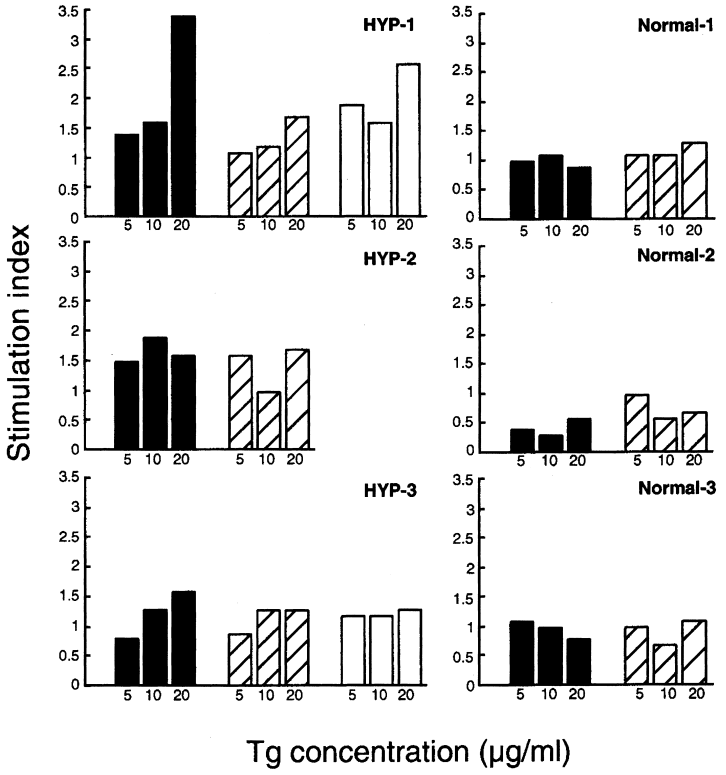

Fig. 1. Mean proliferative responses from replicate assays of PBMC from hypothyroid dogs and normal dogs. The PBMC from hypothyroid dogs (HYP-1, HYP-2 and HYP-3) and normal dogs (Normal-1, Normal-2 and Normal-3) were cultured with final concentrations of 5, 10, or 20 μg/m*l* of cTg. The proliferative responses of the PBMC were determined by the level of incorporated BrdU as detailed in Materials and Methods. The results are expressed as the SI (mean of duplicate cultures). Bar graphs with different patterns denote the result of the first (black bar), second (shaded bar) and third (open bar) assays, respectively.

as percentages.

Statistical analysis: Mean values and standard deviations of the results in the each culture condition were calculated for mean values of the replications in each culture condition in each dog. Spearman's correlation coefficients were used for intercorrelations. A P value of less than 0.05 was considered statistically significant. The software used was Stat View (SAS institute Inc., North Carolina, U.S.A.).

RESULTS

Proliferative responses to cTg were observed in the PBMC from hypothyroid dogs (Fig. 1). The cells from two of three hypothyroid dogs (HYP-1 and HYP-2) showed highly proliferative responses to all concentrations of cTg. The highest responses were observed in the cells from HYP-1 in the presence of 20 μg/m*l* of cTg. The cells from one of the hypothyroid dogs (HYP-3) responded to 10 or 20 μg/m*l* of cTg, but did not react to 5 μg/m*l* of cTg in two of three tests. Mean values and standard deviations of the results from the proliferation assay are shown in Table 2. Dose-dependence of the cTg-induced proliferation was not observed in the cells from hypothyroid dogs. All PBMC were viable, as shown by their proliferative responses to Con A.

Table 2. Proliferative responses of PBMC to Tg in hypothyroid dogs and normal dogs

Antigen	Concentration (μg/m*l*)	Stimulation index	
		HYP (n=3)	Normal (n=3)
Tg	5	1.3 ± 0.3	0.9 ± 0.2
	10	1.4 ± 0.1	0.8 ± 0.3
	20	1.9 ± 0.6	0.9 ± 0.2
ConA	4	18.5 ± 7.6	14.6 ± 4.4

Values are expressed as the mean SI ± SD of the mean values of replications in 3 dogs. Tg, Canine thyroglobulin; ConA, Concanavalin A; HYP, Hypothyroid dog; Normal, Normal dog.

There was a tendency toward a higher number of cells expressing IgG in cultures from the hypothyroid dogs compared to normal dogs (Table 3). The difference in the number of cells expressing Thy-1 was not observed between

Table 3. Percentages of the number of cells expressing Thy-1 and IgG per five hundred of PBMC in the various culture conditions

Antigen	Concentration (μg/m*l*)	Percentage of positive cells			
		HYP (n=3)		Normal (n=3)	
		Thy-1	IgG	Thy-1	IgG
Pre		60.1 ± 5.4	16.6 ± 8.8	57.8 ± 5.6	12.6 ± 1.6
Tg	0	60.8 ± 3.4	20.7 ± 10.7	60.1 ± 4.8	13.3 ± 3.1
	5	64.2 ± 5.6	21.9 ± 7.2	63.0 ± 9.9	13.3 ± 2.9
	10	60.9 ± 4.3	22.5 ± 12.6	58.9 ± 1.6	13.7 ± 2.2
	20	68.7 ± 6.1	21.4 ± 9.1	65.3 ± 8.4	14.2 ± 2.4

Values are expressed as the mean ± SD of the mean values of replications in 3 dogs. Pre, Pretreatment; Tg, Canine thyroglobulin; HYP, Hypothyroid dog; Normal, Normal dog.

Table 4. Percentages of the number of cells expressing CD4 and CD8 per five hundred of PBMC in the various culture conditions

Antigen	Concentration (μg/m*l*)	Percentage of positive cells			
		HYP (n=3)		Normal (n=3)	
		CD4+	CD8+	CD4+	CD8+
Pre		28.5 ± 3.0	22.8 ± 8.0	29.0 ± 3.4	18.4 ± 3.2
Tg	0	29.4 ± 3.2	23.1 ± 6.2	30.1 ± 6.6	19.4 ± 3.1
	5	34.1 ± 3.6	23.7 ± 8.3	30.8 ± 5.6	17.8 ± 2.3
	10	34.1 ± 3.3	22.8 ± 8.9	31.3 ± 6.8	20.3 ± 3.4
	20	39.5 ± 5.8	27.3 ± 8.3	33.6 ± 7.2	18.0 ± 6.0

Values are expressed as the mean ± SD of the mean values of replications in 3 dogs. Pre, Pretreatment; Tg, Canine thyroglobulin; HYP, Hypothyroid dog; Normal, Normal dog.

hypothyroid dogs and normal dogs. The number of $CD4^+$ cells in cultures from hypothyroid dogs increased in the presence of cTg (Table 4). There was a significant ($P<0.05$) positive correlation between the number of $CD4^+$ cells and the concentration of cTg existing in the cultures of cells from hypothyroid dogs. There was a tendency toward a higher number of $CD8^+$ cells in cultures of cells from the hypothyroid dogs as compared with normal dogs.

DISCUSSION

Most studies on the pathogenesis of canine hypothyroidism have provided information on humoral immunity for development of diagnostic methods such as determination of serum TgAA, T3 autoantibody and T4 autoantibody levels [9]. The immunological and molecular pathogenesis of AITD in dogs has not been well characterized. The data reported here indicate that highly proliferative responses to cTg were present in the PBMC from hypothyroid dogs (Fig. 1 and Table 2). There was no such response in normal dogs. The results show the possibility that cTg is one of the major antigens of T cells in canine hypothyroidism. The subpopulation of the responding T cells may be mainly $CD4^+$ cells. It must be indirect evidence of the reactivity of $CD4^+$ cells that the number of $CD4^+$ cells in the PBMC from hypothyroid dogs increased dependent upon the amount of cTg (Table 4). Additionally, the PBMC from hypothyroid dogs contained a higher number of cells expressing IgG as compared with normal dogs (Table 3). The observation also provided indirect evidence of continuous activation of $CD4^+$ cells in the PBMC from hypothyroid dogs. PBMC in the present study contained monocytes, which express MHC class II molecules as antigen presenting cells in the human and other species. Although the contribution of dog MHC subtypes to the disease in dogs are still under investigation [12], activated $CD4^+$ cells may lead to the activation of B cells in hypothyroid dogs. Akasu *et al.* [1] have demonstrated that PBMC and CD8-depleted, CD4-enriched PBMC from patients with HT can proliferate in response to human Tg *in vitro*, and the responses are more apparent in CD8-depleted, CD4-enriched PBMC. These responses were not observed in CD8-depleted, CD4-enriched PBMC from normal controls. They concluded that the function of suppressor $CD8^+$ cells was partially disturbed in the AITD patients. Autoimmune thyroiditis that is induced in athymic mice can be transferred by $CD4^+$ T cells, but a subfraction of normal $CD4^+$ T cells prevented experimental autoimmune thyroiditis in mice, either directly or by inducing suppressor T cells [20]. The subpopulation of $CD4^+$ T cells therefore has an important regulatory role in maintaining tolerance to Tg under normal circumstances [21]. Our results suggest that $CD4^+$ T cells may play an essential role in the development of autoimmune thyroiditis in dogs as well as in human cases.

Our observations in this study provided results for only TgAA positive dogs. The proportion of hypothyroid dogs that have TgAA in serum is generally reported to be around 50% [2, 9, 10, 24]. In a recent study, canine TgAA titers were found to decline during L-thyroxine treatment [8].

Alternatively as proposed in a study with humans receiving L-thyroxine substitution therapy, there could be a reduction of antigenic substances through decreased stimulation of thyroid tissue by circulating TSH [18]. These findings suggest that complete disorder of thyroid functions attributable to long-lasting hypothyroidism may lead to a decline in antigenic substances resulting in reduced TgAA production. The reduction of the antigenic substances may also lead to a decrease in the reactivity of $CD4^+$ T cells. The proliferative responses of PBMC to cTg also need to be evaluated in TgAA negative hypothyroid dogs.

In this study, we selected large dog breeds with hypothyroidism for repeated blood collections in large quantities. Therefore our results are not necessarily representative for all dog breeds with hypothyroidism. A variation in the prevalence of TgAA between different dogs breeds has been reported [9]. That finding suggests the heritability of lymphocytic thyroiditis. There may be a variation of pathologic differences in the disease among different dog breeds. A method for the proliferation assay using small quantities of blood must be developed for investigation of various dog breeds.

This is the first study to determine the reactivity of PBMC to canine Tg in hypothyroid dogs. The findings of the proliferation of the PBMC from hypothyroid dogs indicate a relationship between hypothyroidism and cellular immunity. Loss of self tolerance to thyroid antigens in $CD4^+$ T cells may play an important role in the development of canine hypothyroidism. In the reports regarding the role of T cell epitopes of Tg in the pathogenesis of human AITD and experimental autoimmune thyroiditis in animal models, several pathogenic epitopes have been identified [4, 5, 17]. However, pathogenic T cell epitopes in canine AITD have not been reported, because the amino acid sequence of cTg has not been fully identified. Further investigations of the amino acid sequence of cTg may lead to a better understanding of the etiology of canine hypothyroidism.

ACKNOWLEDGMENTS. The authors are deeply grateful to Nestle Purina PetCare Company for partly supporting our study. This study was supported in part by a Grant-in Aid for Scientific Research No. 14760201 from the Japan Society for the Promotion of Science.

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